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Improving immune function in newborn calves through milk replacer and starter supplementation

Ana Rita Violante Pedro

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Supervisor – Isabel Alexandra Duarte Ferreira Lopes Correia

Category – Assistant Professor

Affiliation – School of Medicine and Biomedical Sciences of the University of Porto

Co-supervisor – Margarida Rosa Garcez Maia

Category – Invited Assistant Professor

Affiliation – School of Medicine and Biomedical Sciences of the University of Porto

Co-supervisor – Manuel João Rua Vilanova

Category – Full Professor

Affiliation – School of Medicine and Biomedical Sciences of the University of Porto

Ana Rita Violante Pedro

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Programa Doutoral da Universidade do Porto (Instituto de Ciências Biomédicas Abel Salazar)

Orientadora – Doutora Isabel Alexandra Duarte Ferreira Lopes Correia

Categoria – Professora Auxiliar

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Co-orientadora – Doutora Margarida Rosa Garcez Maia

Categoria – Professora Auxiliar Convidada

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Co-orientador – Doutor Manuel João Rua Vilanova

Categoria – Professor Catedrático

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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"Não há palavras que consigam transmitir
O orgulho que eu tenho na minha filhota.
És a prova viva de que os sonhos são possíveis de realizar.
É com o coração a transbordar de amor que te digo:
És e vais continuar a ser a minha menina.
Só quero que sejas feliz
E continues,
Com a mesma garra e determinação que tens demonstrado.
As tuas lágrimas são as minhas.
A tua felicidade é a minha.
Por isso: Sê feliz!"

Antónia Pedro
Abril, 2012

Para aqueles que não puderam ver-me concluir esta etapa.
Para o meu pai, restante minha família e amigos.
Para o Hugo e para o meu Francisco,
O meu muito obrigada!

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Abstract

Calf rearing is the most challenging step in dairy production. Since the main diseases affecting calves are multifactorial and of infectious origin, the welfare and health status depend on the balance between the environment, host and pathogenic agent. Feed additives such as probiotics, prebiotics and synbiotics have been used in calf rearing to improve immune function, but the mechanisms that justify their use are still unclear. Yeast and yeast-derived products are the most used dietary supplements in animal production and are mainly composed of β-glucans. These are known to have immunomodulatory properties through recognition by Dectin-1, a receptor expressed on the surface of myeloid cells. Other additives, with growing interest for use in feed supplementation are microalgae. These are sustainable and valuable sources of nutrients and bioactive compounds, such as polyunsaturated fatty acids, pigments with antioxidant capacity and polysaccharides with immunomodulatory properties, although their content varies greatly between species. In this thesis, we stimulated bovine innate immune cells with yeast-derived compounds and three species of digested microalgae (*Chlorella vulgaris*, *Nannochloropsis oceanica* and *Tetraselmis* sp.) to evaluate the induced response and identify the underlying mechanisms. Microalgae were further tested in an intestinal epithelial cell co-culture system to explore the action of these products in a model that mimics the intestinal epithelium. This initial work carried out *in vitro* was followed by an *in vivo* evaluation of milk replacer supplementation with 1% (w/w, dry matter basis) *C. vulgaris* in newborn calves.

Here, we show, for the first time, that bovine monocytes respond in a dose-dependent manner to particulate β-glucans and to particles containing β-glucans through recognition by Dectin-1. Stimulation with these compounds resulted in increased production of pro-inflammatory cytokines and increased expression of the costimulatory molecules CD80 and CD86 on the surface of monocytes. These results point to a possible induction of innate immune memory in bovine cells by particulate β-glucans, a phenomenon already reported in other species. The three species of microalgae induced the expression of pro-inflammatory cytokines and the overexpression of the genes that encode them in macrophages derived from bovine monocytes (MDM), partially through NF-κB signalling. However, pre-incubation of MDM with digested microalgae before stimulation with lipopolysaccharides from *Escherichia coli* evidenced anti-inflammatory effects of microalgae. In the same line, when co-cultures of intestinal epithelial cells (Caco-2/HT29-MTX cell lines) were incubated with digested *C. vulgaris* and *N. oceanica*, there was an increase in the production of reactive oxygen species (ROS). However, co-cultures previously conditioned with microalgae decreased ROS production when

subsequently stimulated with a potent oxidative compound (H_2O_2), suggesting that the digested microalgae have anti-inflammatory effects and are capable of scavenging free radicals. The effects of including *C. vulgaris* in milk replacer of newborn calves were determined by assessing performance, immunological parameters, and faecal fermentation profile and microbiome. Supplementation of the milk replacer with *C. vulgaris* resulted in the increase of indices associated with inflammation (neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, and systemic immuno-inflammation index). Higher serum IL-8 levels were also observed in calves supplemented with *C. vulgaris*. However, no other immunological parameters or performance were altered and MDM from supplemented animals produced significantly lower overall IL-6 levels and increased overall *IL10* mRNA expression after *ex vivo* challenge with various pattern recognition receptor agonists. Faecal proportions of valeric and branched short-chain fatty acids were increased in supplemented calves, which was probably due to the higher protein intake by calves supplemented with *C. vulgaris*, but no differences were observed in either the abundance or diversity of the microbiota.

Taken together, these results show that yeast-derived particulate β-glucans are recognized by bovine innate immune cells. This result may explain some of the already described beneficial effects of including supplements rich in β-glucans in cattle diets. Furthermore, although microalgae induced MDM activation *in vitro*, we did not observe significant effects on immunity and health of newborn calves after dietary supplementation with *C. vulgaris*. Thus, it seems pertinent to carry out more comprehensive future studies that can explore in greater detail the observations reported here.

Resumo

A criação de vitelos é a etapa mais desafiante na produção de bovinos de leite. Como as principais doenças que afetam os vitelos são multifatoriais e de origem infeciosa, o bem-estar e o estado de saúde do animal dependem do equilíbrio entre ambiente, hospedeiro e agentes patogénicos. Os suplementos alimentares, como probióticos, prebióticos e simbióticos, têm vindo a ser usados na criação de vitelos com vista a melhorar a função imunológica, mas os mecanismos que justificam o seu uso ainda não são claros. As leveduras e produtos derivados de leveduras, os suplementos dietéticos mais utilizados na produção animal, são constituídos principalmente por β-glucanas. Estas são conhecidas por apresentarem propriedades imunomoduladoras através do reconhecimento por Dectin-1, um recetor expresso à superfície de células mieloides. Outros suplementos alimentares com interesse crescente são as microalgas. Estas são ingredientes sustentáveis e ricas em nutrientes e compostos bioativos, como ácidos gordos polinsaturados, pigmentos com capacidade antioxidante e polissacarídeos com propriedades imunomoduladoras, ainda que os seus conteúdos variem grandemente entre espécies. Nesta tese, estimulámos células imunitárias inatas de bovino com compostos derivados de leveduras e três espécies de microalgas digeridas (*Chlorella vulgaris, Nannochloropsis oceanica e Tetraselmis* sp.) para avaliar a resposta induzida e identificar os mecanismos subjacentes. As microalgas foram ainda testadas num sistema de co-cultura de células epiteliais intestinais para explorar a ação destes ingredientes num modelo que mimetiza o epitélio intestinal. A estes trabalhos iniciais realizados *in vitro* seguiu-se a avaliação *in vivo* da suplementação do leite de substituição com a 1% (p/p, na matéria seca) de *C. vulgaris* em vitelos recém-nascidos. Aqui, mostramos, pela primeira vez, que os monócitos de bovino respondem de forma dependente da dose a β-glucanas particuladas e a partículas contendo β-glucanas através do reconhecimento por Dectin-1. A estimulação com estes compostos resultou no aumento da produção de citocinas pró-inflamatórias e no aumento de expressão das moléculas coestimuladoras CD80 e CD86 à superfície dos monócitos. Estes resultados apontam para uma possível indução de memória imunitária inata em células de bovino por β-glucanas particuladas, um fenómeno já reportado noutras espécies. As três espécies de microalgas digeridas induziram a expressão de citocinas pró-inflamatórias e a sobre-expressão dos genes que as codificam em macrófagos derivados de monócitos de bovino (MDM), parcialmente através da sinalização por NF-κB. No entanto, a pré-incubação de MDM com microalgas digeridas antes da estimulação com lipopolissacarídeos de *Escherichia coli* evidenciou efeitos anti-inflamatórios das microalgas. No mesmo sentido, quando co-culturas de células epiteliais intestinais

(linhas celulares Caco-2/HT29-MTX) foram incubadas com *C. vulgaris* e *N. oceanica* digeridas, verificou-se um aumento da produção de espécies reativas de oxigénio (ROS). No entanto, as co-culturas previamente condicionadas com as microalgas diminuíram a produção de ROS quando estimuladas subsequentemente com um potente composto oxidativo (H_2O_2), o que sugere um efeito anti-inflamatório das microalgas digeridas e capacidade em eliminar radicais livres. Os efeitos da inclusão de *C. vulgaris* no leite de substituição de vitelos recém-nascidos foram determinados através da avaliação do desempenho, parâmetros imunológicos e perfil de fermentação e microbioma fecais. A suplementação do leite de substituição com *C. vulgaris* resultou num aumento de índices associados à inflamação (razão neutrófilos:linfócitos, razão plaquetas:linfócitos e índice de imuno-inflamação sistémica). Também se observaram níveis mais elevados de IL-8 sérica em vitelos suplementados com *C. vulgaris*. No entanto, nenhum outro parâmetro imunológico ou desempenho foi afetado e os MDM de animais suplementados produziram níveis globais significativamente mais baixos de IL-6 e uma expressão global aumentada de mRNA de *IL10* após o desafio *ex vivo* com vários agonistas de recetores de reconhecimento de padrões. As proporções fecais de ácido valérico e ácidos gordos voláteis de cadeia ramificada aumentaram em vitelos suplementados com *C. vulgaris*, o que poderá ser devido à maior ingestão de proteína, contudo não foram observadas diferenças quer na abundância, quer na diversidade do microbioma.

No seu conjunto, estes resultados mostram que as β-glucanas particuladas derivadas de leveduras são reconhecidas por células da imunidade inata de bovinos. Este resultado pode explicar alguns dos efeitos benéficos já descritos da inclusão de suplementos ricos em β-glucanas na dieta de bovinos. Adicionalmente, embora as microalgas tenham induzido a ativação de MDM *in vitro*, não observámos efeitos significativos na imunidade e na saúde de vitelos recém-nascidos após suplementação da dieta com *C. vulgaris*. Assim, parece pertinente a realização de estudos futuros mais abrangentes que possam explorar em maior detalhe as observações aqui reportadas.

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List of Abbreviations

16S rRNA	16S Ribosomal ribonucleic acid
ADG	Average daily gain
ALR	AIM2-like receptor
APC	Antigen-presenting cell
AP-1	Activating protein 1
ATCC	American Type Culture Collection
B2M	β2 Microglobulin
BOMAC	Bovine macrophage cell line
bp	Base pairs
BRD	Bovine respiratory disease
BSA	Bovine serum albumin
$CaCl_2(H_2O)_2$	Calcium chloride dihydrate
CCIVV	Clinical and Research Veterinary Center of Vairão
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLEC7A	C-type Lectin domain containing 7A
CGF	Chlorella Growth Factor
CLR	C-type Lectin receptor
ConA	Concanavalin A
COVID-19	*Coronavirus* disease 2019
CR3	Complement receptor 3
CV	*Chlorella vulgaris*
CXCL8	CXC Motif chemokine ligand 8
DAPI	4′,6-Diamidino-2-phenylindole
DC	Dendritic cell
DCF	Dichlorofluorescein
DCFH-DA	2’,7’-Dichlorofluorescein diacetate
DC-SIGN	dendritic cell-specific ICAM-grabbing non-integrin
DGAV	Direção Geral de Alimentação e Veterinária
DHA	Docosahexaenoic acid
DMEM	Dulbecco’s modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
ECACC	European Collection of Authenticated Cell Cultures

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
EU	European Union
F-actin	Filamentous actin
FACS	Fluorescence-activated cell sorting
FcRn	Neonatal Fc receptor
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FOS	Fructooligosacharides
FTPI	Failure of transfer of passive immunity
FVD	Fixable Viability Dye
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GOS	Galactooligosacharides
H_2DCFDA	2',7'-dDchlorodihydrofluorescein diacetate
HBSS	Hank’s balaced salt solution
HCl	Hydrochoric acid
HEPES	N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
HSC	Haematopoietic stem cell
IEC	Intestinal epithelial cell
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
ILC	Innate lymphoid cell
IκB	Inhibitor of κB
LDH	Lactate dehydrogenase
LEfSe	Linear discriminant analysis effect size
LPS	Lipopolysaccharide
M cells	Microfold cells
mAb	Monoclonal antibody
MAIT	Mucosa-associated invariant T cell
MARVELD1	MARVEL domain containing 1
MEM	Minimum essential medium
MHC	Major histocompatibility complex
MINCLE	Macrophage inducible Ca^{2+} - dependent lectin receptor

MDM	Monocyte-derived macrophages
MLN	Mesenteric lymph node
MR	Milk replacer
mRNA	Messenger ribonucleic acid
mRNA	microRNA
MOS	Mannan oligosaccharides
Mϕ	Macrophage
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NCD	Neonatal calf disease
nd	Not detected
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NK	Natural killer cell
NKT	Natural killer T cell
N/L	Neutrophil to lymphocyte ratio
NLR	NOD-like receptor
NO	*Nannochloropsis oceanica*
NTC	Negative template control
ORBEA	Animal Ethics Committee of School of Medicine and Biomedical Sciences, University of Porto
OTU	Operational taxonomic unit
P3C	Pam3CSK4 [Pam3Cys-Ser-(Lys)4]
PAMP	Pathogen associated molecular pattern
Papp	Apparent permeability coefficient
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PerCP-Cy5.5	Peridinin-chlorophyll protein-cychrome 5.5
PHA	Phytohemagglutinin
PI	Propidium iodide
P/L	Platelet to lymphocyte ratio
PMA	Phorbol myristate acetate
PPIA	Peptidylprolyl isomerase A
PRR	Pattern recognition receptor
PUFA	Polyunsaturated fatty acids
qPCR	quantitative polymerase chain reaction
RLR	RIG-I-like receptor
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RPE	R-Phycoerythrin
RPMI	Roswell Park Memorial Institute (media)
RT-PCR	Reverse transcription polymerase chain reaction
SCFA	Short-chain fatty acid
SEAP	Secreted embryonic alkaline phosphatase
SEM	Standard error of the mean
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SII	Systemic immune inflammation
siRNA	Small interference ribonucleic acid
SR	Scavenger receptor
SSF	Simulated salivary fluid
T	*Tetraselmis* sp.
TAE	Tris acetate EDTA
TCR	T-cell receptor
TED	Transepithelial dendrite
TEER	Transepithelial electrical resistance
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor Alpha
TPCA-1	2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide
Treg	Regulatory T-cell
TRITC	Tetramethylrhodamine
TSP	Total serum proteins
UNC	MISSION® siRNA Universal Negative Control #1
UK	United Kingdom
US	United States
UXT	Ubiquitously expressed prefoldin like chaperone
XOS	Xylooligosaccharides
WGP-D	Whole glucan particles - dispersible
WGP-S	Whole glucan particles – soluble
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
Zym	Zymosan
γδT	Gamma delta T cells

CHAPTER I

State of the Art

1. Introduction

Over the past few decades, livestock farming has undergone profound changes towards more complex and specialized structure and management procedures (1). This evolution was particularly notorious in the dairy sector and emerged as a result of pressures imposed by the growing demand for food, global market prices, policy frameworks and consumers expectation of sustainable animal products (1-3). Such contrasting challenges led to a decline in dairy farm numbers, while technological progress and improvements in animal genetics and feed efficiency promoted specialization and increased herd size and milk production (4, 5).

Major advances have also been attained in cattle housing, nutrition, biosecurity, breeding, and implementation of veterinary herd health management programs, which allowed the reduction of metabolic and infectious diseases incidence (1, 6). Health status depends on the complex and intricate interaction between host, environment, and microbial agent, each of which is influenced by management practices (6). This fragile balance defines the outcome for the host: health or disease (Figure 1). Addressing the multifactorial nature of disease has been a cornerstone to promote dairy cattle health through disease prevention rather than disease treatment. Achievements include reduction in the incidence of milk fever, clinical respiratory disease in adult cattle, mastitis, parasitism, and contagious disease. Moreover, control and recognition of sub-clinical diseases such as subacute ruminal acidosis, ketosis, subclinical mastitis, and endometritis also contributed to disease prevention (6).

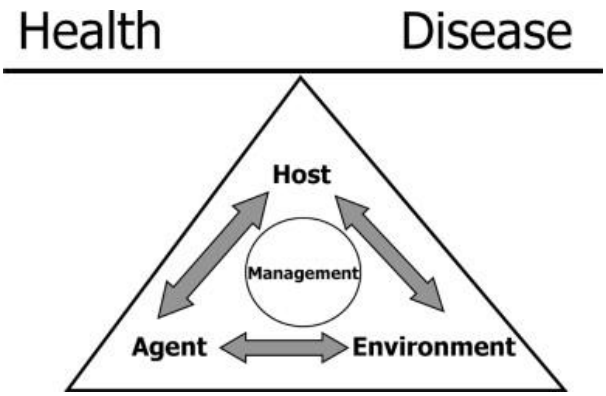

Figure 1 – Interplay between main determinants for health status: host, environment, and the microbial load, all affected by management practices. The balance of this interplay defines animal health or disease. Reprinted with permission from (6).

Despite advances on cattle management practices, calf rearing is still characterized by high incidences of enteric and respiratory disorders, particularly severe until weaning, which are the main causes of calf morbidity and mortality in bovine herds (7). These

multifactorial syndromes are mostly of infectious origin, and usually result from the imbalance between the immune system's ability to respond to pathogens and the pressure of infection that naturally exists in the surrounding environment (8-10). Calf diseases have a major impact on the economic viability of farms, due to costs of treatment, loss of animals, reduction of the genetic pool, and decreased performance of the surviving animals. In addition, high rates of morbidity are related to increased use of antibiotics. This excessive use of antibiotics was identified as one of the major causes for the increased incidence of multidrug-resistant bacteria infections in humans (11, 12) and animals, namely calves (13, 14). Indeed, in 2015, in the United States (US), 80% of the antibiotics were sold for veterinary use. They were mainly used as growth promoters, but also as metaphylactic and/or prophylactic, which may contribute to the emergence and spread of resistant bacteria (11, 12, 15). In the European Union (EU), although the use of antibiotics as growth promoters has been banned since January 2006, the incidence of zoonotic infections caused by multidrug-resistant bacteria is high (16, 17). In addition, the prevalence of antibiotic-resistant bacteria with zoonotic potential was considered to be unsettling (18), reinforcing the need of reducing the use of antibiotics in animal production, as well as finding alternatives to prevent their use.

Management practices commonly adopted on-farm pose challenges to calf health as physiological stressors are induced to young animals with poor ability to fight infections due to their immature immune system (7, 19). Hence, an important aspect to consider regarding incidence of disease is the pathogen load in the surrounding environment since most agents for neonatal diarrhoea infect through faecal-oral route (20), and the causes of respiratory disease (albeit complex) are also related to the pathogen load in the facilities (8). It is thus particularly important to ensure effective cleaning and hygiene operations in the rearing areas, as well as isolation and quick treatment of sick calves. Additionally, it is essential to leverage the immune system of calves, so they can respond more quickly and efficiently to infectious agents, preventing the occurrence of disease and the use of pharmaceutical compounds while promoting food security, animal welfare and human health. To overcome these challenges a holistic approach to calf rearing must be addressed, embracing the One Health concept, as human health, animal health and welfare, and environment are interconnected and interdependent. The first and foremost strategy to improve immune function in young calves should be the optimization of nutrition planes and feeding management (21, 22). Lorenz *et al*. have associated feeding high planes of nutrition with lower risks for calf diarrhoea (23). Hammon *et al*. demonstrated, by whole transcriptome sequencing, that calves fed conventional (restricted) planes of nutrition presented lower gene activation (mostly genes related to immune function) in the jejunal mucosa than calves fed milk replacer *ad libitum* (24). The

second line would then be the use of feed additives such as probiotics and prebiotics to enhance the immune function (21).

2. Calf Health and Welfare

Calf rearing is one of the most sensitive processes on dairy farms, with calf health and welfare being commonly assessed through growth performance and mortality rates (25). Dairy calf rearing may represent 15 to 20% of the total costs, with no immediate income since return on investment only occurs after calving (3). Thus, although advances have been achieved over the last decades, poor management practices are still one of the major causes for calf morbidity and mortality.

Morbidity and mortality rates described are quite variable depending on the above-mentioned interactions that determine risks for disease (Figure 1), which are different depending on the calf (breed, age), overall management, housing, colostrum management and incidence of failure of transfer of passive immunity (FTPI), and season (weather conditions) (26, 27). Differences may also be explained by case definition, study design and geography/climate zones where data was collected (27).

In the US, from 1991 to 2007, mortality rates in heifers until weaning were estimated to be 10% (28, 29). A study published in 2014 reported overall mortality rates of 3.5%, in heifers up to 3 months (27), whereas overall calf mortality was estimated to be 6.2% in 2015 (30) and 5% in 2018 (31).

In Europe, a British study reported mortality rates in dairy calves, up to 3 months of age, of 6% (32) and in beef calves of 2.86% (32). In Switzerland, overall mortality rates in calves of 22 different breeds, up to 4 months-old, was 4.8% (33). In Norway, overall mortality rates of dairy calves were reported to be 4.6% (34), and in the Netherlands, reported mortality rates, in calves up to 14 days of age, was 3.3%, in calves from 15 to 55 days of age was 4.5%, and in weaned calves from 56 days of age up to a year old was 3.1% (25).

In Portugal, two studies reported dairy calf mortality rates of 10 and 20% (35, 36). One study assumed a mortality of 5.7% in beef calves until weaning (37) and another study reported mortality rates of 3.2% and 2.3% in Alentejana and Mertolenga calves, respectively (38).

Main diseases affecting young calves and contributing to mortality rates are the multifactorial neonatal calf diarrhoea (NCD), bovine respiratory disease (BRD) and navel infection (omphalitis/omphalophlebitis) (26, 27, 39, 40). According to a cohort study recently performed in 11 commercial UK dairy farms, NCD, BRD and navel infections were recorded in 48.2%, 45.9% and 28.7% of the 492 heifers monitored from birth to

nine-weeks old (26). In Germany, a cross-sectional study performed in 731 dairy farms, recorded an overall morbidity rate of 42% in preweaned calves, mostly due to omphalitis, diarrhoea, and respiratory disease (41). An interesting observation was that co-morbidities were quite common, with 7.1% of the calves being affected by more than one disorder at the same time (41). While enteric infections are more frequent in calves less than one month of age, pneumonia is more frequent in calves over one month old (7). Other causes of calf mortality may be calving-related (calving associated anoxia), weather-related and, quite frequently, unknown (14.2%) (30).

2.1. Neonatal Diarrhoea

One of the major challenges affecting cattle health between one and three weeks old, with a peak incidence at two weeks old, is NCD (20). The most common causes of NCD are *Escherichia coli*, Rotavirus, Bovine Coronavirus, *Cryptosporidium parvum,* and *Eimeria* spp. (coccidiosis) infections (20, 39, 42-45). Other pathogens that may play a role in the development of neonatal diarrhoea are *Clostridium perfringens*, *Clostridium difficile*, *Salmonella* spp., *Giardia lamblia*, bovine viral diarrhoea virus, bovine torovirus and caliciviruses (norovirus and nebovirus) (10, 20, 42, 46). Depending on the infectious agent and the occurrence of co-infections, clinical signs vary from mild – if there are slightly less consistent faeces and no alterations on the clinical examination – to severe – if watery and frequent diarrhoea accompanied by dehydration are present. In the severe, acute cases, dehydration left untreated will rapidly escalate to acidosis and electrolytic imbalance, and death may occur if no immediate veterinary intervention is assured (42). In Europe, morbidity rates are highly variable, ranging from as low as 2.7% (47) to approximately 20% (41, 44, 48), and may be as high as 50% (26). Two studies report an incidence of 23% (27, 49) in Canada and in the US. Additionally, according to the United States Department of Agriculture (USDA), in 2017, NCD accounted for 15.4% of the mortality in calves, the figure rising to 28.1% in dairy unweaned calves (30).

2.2. Respiratory Syndromes

The BRD is of complex and multifactorial origin. Host, management, environment and microbial load converge to dictate the incidence and severity of disease (8, 50, 51). Host factors are related to passive immunity (adequate colostrum intake) and artificial (vaccine-induced) active immunity, genetics, and resident respiratory microbiome. These factors determine the ability of the immune system to respond to pathogens and inflammatory triggers (51). There are infectious agents that play a major role on the incidence of BRD, namely viral (bovine respiratory syncytial virus, bovine parainfluenza

virus, bovine herpesvirus, bovine viral diarrhea virus, adenovirus, rhinovirus, enterovirus, bovine coronavirus) and bacterial (*Mannheimia haemolytica*, *Pasteurella multocida*, *Histophillus somni*, *Mycoplasma bovis*, *Trueperella pyogenes*, *Streptococcus pneumoniae* and *Staphylococcus aureus*) (8, 50). Management practices (poor nutrition, dehydration, veterinary procedures, weaning, transport, regrouping), and environmental causes, such as weather (extreme cold or heat) or housing conditions (inadequate ventilation with accumulation of ammonia, dust and infectious agents) may lead to BRD (8, 50, 51). This could result from respiratory epithelium damaging or stress induction that impairs immune response to infectious agents due to increased endogenous corticosteroids (52). European studies reported BRD morbidity rates of 5.7% (47), 8.7% (41), 31.3% (48), and up to 45.9% (26). In Canada, a study reported morbidity rates of 17% and in the US, 22% of the calves enrolled in an observational study were treated for BRD (27, 49). BRD is also a major cause of calf mortality. It is estimated to account approximately 22.5% to 32.7% to the overall mortality (29).

2.3. Navel infections

The umbilical cord is a structure that provides foetal-maternal connection, allowing the elimination of metabolites and passage of nutrients. It consists of umbilical arteries, an umbilical vein and the uracus (53, 54). These structures regress after birth, and drying period may range from 1 to 8 days, although at 5th day almost all calves have the umbilical cord dry (54, 55). During the drying period there is a risk for colonization and infection of the umbilical cord (omphalitis), either from the environment or as a cause of generalized bacteraemia (54). The main pathogens involved in this disease are *E. coli* and *Trueperella pyogenes* (54). Infection may involve the umbilical vein (omphalophlebitis) and the umbilical arteries (omphaloarteritis) and also lead to umbilical abscesses (56, 57). Morbidity rates of omphalophlebitis or umbilical abscess are reported to be of 1.3% (58), 9.8% (48), 28.7% (26), 29.9% (59) and 33% (60). Van Camp *et al*. 2022 suggested that different case definitions (some authors use observation of an abnormal umbilicus to define the case, while others confirm clinical findings with microbial culture or use a scoring system to identify this disease) could be a factor for such discrepancies in the reported incidences (57).

2.4. Impact of infection on animal performance

Besides welfare and wellbeing concerns, incidence of disease in young calves has short and long-term economic impact on commercial farms (9, 61). (25). Dairy calf rearing may represent 15 to 20% of the total costs and has no immediate return on investment (3).

Rossini (2004) reported that occurrence of NCD increased the risk of BRD by 2-fold (62). In the same study, the author reported age at first calving increased approximately 15 days in calves that have had an episode of BRD, and age at first calving may increase up to one and a half month, comparatively to healthy calves (62). Bach (2011) did not find an association between the incidence of NCD, BRD and navel infections with chances of finishing the first lactation. However, heifers that have had four or more respiratory infection episodes before calving, were estimated to be 1.8 times more likely not to finish the first lactation. Besides, days-in-milk decreased significantly and linearly as the episodes of BRD increased during calfhood (63). In a Systematic Review and Meta-analysis study, the authors reported that episodes of BRD in calfhood increased 2.85 times the odds of heifer death before first calving. Additionally, heifers that survived showed lower average daily gains (0.067 kg/day) and produced less milk in the first lactation (121.2 kg) (64). Thus, it is crucial to improve calf's health status and welfare. Mortality and morbidity rates of calves, in particular those caused by NCD and BRD, were reduced with improvement of housing conditions, appropriate nutrition and feeding strategies, and enhancement of the immune system of animals (26, 27, 31), pinpointing the need to address these factors in a holistic approach.

3. The Immune System

The immune system is classically divided in two branches, respectively mediating the so-called innate and adaptive immune responses, according to the speed and specificity of the response (65, 66). Although defined in separate branches, there is an intricate and complex interplay between innate and adaptive immune mechanisms (65).

3.1. Overview of the Immune System

3.1.1. Innate immune system

3.1.1.1. Physical, chemical and microbiological barriers

Anatomic/physical and physiological barriers such as intact skin, epithelia, and mucosal secretions, are the first line of defence against pathogens (66). Respiratory and intestinal mucosae are complex and particularly vulnerable, since they must allow passage of molecules (either gases or nutrients) while in constant interaction with pathogens (67). Thus, epithelial layers are equipped with mechanisms to prevent colonization by pathogens. Epithelial cells provide an effective physical and functional barrier and the mucous layer coating mucosal epithelium provides both physical and chemical protection. The latter prevents adhesion of pathogens directly to the epithelial cell

surface, facilitates clearance of pathogens and allergens, and provides a scaffold for antimicrobial peptides and IgA (67, 68). Commensal bacteria present in the skin and in the mucosal surfaces, additionally provide protection against pathogen colonization through competitive mechanisms (69). If physical and physiological barriers are not able to contain a pathogenic microorganism, so that this line of defence is breached, then other innate immune mechanisms will take pace very rapidly to contain the infection and to activate the slower, yet antigen-specific, adaptive immunity (66, 70).

3.1.1.2. Cells and soluble components

Several soluble proteins and small molecules contribute to the innate immune response. Those include complement proteins, antimicrobial peptides, cytokines, chemokines, lipopolysaccharide (LPS)-binding protein, C-reactive protein, lipid mediators, reactive free radical species and enzymes. Haematopoietic cells associated with innate immunity include neutrophils and other granulocytes, innate lymphoid cells (ILCs) and natural killer (NK)-cells, dendritic cells (DCs), and macrophages (Mϕ). Some T lymphocyte populations are associated with an innate-like response, including gamma delta T (γδT) cells, NKT cells and mucosa-associated invariant T (MAIT) cells, (66, 71-73). Non-haematopoietic cells such as epithelial cells, endothelial cells, and fibroblasts also contribute to the innate immune defence (74).

3.1.1.3. Recognition of pathogens and induction of inflammation

The promptness of the innate immune response relies on the ability of the cells to recognize conserved microbial structures, denominated pathogen associated molecular patterns (PAMPs), through a limited but effective repertoire of cellular receptors, collectively designated as pattern recognition receptors (PRRs) (66). PRRs include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and extracellular or soluble pattern recognition molecules, such as collectins, pentraxins and ficolins (74, 75). TLRs are one of the best characterized PRR families and encompass 10 different functional TLRs in the bovine species (TLR-1 to 10) (70, 76). Cell surface TLRs (1, 2, 4, 5 and 6) mainly bind bacterial and fungal structures, whereas intracellular TLRs (3, 7 ,8 ,9) bind viral and bacterial nucleic acids (70, 73). RLRs, NLRs and AIMs detect intracellular PAMPs (75). CLRs comprise Dectin-1, Dectin-2, mannose receptor, dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN) and macrophage inducible Ca^{2+} - dependent lectin receptor (MINCLE), and recognize carbohydrates by a carbohydrate-binding domain (74, 77).

Activation of PRRs triggers intracellular signalling cascades that lead to upregulation of

genes involved in pro-inflammatory responses. One of the major signalling pathways activated upon ligand recognition by PRRs is the NF-κB pathway, which is normally inactive due to the binding of an inhibitory protein (IκB) (75). Activation of IκB kinase (IKK) phosphorylates IκB and releases the transcription factor NF-κB, which is then translocated to the nucleus, leading to the transcription of pro-inflammatory genes (75). Activating protein 1 (AP-1) is another family of transcription proteins, composed of Jun, Fos and activating transcription factor proteins, that mediate gene expression, thereby regulating cytokine production, cell proliferation, differentiation and apoptosis. (78)

The pro-inflammatory mediators produced after PRRs' signalling activation, such as cytokines and chemokines, will then orchestrate a rapid immune response (74, 79). Tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 are pro-inflammatory cytokines that modify vascular endothelial permeability, recruit leukocytes to the site of infection and induce the production of acute-phase proteins (74). CXC motif chemokine ligand 8 (CXCL8) or IL-8 is a chemokine that mainly attracts neutrophils to the site of infection (80). TGF-β and IL-10 promote anti-inflammatory responses to avoid a counterproductive and excessive immune response, which can be harmful for the host (73, 81, 82). Cytokines produced by innate immune cells will also play an important role in initiating and regulating the adaptive immunity.

3.1.1.4. Innate immune memory

Even though the innate immune system has limited specificity and has long been regarded as having no long-lasting memory, recent research has proved otherwise (83). In fact, innate immune cells show enhanced response to antigen stimulation, induced by epigenetic changes that result in transcriptional and metabolic reprograming, after previous contact with non-related stimuli (83, 84). This effect, named innate immune memory or trained immunity, can be induced by infection or treatment with certain PAMPs, followed by ligand recognition by PRRs (85). A particular PRR has been extensively studied and reported to induce innate immune memory, the C-type Lectin Receptor, Dectin-1 (86). Long-lasting innate immune memory may thus be initiated by recognition of β-glucans by Dectin-1 (86, 87). Monocytes primed *in vitro* with β-glucans, and allowed to rest for 6 days, show enhanced production of pro- and anti-inflammatory cytokines upon restimulation with non-related stimuli (88). The inverse phenomenon also happens, and the best characterized is the "LPS-tolerance". It is induced by exposure to low doses of LPS that may lead to lower inflammatory responses after stimulation with a non-related stimulus (89).

3.1.2. Adaptive immune response

Adaptive immunity comprises antibody-mediated and cell-mediated immunity, carried out by B and T lymphocytes, respectively (90). This branch of the immune system is characterized by the establishment of antigen-specific effector pathways that lead to the elimination of specific pathogens (or their products) and pathogen-infected cells. Activation of cell-mediated adaptive immunity requires antigen presentation to T cells by specialized innate immune cells (antigen-presenting cells – APCs), mainly dendritic cells, but also macrophages, that had previously phagocytosed and processed the antigen (91). Antigen-presentation occurs through interaction of major histocompatibility complex (MHC) class I or II molecules, harbouring processed peptides, on these cells with T-cell αβ receptors (TCR) on $CD8^+$ and $CD4^+$ T cells, respectively (71, 73, 91). Activation of T cells also involves binding of the co-stimulatory molecules CD80 and CD86 to CD28 molecule on the surface of T cells (92, 93). Antibody-mediated responses initiate after antigen binding to the B cell receptors on the surface of naïve B cells. This binding results in intracellular signalling that leads to the endocytosis, processing and loading of the antigen on MHC class II molecules, and migration of the responding B cell to T cell zones in secondary lymphoid tissues. Following interaction with antigen-specific primed T helper cells, B lymphocytes can differentiate into short-lived plasma cells producing IgM antibodies or into long-lived plasma cells or B memory cells that have undergone antibody affinity maturation and isotype switch (94). Bovine immunoglobulins comprise five heavy chain types (α, δ, ε, γ and μ) that define five immunoglobulin classes (IgA, IgD, IgE, IgG, and IgM), three IgG subclasses, and two IgM subclasses. Of the two possible light chain types (λ and K), the former predominates in bovine antibodies (73, 95).

Adaptive immunity has the distinctive characteristic of generating antigen-specific long-term immunological memory. Upon a first contact with an antigen, sets of long-lived memory T and B cells are generated (65, 90). Short and long-lived plasma cells are also generated from terminally differentiated antigen-specific B lymphocytes. The specificity of this memory is based on irreversible DNA modifications in antigen-stimulated naive lymphocytes that can be transmitted to daughter cells during the clonal expansion (96). In a second contact with the same antigen, memory B and T cells and antigen-specific immunoglobulins produced by B-cell derived plasma cells, mount a quick and robust immune response (65, 90).

3.2. The Immune System and the Gut

The gastrointestinal (GI) mucosa is one of the largest body surfaces exposed to the

surrounding environment (42). It not only functions as a digestive and absorption system, but also serves as a line of defence against pathogens (97, 98). Gut-associated lymphoid tissue (GALT) is one of the largest lymphoid organs (99) and it is mostly located in the mucosa, in close contact with food/feed and microbial products, allowing antigen sampling from the gut lumen (73). It is known that interaction between the intestinal epithelium, microbiome, and the local immune system is complex and impacts systemic immunity (42, 97). In human medicine, for example, several studies have addressed the composition of microbiome, innate and adaptive immune responses, and the interaction between them as key to the pathogenesis of several immune disorders such as systemic lupus erythematosus and rheumatoid arthritis (100, 101). Although essential for host health (102), microbiota colonization presents a risk of inflammation and infection if mucosal homeostasis is compromised (103). In order to establish an infection in the gut, pathogens need to overcome four barriers: local microbiome, the mucous and epithelial layers, and the mucosal immune system (73, 97). Overview of gut structure, including microbiome (commensal bacteria), mucous and epithelial barriers and cells and secreted products of local immune system, is depicted in Figure 2.

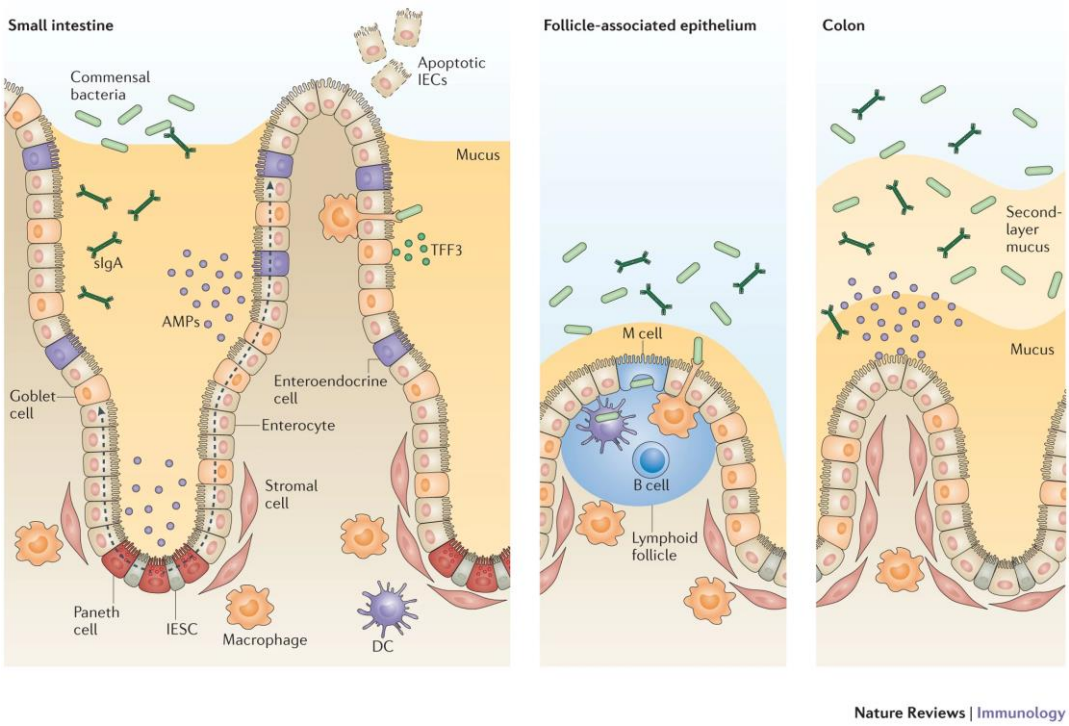

Figure 2 – Intestinal epithelial cells form a barrier that separates luminal contents from the immune cells present in the mucosa. Intestinal epithelial stem cells guarantee renewal of the epithelial layer by migrating towards the villus. Goblet cells secrete mucus, and M cells transport antigens from the lumen, to be processed by APCs and presented to T cells. These antigens are also made available to B-cells in the lymphoid follicles (Peyer's Patches). Subepithelial macrophages and dendritic cells may also sample antigens from the lumen through transepithelial dendrites. Reprinted with permission from (103).

3.2.1. Microbiome

Gut microbiome and mammals have evolved together and present strong mutualism (104). The host provides habitat and nutrients for the microbes, and they, in turn, contribute to dietary digestion, provide competitive protection against pathogens and regulate host physiology and homeostasis (105). Bovine faecal microbiome is mainly composed of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria* phyla (73). Microbial colonization occurs immediately after birth, from the vaginal canal, faecal material, colostrum, skin, and saliva from the dam and from bedding and environment (106). Development and establishment of the microbiome is a dynamic process which can be influenced by internal factors, such as functional maturity of the gut and the immune system, and external factors, namely the nutritional status and the environment (73).

Microbiome functions include fermentation of carbohydrates and production of metabolites, synthesis of amino acids and vitamins, cross-talk with intestinal epithelium and local immune cells (contributing to local homeostasis and systemic immunity) and competition with pathogenic microorganisms for resource accessibility (either nutrients or local sites for adhesion), thus preventing gut pathogen colonization and intestinal damage (98, 107, 108). Microbiome also stimulates enterocyte turnover, metabolic activity, production of antimicrobial peptides and IgA (109). Intestinal epithelial cells (IECs) respond to microbiome signals through PRRs (110). Upon stimulation, IECs produce cytokines and chemokines that will be sensed by the local immune cells which, in turn, will produce molecules that will modulate IECs' response to luminal bacteria (103, 110). This complex bidirectional crosstalk regulates gut homeostasis and maintains gut health (97, 110). Other mechanisms by which microbiome interacts with epithelial cells, shaping local immune responses, is through the synthesis of metabolites, such as short-chain fatty acids (SCFA). Acetic, butyric and propionic acids are the major SCFA produced in the gut (105, 111). It was reported that butyric acid, for example, is able to stimulate the maturation and expansion of regulatory T cells (Tregs) that control inflammatory responses and contribute to gut homeostasis (112, 113). SCFA may also play a role in the induction of innate immune memory and in the modulation of hematopoietic precursors in the bone marrow (114-116). Gut microbiome and microbial products are sources of PAMPs that can be recognized by PRRs of local innate immune cells, such as DCs, Mϕ and NK cells (Figures 2 and 3) (103, 105). Some microbial products may also reach the bone marrow and induce long-term effects on haematopoietic precursors (Figure 3) (105, 117).

It is thus possible that supplementation with prebiotics or probiotics that reach the gut

and are sampled by local immune cells may play a direct role in the induction of local and systemic innate immune memory (105).

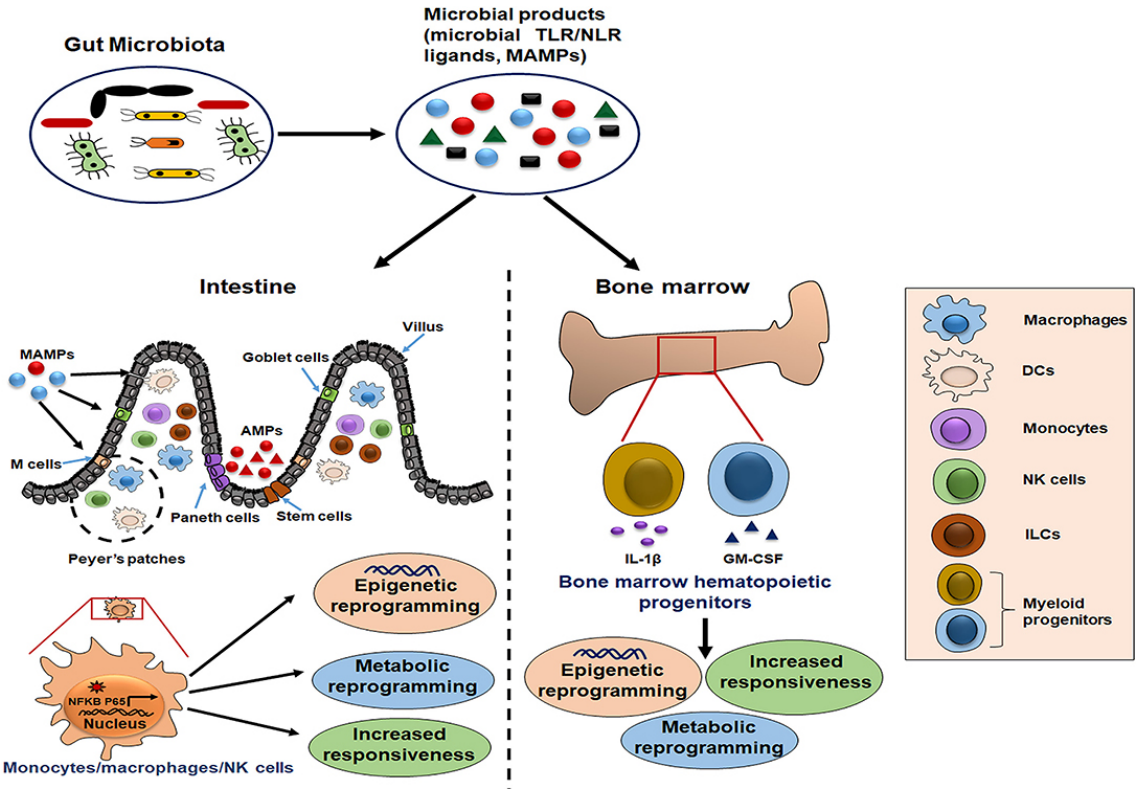

Figure 3 – Induction of innate immune memory in local immune cells and haematopoietic precursors in the bone marrow by gut microbiome and microbial products. Microbiome and microbial products are recognized through PRRs, leading to epigenetic and metabolic reprogramming of innate immune cells and haematopoietic precursors in the bone marrow. Adapted with permission from (105).

Interestingly, there is also evidence that modulation of the gut microbiome through the use of prebiotics probiotics and synbiotics is linked to lower incidences of respiratory disorders in humans (118). Several reports have addressed the gut-lung axis, where it is suggested that primed-cells in the gut can migrate to distant locations through lymph ducts, namely thoracic duct, and play a role in the respiratory immune system (97, 119). Hence, it could be possible to modulate immune function in the respiratory tract through dietary supplementation.

3.2.2. Mucosa

The intestinal epithelium is organized in villi and crypts, and is composed of a monolayer of IECs, joined together by tight junctions. It forms a physical barrier that separates gut commensals from the underlying tissues (42). The epithelium is constantly renewed by pluripotent intestinal epithelial stem cells located in the crypts (103). IECs comprise

enterocytes, with digestive and absorptive capacity, and secretory cells, namely enteroendocrine cells, goblet cells and Paneth cells (73, 103). Goblet cells secrete mucus, which is one of the first physical lines of protection against pathogen colonization, by preventing direct contact of commensal bacteria and pathogens with epithelial cells (103, 120). Mucus is organized in two layers, a less dense outer layer, extensively colonized by microbiome, and a denser adherent inner layer (42). The inner layer is more resistant to microbial colonization due to high concentration of antimicrobial compounds such as mucins (secreted by goblet cells), antimicrobial proteins and peptides (secreted by Paneth cells) and immunoglobulins (mainly IgA) produced by plasma cells located in the lamina propria and transported across the epithelial barrier by IECs (97, 103, 121). Bovine IECs are also an important and effective mechanism from the innate immune arm. Not only do they provide a physical and chemical barrier, they also sense microorganisms through pattern-recognition receptors (PRRs), such as TLRs (73). Upon recognition, these epithelial cells produce chemokines and cytokines (pro- and anti-inflammatory, depending on the nature of the antigen) needed to orchestrate a protective immune response by cells of the gut-associated lymphoid tissue, thus informing local immune cells of a potential breach in the gut barrier (73). However, it is important to note that the tremendous load of antigens and microbial products in the gut lumen requires IECs to be in a state of altered responsiveness to microbial products. Indeed, although a pro-inflammatory effect of PRR signalling was first evaluated in IECs, recent research, particularly in mice and humans, have been focusing on the contribution of these signalling pathways to gut homeostasis and immune tolerance (103)

3.2.3. Gut-Associated Lymphoid Tissue

The Gut-Associated Lymphoid Tissue (GALT) is one of the most complex immune tissues and protects the gastrointestinal tract from infections with enteric pathogens (122). The GALT consists of scattered immune cells positioned in the basolateral spaces between luminal IECs (intraepithelial lymphocytes) or dispersed in the lamina propria, immune cells organized in individualized follicles or in more complex structures named Peyer's patches (where antigen presentation occurs), and mesenteric lymph nodes (MLNs) (42, 73, 123). Peyer's patches are distributed in the jejunum and in the ileum. While in jejunum they are dispersed along the organ, functioning as inductor and effector sites, in the distal ileum there is a continuous Peyer's patch which is also considered a primary lymphoid organ, responsible for development and selection of B cells (42, 124). In the epithelium lining of Peyer's patches there are microfold (M) cells, which are highly specialized in sampling antigens from the lumen, through pinocytosis. These antigens are further processed by APCs and presented to T cells and also made available to B-

cells in the Peyer's patches (73, 125). Antigen sampling not only occurs through M cells. Subepithelial DCs and Mϕ can directly sample luminal contents by projection of transepithelial dendrites (TEDs), without compromising the integrity of the epithelial barrier, due to the expression of tight-junction proteins (occluding, claudin and zonula occludens 1) (126, 127). Intestinal dendritic cells can transport processed antigens along the lymphatic vessels to MLNs, where they present them to T cells that initiate clonal expansion. Sampled antigens can also reach the MLNs via lymphatics, captured by subcapsular Mϕ and transferred to the follicles, where they are bound by antigen-specific B cells (128). These activated B and T cells can then leave the MLNs via bloodstream and migrate to the lamina propria and Peyer's patches. B cells primed in the gut and MLNs predominantly produce IgA that is transcytosed into the intestinal lumen by IECs (42, 73, 103). Some of the activated/memory B and T lymphocytes express homing receptors that allow them to leave the GALT, enter the bloodstream, and migrate to other mucosal sites and also other tissues, a concept known as "common mucosal immune system" (73). Microbiome and microbial products activate innate immune cells in the gut through PRRs signalling and it is plausible it may also induce epigenetic and metabolic reprogramming in these cells and lead to increased responsiveness upon contact with subsequent antigens (105, 129). Reprogramming involves transcriptional changes. After recognition and activation of intracellular signalling cascades, different metabolic pathways are upregulated. Metabolites produced from these metabolic pathways influence the activity of enzymes involved in histone methylation and acetylation of genes of the innate immune responses (83). These chromatin modifications ease gene transcription in cells stimulated for the second time, leading to more rapid gene transcription and enhanced responses to those second triggers (83, 130). With respect to innate immune memory, particular important subsets are ILCs groups 1 to 3. However, ILCs have not been confirmed in cattle yet (73, 131), with the exception of NK-cells, belonging to group 1 ILCs (132). In human and mice, ILC3 mainly reside within tissues, particularly in mucosal surfaces, and have recently been proven to be "trained" and persist for months upon exposure to a pathogen. ILC3, devoided of PRR, can be activated by cytokines (namely IL-1β and IL-23) produced by epithelial and immune cells (133) and, when rechallenged, show enhanced IL-22 production, a cytokine involved in epithelial integrity maintenance and mucus production (134). They also show higher proliferative ability and increased ability to control infection (135). SCFA produced by the microbiome increase the activity of ILC3, which are important regulators of intestinal barrier both during homeostasis and in response to infection. These cells are also known to regulate the microbiota (136).

Intestinal microbiota and events in the gut may impact systemic immune responses

(137), and gut-liver, gut-lung and gut-brain axes, which have been well documented (138). Modulation of the immune system using a dietary approach may thus impact gut immunity and calf's health, preventing enteric and respiratory diseases. However, research is needed to understand the mechanistic links between nutrients, microbiome, and immune system.

3.3. Immunology of the Calf

3.3.1. Particularities of the calf's immune system

There is a general perception that "calves are born without immunity", but bovine immune system starts developing days after conception, progressing in small steps during foetal development and the first months of life, until it reaches mature levels at 5 to 8 months of age (139, 140). The calves are, in fact, born immunocompetent: they are able to mount an effective immune response to antigens, either to natural-occurring pathogens or vaccines (139). However, one particularity of the foetal development in ruminants, is its characteristic synepitheliochorial placentation which impairs the passage of macromolecules from the dam to the foetus (141, 142). The protective environment conferred by the dam during gestation protects them from contact with pathogens, but it also prevents the development of adaptive immunity or activation of innate immune mechanisms through interaction with antigens (19, 139). Additionally, the hormonal influence of calving compromises the performance of the calf's immune system during the first weeks of life, due to high concentrations of maternal and foetal cortisol, characteristic of this period and necessary for foetal development and maturation and for the dynamics of calving (139, 143).Hence, although immunocompetent, the newborn calf is practically agammaglobulinemic at birth (139). These general aspects mean calves mostly rely on adequate colostrum intake as the most important defence mechanism to face pathogens in their first weeks of life (19).

After birth, the immune system continues to develop, and only reaches full functional levels at 6 months old approximately (Figure 4). Circulating B-cells reach adult levels at 20 days old and production of immunoglobulins, namely IgA and IgG, reaches functionally significant levels at 16 to 32 days old. The activity of the complement system in 1 month old calves is still about 50% of the activity recorded in adult cattle, and although neutrophils in 7 day-old calves have phagocytic ability, it is only at 5 months old that functional levels reach those found in adults (139). Newborn calves have particularly high numbers of circulating gamma-delta (γδ) T cells (60% of the lymphocyte population), which is proposed to be a compensatory mechanism for the immature immune response (73, 139). Guzman *et al*., 2014 have presented evidence that γδT cells are the main

regulatory T cell subset in the bovine species (144). Bovine γδT cells secrete the anti-inflammatory cytokine IL-10, proliferate when in contact with APCs and inhibit proliferation of $CD4^+$ and $CD8^+$ cells (144). These cells may thus contribute to the control of exaggerated and detrimental immune response in the calf.

The GALT of the young calf also presents some particularities. Parsons *et al*. (1989) showed that B cells were significantly increased in the ileum of the calf, comparatively to adult animals. However, T-cells were decreased in all lymphoid areas and in the intestinal mucosa of the calf comparatively to adults (145). These particularities of the bovine immune development mean colostrum intake is, by far, the most important defence mechanism against pathogens in the first weeks after birth. But it also evidences an increased risk for disease in young calves until the immune system fully develops, even if colostrum management guarantees passive transfer of immunity. This will be addressed below in “Risk Factors in Adequately Managed Calves”.

3.3.2. Composition of Colostrum – Impact on the Immune System

Colostrum is secreted by the mammary gland in the final stage of gestation and in the first days after calving (146). It is rich in macro and micronutrients but also in immunological and biologically active compounds such as growth-factors, leukocytes, hormones, cytokines, and antimicrobial factors (lactoperoxidase, lysozyme, lactoferrin) (146-149). Colostrum is an important source of immunoglobulins that confer passive immunity and protect the newborn until its immune system is able to produce a quick and effective response against pathogens (149, 150). Indeed, colostrum may modulate immune response in the respiratory system and, thus, prevent respiratory disease (149), being commercially available as a dietary supplement and studied for prophylaxis and treatment of human digestive disorders such as inflammatory bowel disease (149).

Colostrum has high protein content (immunoglobulins, growth factors, cytokines, antimicrobial factors and casein) (146, 147). Immunoglobulins are the main component of the protein fraction, namely IgG1 (140, 147, 151). Caseins have also been reported to present immune modulating effects *in vitro* (152). Trypsin inhibitor is present in colostrum to protect immunoglobulins and other biologically important proteins from hydrolysis (147, 150) and diverse cytokines play a role in the modulation of the calf’s immune system (147). Growth factors stimulate gut growth and maturity, helping stablish gut permeability and a barrier effect quickly (147) and are anabolic compounds (153) that stimulate cell proliferation and differentiation (154), inhibit IECs apoptosis, thereby stimulating development and growth of intestinal mucosa and increased absorptive capacity by the newborn (147, 151, 155). Leukocytes, such as neutrophils, lymphocytes, and macrophages, in bovine colostrum are in very similar concentrations comparatively

to peripheral blood (139). According to previous studies, cell-mediated immunity may also be transferred to neonates through colostrum intake, along with immunoglobulins, to protect the newborn calf against antigens which the dams had previously responded to (150, 156). Colostrum has antimicrobial factors such as lactoferrin, an iron-binding glycoprotein displaying antimicrobial (157), immunomodulatory (158), and antioxidant (159) activities, and lysozyme, an antimicrobial enzyme that induces lysis of gram-positive and gram-negative bacteria. Crude fat percentage in colostrum is approximately twice that in whole milk, which helps in thermogenesis, critical in newborns (148, 150). Colostrum also contains fatty acids such as ω-3 and ω-6 polyunsaturated fatty acids, conjugated linoleic acid and short chain fatty acids (147). Colostrum is rich in vitamins A, E, B2, B9 (folic acid), vitamin B12 (cobalamin), choline and in minerals such as calcium, magnesium, iron, copper, manganese, phosphorus, zinc, and selenium, which are important to support immune function (147, 150). Bovine colostrum and bovine milk also contain micro RNAs (mRNAs) (160). It is suggested that milk mRNAs allow signalling from the mother to the calf, stimulate cell proliferation, differentiation and intestinal epithelial development (150, 161).

3.3.3. Failure of Passive Transfer (FPT)

Colostrum intake must occur very rapidly. During the first 24 hours of life, calf's intestinal epithelium absorbs dietary colostrum molecules non-selectively, by pinocytosis (150), with maximum absorption occurring in the first 4 to 6 hours after birth. Specific receptors for immunoglobulins, such as FcRn are absent in the calf's intestinal epithelium (19). The absorption process declines progressively and linearly immediately after birth (150), ceasing at 24-36 hours of life, in a process denominated by "gut closure" (162). This occurs due to the increased expression of tight-junction proteins (73). Although the mechanisms are not clear, it seems that stimulation of the gut by ingestion and digestion processes begins to change the population of epithelial cells (140). It is also proposed that establishment of microbiome in the intestinal mucosa and the interaction between them may play a role in the process (163). There is also a transition from non-proteolytic to proteolytic processes in the gastrointestinal epithelium, and intestinal crypt cells begin to express FcRn to export IgG1 from the circulation into the gut to protect the calf from enteric pathogens (162).

Hence, colostrum is a source of immunoglobulins and memory cells, allowing calves to rapidly contain infections. However, transfer of passive immunity is only effective when colostrum intake occurs adequately. Research has identified 5 major aspects to consider regarding colostrum management: quantity, quality, quickness, cleanliness and quantification. A conventional suggestion is to feed the calf with approximately 10% to

12% of its birth weight (150). However, there is not a thumb rule in terms of volume, but rather a requirement regarding quantity of IgG ingested (concentration of IgG plus volume administered) to aim at least 10 mg/mL of serum IgG in the newborn. This means that, in average, a 45 kg calf should ingest at least 160 g of IgG in the first feeding (163). Calves that were adequately fed colostrum had lower rates of respiratory disease (164). It is also reported that calves with failure of passive transfer of immunity present 4.6 times higher risk of mortality compared to calves with adequate transfer of immunity (143). Although progresses have been made regarding colostrum management (165), it seems that failure of passive transfer of immunity still affects between 40 to 50% of the calves (30, 166-168). This highlights the need of improving management practices and pinpoints the importance of adopting strategies to enhance calf's immune system, namely by dietary supplementation.

3.3.4. Risk Factors in Adequately Managed Calves

There is always a risk for disease in well managed calves due to the particularities of the immune development. Even though passive immunity plays a key role in the first 2 to 4 weeks of life (139), active immunity takes time to develop (28). Hence, there is a period of greater susceptibility that results from decreased levels of circulating maternal immunoglobulins and maternal immune cells before the calf is able to mount a self-sufficient immune response (Figure 4). This is when calves are at most risk for enteric disease (28). Another factor potentiating the incidence of disease in well managed calves is the circulating cortisol concentrations around calving and after stressor events, which may affect immune responses (19, 28). This hormone is typically described as anti-inflammatory, and known to depress immune function, although high persisting levels of cortisol may result in enhanced inflammation in response to acute stress (169, 170). Farm procedures and management operations may also induce stress and contribute to cortisol release and immune depression in young animals, namely transportation, castration, dehorning, regrouping, and weaning (progressive withdrawal of milk or milk replacer) (28). It was also reported that heat stress impairs immune development in pre-weaned calves (171). Additionally, redox balance, namely the exposition to oxidative stress that overwhelms the antioxidant capacity, may play a major role on the susceptibility to disease. (172). Colostrum is an unavoidable source of reactive-oxygen species (ROS) and, together with endogenous production of ROS by the calf, that increases in the first days of life, may overload calf's antioxidant capacity and lead to oxidative stress. In a study performed a few years ago, it was reported that colostrum redox balance significantly impacted the oxidative status and transfer of passive immunity in calves (172). Recently, a study reported that calves exposed to high

oxidative stress in their first month of life had higher plasma IL-4 and lower plasma interferon gamma (IFN-γ). Circulating peripheral blood mononuclear cells (PBMCs) presented higher *IL4* and *IL10* mRNA expression and lower *IFNG* and *IL2* expression (173). *In vitro*, it was demonstrated that lymphocyte activation, production of immunoglobulins as well as protein and gene expression of key cytokines were altered according to redox balance of colostrum (173). Further studies are still needed to understand the effects of colostrum redox balance and oxidative stress on immune responses of calves. It is hence possible that management is performed accordingly and outbreaks of enteric or respiratory disease occur. Modulation of calf's immune system through dietary supplementation may thus be helpful in preventing disease.

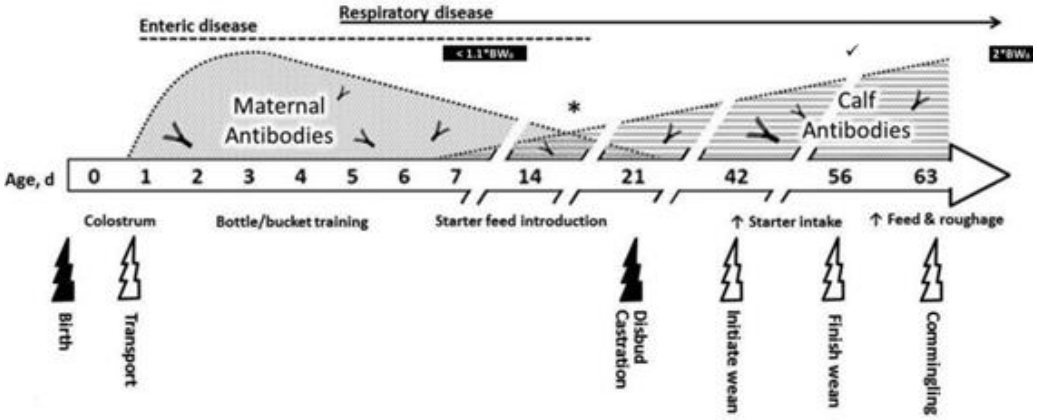

Figure 4 – Immune development and susceptibility in the first weeks of life. Passive immunity is key for preventing disease in young calves, however, there is a period of greater susceptibility that derives from the decreased maternal antibodies and immune cells before active immunity is fully functional. This period usually overlaps management procedures and interventions that may depress calf's immune system, predisposing to disease. Reprinted with permission from (28).

4. Dietary Modulation of Calf's Immune System

4.1. Development of the digestive system

At birth, calves are physiologically non-ruminants. Digestive physiology of the calf, from birth to weaning, is extremely complex, and it develops progressively in three phases: the pre-ruminant phase, from birth to 2-3 weeks old; the transition phase, from 3 weeks old until weaning; and the ruminant phase (174).

In the pre-ruminant phase, calves depend on milk intake to meet nutritional requirements. At this stage the reticulum-rumen and omasum are underdeveloped (175), but reticulum-rumen microbiota colonization occurs at birth, from the vaginal canal, faecal material, bedding and environment, colostrum, skin, and saliva from the dam (106). As the calf

begins to eat increasing amounts of starter feed, it enters the transition phase. During this transition phase, the reticulum-rumen and its microbial ecosystem develop, as the readily fermentable (non-structural) carbohydrates are fermented to SCFA, mainly butyric and propionic acids. These SCFA (particularly butyric), promote the development of ruminal epithelium into the characteristic stratified squamous. Surface area is thus increased by development of ruminal papillae, to enhance SCFA absorption (106). Reticulum-rumen volume and musculature develop as the calf ingest increasing amounts of starter and fibrous feed during the transition period (176). When these feeds cover nutritional requirements, the animal can be weaned without significant growth slumps (177). After weaning, the reticulum-rumen is fully functional and, from this point on, the calf is functionally and physiologically a ruminant, with the ability to meet their nutritional requirements for maintenance and growth through rumen microbiota fermentation of solid feed, namely grass, forage and compound feed (177), similarly to adult ruminants.

4.2. Milk and Immune Function

Calves are particularly susceptible to diseases during the pre-ruminant and transition phases, although milk and milk replacers can be good sources of bioactive compounds with immunomodulatory properties, such as polyunsaturated fatty acids (PUFA), organic minerals and vitamins. Milk fatty acid profile may affect innate and adaptive immune functions through modification of immune cell membranes, which can affect membrane fluidity and cell function (178, 179). It was reported that differences in dietary PUFA have an impact on the formation of membrane lipid rafts, which are specialized glycolipoprotein microdomains that affect membrane protein trafficking and immune cell signalling (178, 180). Long-chain ω-3 PUFA, such as eicosapentaenoic acid (EPA) and docosahexaenoic (DHA), have anti-inflammatory properties through inhibition of the NF-κB pathway. Conversely, saturated fatty acids (such as lauric, myristic, and palmitic) seem to activate NF-κB pathway through TLR-4 signalling (179). Hence, fatty acid profile may modulate PRR-mediated inflammation and the inflammatory response (179, 181). Fatty acid composition also influences the production of oxylipids, which are synthesised from linoleic and arachidonic acids (ω-6 PUFA) or EPA and DHA (ω-3 PUFA), that modulate the immune response (178). Oxylipids derived from ω-6 PUFA, such as prostaglandins, leukotrienes and thromboxanes are usually proinflammatory and those derived from ω-3 PUFA tend to be anti-inflammatory (182). Milk micronutrients also affect immune function mainly through their antioxidant properties (179). Copper, selenium, cobalt, and vitamin E deficiencies affect immune cell activity. Copper also affects antibody and TNF production. Vitamin E is a potent antioxidant but it also affects cell

membrane integrity, cell signalling and cell proliferation (183), and selenium regulates immune function through the activity of selenoproteins, such as glutathione peroxidase, that controls excessive production of free radicals (184), selenoprotein S, that regulates cytokine production (185), and selenoprotein P, that transports selenium, thereby regulating its homeostasis (186).

4.2. Dietary supplementation

Nutraceutical supplementation of milk replacer and starter feed is an effective strategy to promote calf's gut immune maturation and decrease morbidity and mortality (187-189). Probiotics, prebiotics and synbiotics are commonly used as dietary supplements, although the mechanistic mode of action remains greatly unclear.

4.2.1. Probiotics

Probiotics are cultures of live microorganisms, described to enhance the development of a healthy microbiome by preventing colonization of the gut by enteric pathogens, while improving digestive efficiency, lowering the pH, and improving local immunity by directly interacting with enterocytes, M-cells and intraepithelial innate immune cells such as DCs (122, 126, 190, 191). There is evidence that probiotics enhance regulation of intestinal mucosal immune responses by stimulating the production of signalling molecules and antimicrobial compounds by IECs, increasing dendritic cell-induced T cell hypo-responsiveness, and regulating TLR, NLR and signalling pathways (122, 191, 192). It is worth pinpointing that besides its immunomodulating properties, probiotics promote gut health directly by competing with pathogenic bacteria for nutrient resources, and indirectly by producing compounds such as lactic acid, SCFA and bacteriocins which prevent proliferation and activity of pathogenic species (191, 193). Moreover, probiotic effects may extend to other locations, such as the respiratory system and even systemically (194, 195). Different probiotics may present distinct immunomodulatory effects, inducing different cytokine production profiles, depending on the strains used (196). Thus, probiotics may help regulate immune responses and maintain homeostasis through the induction of pro- and anti-inflammatory cytokines, balancing the effector and regulatory responses of T cells (197-199).

Most probiotics studied for animal supplementation belong to *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Propionibacterium*, *Bacillus* and *Saccharomyces* (200, 201). The most used probiotics in young calves are strains of *Lactobacillus* and *Bacillus*. Probiotics supplementation in newborn calves is a common strategy used on-farm shortly after birth, to stabilize gut microbiome and prevent

adherence of pathogenic microorganisms to the mucosa. However, despite its use, it is still unclear the exact mechanisms by which probiotics interact with the whole microbiome and with the immune system (190, 191). In addition, probiotic effects may differ according to microorganisms and strains composition, animal microbiome composition and health status, and interactions between these factors (191).

4.2.2. Prebiotics

Prebiotics are compounds not absorbed by the animal that can be used as a substrate by the intestinal microbiome, stimulating the growth and activity of commensal bacteria and promoting local and systemic immune responses with benefit to the host (190, 202). The most commonly used prebiotics are mannanoligosaccharides or mannan oligosaccharides (MOS), fructooligosacharides (FOS), galactooligosacharides (GOS), xylooligosaccharides (XOS), glucans and yeast-cell wall (98, 122, 190). MOS, FOS, and β-glucans are, in fact, called immunosaccharides by some authors, due to their particular immunostimulant properties (203). Mechanisms of action include prevention of pathogen bacteria binding to mucosal sites, nourishment of the commensal microbiome, and activation of innate immunity by interaction with PRRs on the surface of IECs and antigen-presenting cells such as DCs and Mϕ. Thus, local immune effects include enhanced cytokine and ROS production, neutrophil recruitment and migration, increased phagocytic activity and lymphocyte proliferation (122, 202, 204-206). When included in the milk, prebiotics surpass the reticulum-rumen through the oesophageal groove (190) and are fermented by the intestine microbiome to SCFA, such as acetic, butyric, and propionic acids, which lower the pH, nourish the intestinal epithelium and function as mediators of the intestinal immune response of young calves (207, 208). Although small effects can be observed in healthy calves (190), supplementation with prebiotics may stabilize the intestinal microbiome and, upon an immune challenge, prevent the incidence of disease (190). After weaning, prebiotics are usually fermented by the ruminal microbiome and may fail to reach the intestinal lumen to exert its effects. Nevertheless, administration to weaned calves may be advantageous if it enhances the development of a desirable ruminal and/or intestinal microbiome (190).
Despite goals and overall effects of prebiotic supplementation being similar, composition and mechanisms underlying the mode of action of each one of them may be different.

4.2.2.1. Yeast-cell walls

Yeasts have long been used in animal feeding, firstly as sources of proteins and group B vitamins, and more recently as feed additives with immunomodulating activities (209). These supplements are mainly obtained from *Saccharomyces cerevisiae* (210), and their

immune effects are mostly attributed to the cell wall composition: approximately 50% β-1,3-glucans, 40% mannoproteins, 10% β-1,6-glucans and 2% chitin (209, 211). Yeast-cell wall directly interact with components of the immune system, such as β-glucan receptors (212), and induces the expression of pro-inflammatory cytokines, including *IL8*, by immune cells (213). Some authors reported yeast-cell wall supplementation was able to mitigate deleterious effects of the acute phase response upon LPS challenge (214, 215), and to improve health and probability of recovery of cattle exposed to stress (216, 217).

4.2.2.2. β-glucans

Yeast-derived β-glucans are probably one of the most promising and studied dietary supplements with immunomodulatory properties (205). These oligosaccharides are composed of D-glucose monomers, linked by β-1,3 glycosidic bonds with side chains of D-glucose attached by (1,6)-β linkages (218). β-glucans are known to increase, *in vitro*, phagocytosis, lymphocyte proliferation, cytokine production, and expression of co-stimulatory molecules such as CD80/CD86 through Dectin-1 signalling (219, 220). β-glucans are being extensively studied due to their ability to induce trained immunity in innate immune cells, enabling them to respond better to second, non-related stimuli (221, 222). It was also reported that β-glucans may increase the density of intraepithelial lymphocytes in the intestine (223). These compounds are recognized by innate immune cells mainly through Dectin-1 receptor (224), however, mechanisms of response to β-glucans in bovine innate immune cells are not fully elucidated.

4.2.2.3. Mannan oligosaccharides

Mannan oligosaccharides are composed of mannose units and are obtained by partial hydrolysis of mannan polysaccharides (225). These can be of fungal or vegetable origin. Studied MOS are mostly from *S. cerevisiae* cell walls (226). Mechanisms proposed to explain the beneficial activity of MOS include increased serum immunoglobulin production upon infection, enhanced phagocytosis, and improved complement system activity (227-229). MOS also provide alternative binding sites for pathogenic microorganisms, namely gram-negative bacteria, that bind to D-mannose residues in the intestinal epithelium through type-1 fimbriae (230, 231). Some authors also proposed that MOS improve villous height to crypt depth ratio, suggesting a greater absorption by supplemented animals (232-234), and increased production of IgA in the gut (235).

4.2.2.4. Fructooligosaccharides and inulin

Fructooligosaccharides and inulin are fructose polymers linked to a glucose residue. While FOS are composed of 3 to 10 units, inulin may have higher degree of

polymerization, of up to 60 fructose units (236). Inulin is mainly extracted from chicory roots, and most FOS are obtained from inulin hydrolysis (237). These are highly soluble fibres, which are fermented by the gut microbiota, leading to increased concentration of butyric, acetic and lactic acids (238). The latter may be further converted to butyric acid, which besides being used as an energy source by the intestinal epithelial cells, also has immunomodulating properties (239, 240). However, the extent of fermentation greatly depends on the degree of polymerization (236, 241). By stimulating the growth of *lactobacilli* and *bifidobacteria*, FOS decrease faecal pH and serum cholesterol, increase IgA secretion, and have been suggested to improve mineral absorption (242, 243). Furthermore, inulin fermentation products were reported to induce the upregulation of genes encoding tight and adherens junction components, suggesting metabolites produced reinforce barrier function, as observed on an intestinal porcine enterocyte cell line (241).

4.2.2.5. Galactooligosaccharides

Galactooligosaccharides are soluble carbohydrates, mainly synthesized from bovine milk-derived lactose by enzymatic treatment (244). They are very similar to oligosaccharides found in human breast milk and are often added to formula milk to promote infant's microflora and immune system (245, 246). Similarly, inclusion of GOS in milk-fed calves may promote growth performance and health benefits, namely increased average daily gain (ADG), body weight, feed efficiency, total serum proteins and reduced the incidence of diarrhoea (247). In another study, calves fed GOS-enriched milk replacer had higher relative abundance of *Lactobacillus* and *Bifidobacterium* in colon contents, and more developed intestinal epithelial structures (208). The growth of these beneficial bacteria in the gut help controlling the proliferation of pathogenic microorganisms by competing for nutrients, adhesion sites and promote the production of SCFA, mainly butyric acid, as well as antimicrobial compounds (244, 248).

4.2.2.6. Xylooligosaccharides

Xylooligosaccharides are prebiotics composed of xylose units that occur naturally in some fruits, vegetables, honey among other feedstocks (249). Commercial XOS are mostly extracted from lignocellulosic biomass, such as corn cobs, bagasses, straws, hardwoods and hulls, either by enzymatic or chemical processes (249, 250). XOS selectively enhance the growth of beneficial bacteria in the gut, namely *bifidobacteria*, *lactobacilli* and *eubacteria* (251, 252), thereby restraining growth of pathogenic bacteria, namely through the production of SCFA (253). The use of XOS in young calves is still in its infancy, but its use in monogastric livestock has been reported as promising.

Supplementation of XOS to broilers fed a conventional corn-soybean meal, induced higher feed conversion ratios and decreased duodenal crypt depth. However, no effect was observed in serum immunoglobulin titres against Newcastle disease and H5N1, nor in blood T-lymphocyte proliferation (254). Similarly, 30-days old piglets supplemented with XOS showed increased growth performances, antioxidant capacity, serum immunoglobulins and IL-10 while serum IL-1β was decreased (255). In another study, piglets fed XOS in their diets had decreased episodes of diarrhoea and increased serum IgA, intestinal villus height and antioxidase activity (256). Other authors also reported antioxidant properties for XOS (257), while others described XOS to be able to control hyperglycemia, hyperlipidemia and oxidative stress induced by high-fat diets in rats (258).

4.2.2.7. Microalgae

Microalgae are a highly diverse group of ubiquitous prokaryotic and eukaryotic photosynthetic microorganism, composed of over 40,000 strains (259). Microalgae have been used as dietary ingredients for some centuries. However, only recently has the large-scale production allowed higher availability and diversity of microalgae as an ingredient, food or feed supplement (260). When supplemented as dry biomass, microalgae can be considered as prebiotics (261, 262).

Microalgae are valuable sources of nutrients and bioactive compounds, including proteins, PUFA, organic minerals, vitamins, antioxidants, and polysaccharides (263). The immunomodulatory potential of some of these constituents has leverage the interest of microalgae in calf's diet. These include polysaccharides, namely β-glucans, which exert immunomodulatory effects (264), proteins and bioactive peptides released during digestion of proteins (264, 265), PUFA such as EPA and DHA (266, 267), pigments like astaxanthin, with potent anti-inflammatory and antioxidant activities (264), antioxidants, such as carotenoids and phenolic compounds, important to maintain a balanced immune response, since they protect host cells from oxidative stress (263), and organic minerals and vitamins (267). Furthermore, microalgae could be an interesting source of antibacterial, antifungal, and antiviral substances (268). Antimicrobial activities may be due to halogenated compounds, terpenes, phenols and polysaccharides present in some microalgae species (265). However, microalgae constituents vary greatly between species and accordingly to growth stage and culture conditions (263, 269), which may impact on their immunomodulatory effects.

4.2.3. Organic Acids

Organic acids are weak carboxylic acids such as acetic, propionic, butyric, lactic, formic,

citric, fumaric, and sorbic acids (270). These compounds have antimicrobial properties and are thus used in poultry and swine feed as preservatives (271, 272), but also have beneficial effects by decreasing gut pH, improving nutrient digestibility, mitigating pathogen growth and enhancing local immune response, namely by increasing populations of intestinal γδ T cells (270, 272-274). In calves, the main organic acid used is butyric, in the form of sodium butyrate, which has been shown to improve growth and feed conversion rates (275, 276).

4.2.4. Phytonutrients

Phytonutrients are a vast group of plant extracts that include polyphenols, carotenoids, resveratrol, terpenoids and essential oils, such as cinnamaldehyde, eugenol, among others (277). These compounds induce identical immune responses, although its chemical structures are significantly different (277). Polyphenols, such as flavones, flavonols and proanthocyandins, exert their activity through its potent antioxidant activities, but they also increase serum immunoglobulins, modulate immune response by supressing inflammatory cytokines, and improve intestinal barrier function (277-279). Proanthocyandins were suggested to neutralize enterotoxins *in vitro* (280), to present antimicrobial properties (as observed in the control of enteric infections in piglets and coccidia in small ruminants) and to modulate gut microbiota (281-283).

4.2.5. Synbiotics

Synbiotics contain both probiotic, such as *bifidobacteria*, *lactobacilli, enterococci,* and *S. cerevisiae var boulardii,* and prebiotic compounds, namely FOS, inulin, MOS, GOS and XOS (284, 285). A synergy effect between these compounds occur, in which the prebiotic fraction increases the stability of probiotics by favouring the survival and growth of the viable microorganisms along the gastrointestinal tract (286). Hence, commercial synbiotics are commonly used in calves' feeding, such as combination of *Enterococcus faecium* and FOS (284), although the mechanistic effects remain to be identified.

5. Aims and Thesis Outline

This thesis emerged from the need to address particular challenges in rearing calves, namely of infectious origin, such as neonatal diarrhoea and pneumonia. Despite morbidity and mortality rates being extremely variable worldwide, they are generally quite high and may compromise not only calf's health and welfare, but also the economic sustainability of the farms. Besides, treatment protocols for diseased calves frequently include antimicrobial drugs, which pose a risk for the emergence of antibiotic-resistant bacteria.

Adequate management is of paramount importance to prevent disease in newborn calves. However, even in well-managed farms, there is always a higher risk of infection in the first weeks of life, due to particularities of the immune development in ruminants. The hypothesis behind this thesis work plan is based on the evidence that dietary supplementation may be a sustainable strategy to modulate the immune system and increase resistance to infection. Even though some dietary supplements are currently used to improve fitness and resistance to disease, namely in the bovine species, most mechanisms of action are not yet clarified.

Hence, our main goals were to:

1. Evaluate and mechanistically characterize the *in vitro* effects of different supplements, such as yeast-derived products and microalgae, on bovine innate immune cells and on intestinal epithelial cell lines;
2. Conceptualize and design a comprehensive intestinal *in vitro* model for dietary compounds screening that could provide more translational results than the simpler available *in vitro* models;
3. Evaluate the immune function and health of newborn calves supplemented with the most promising compounds, selected according to previously obtained results.

This thesis is organized in 6 chapters:

- Chapter I presents a review of the current knowledge regarding calf health and welfare, bovine immune development, calf nutrition, and dietary strategies to enhance immune function.

- Chapter II addresses the mechanisms of yeast-derived β-glucan recognition by bovine peripheral blood monocytes, and the resulting inflammatory response, with a particular focus on the Dectin-1 receptor.

- Chapter III covers the pro-inflammatory response and antimicrobial mechanisms of bovine peripheral blood monocyte-derived macrophages to *in vitro* digested microalgae *Chlorella vulgaris*, *Nannochloropsis oceanica* and *Tetraselmis* sp.

- Chapter IV addresses the metabolic activity and oxidative status of human intestinal epithelial co-cultures, namely Caco-2 and HT29-MTX, cultured with digested microalgae and proposes a novel intestinal *in vitro* model, using these intestinal epithelial cell lines and human B and monocyte cell lines (Raji B and THP-1 cells, respectively). This model of intestinal inflammation conjugates epithelial cells and immune cells and may be a helpful tool for screening of anti-inflammatory, antioxidant and immunomodulatory activity of dietary compounds.

- Chapter V presents the effects of milk replacer supplementation with *C. vulgaris* on immune parameters and health of Holstein-Friesian calves.

- Chapter VI consists of a general discussion, integrating all the information gathered in the Chapters II-V, and the concluding remarks.

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CHAPTER II

Dectin-1-Mediated Production of Pro-Inflammatory Cytokines Induced by Yeast β-Glucans in Bovine Monocytes

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Dectin-1-Mediated Production of Pro-Inflammatory Cytokines Induced by Yeast β-Glucans in Bovine Monocytes

Ana R. V. Pedro[1,2,3], Tânia Lima[1], Ricardo Fróis-Martins[1], Bárbara Leal[4,5], Isabel C. Ramos[6], Elisabete G. Martins[7,8,9], Ana R. J. Cabrita[3], António J. M. Fonseca[3], Margarida R. G. Maia[3], Manuel Vilanova[1,2] and Alexandra Correia[1,2,*]

[1] Immunobiology Group, i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal

[2] Laboratório de Imunologia, DIMFF, ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[3] LAQV, REQUIMTE, ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[4] Laboratório de Imunogenética, DPIM, ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[5] UMIB, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[6] Animal Nutrition Division, Cooperativa Agrícola de Vila do Conde, Vila do Conde, Portugal

[7] ADM Portugal, SA, Murtede, Portugal

[8] EPIUnit, Instituto de Saúde Pública, Universidade do Porto, Porto, Portugal

[9] Department of Veterinary Medicine, Escola Universitária Vasco da Gama, Coimbra, Portugal

* Corresponding author

Abstract

Yeast-derived products containing β-glucans have long been used as feed supplements in domesticated animals in an attempt to increase immunity. β-glucans are mainly recognized by the cell surface receptor CLEC7A, also designated Dectin-1. Although the immune mechanisms elicited through Dectin-1 activation have been studied in detail in mice and humans, they are poorly understood in other species. Here, we evaluated the response of bovine monocytes to soluble and particulate purified β-glucans, and also to Zymosan. Our results show that particulate, but not soluble β-glucans, can upregulate the surface expression of costimulatory molecules CD80 and CD86 on bovine monocytes. In addition, stimulated cells increased production of IL-8 and of *TNF, IL1B,* and *IL6* mRNA expression, in a dose-dependent manner, which correlated positively with *CLEC7A* gene expression. Production of IL-8 and *TNF* expression decreased significantly after *CLEC7A* knockdown using two different pairs of siRNAs. Overall, we demonstrated here that bovine monocytes respond to particulate β-glucans, through Dectin-1, by increasing the expression of pro-inflammatory cytokines. Our data support further studies in cattle on the induction of trained immunity using dietary β-glucans.

Keywords

Dectin-1, β-glucans, bovine, monocytes, cytokines, siRNA, CLEC7A

Introduction

Immune modulation by natural compounds has long been studied in domesticated animals such as poultry, fish, and livestock, to enhance immunity and improve animal welfare and wellbeing, ultimately reducing the incidence of disease and the overuse of pharmaceutical compounds, such as antibiotics. Dietary supplementation with yeasts (1-6) and yeast-derived compounds, such as mannan-oligosaccharides (MOS) and β-glucans (1, 4-7), is one of the most used strategies to enhance immunity in domesticated animals. β-glucans are naturally occurring polymers present in the cell wall of fungi, bacteria, algae, and plants. Yeast β-glucans are usually composed of linear molecules of D-glucose units linked by β-1,3 glycosidic bonds with β-1,6 branching (8, 9). β-glucans are recognized by immune cell surface pattern recognition receptors (PRR) such as C-type lectin domain family 7 member A (CLEC7A), also designated as Dectin-1, complement receptor-3 (CR3), scavenger receptors, and lactosylceramide (10). In addition, Toll-like receptor (TLR)-2 and TLR-6 can synergistically contribute to the recognition and elicited biological effects of particulate β-glucans, such as Zymosan (11-

14). Biological activities of β-glucans depend on their recognition and downstream cell signalling, which in turn depend largely on the structure, conformation, and physical properties of the different β-glucans (9, 15). Although both particulate and soluble β-glucans bind Dectin-1, only the particulate form can induce Dectin-1 signalling and generate a “phagocytic synapse” (16). Activation of Dectin-1 triggers an intracellular signalling cascade eliciting phagocytosis, production of cytokines, and reactive-oxygen species (ROS) (14-19).

The immune recognition of β-glucans and elicited response has been extensively studied in mice and humans at mechanistic level (15, 20, 21). However, in other species, including cattle, the effects of β-glucans on the immune system are mainly supported by observational reports and *in vivo* studies (22-25). A homologous transcript for human Dectin-1 has been described in bovines (boDectin-1) (26). However, β-glucan recognition and its effects on bovine leukocytes were not fully elucidated.

Bovine monocytes express *CLEC7A* (26) and this cell type is the most used in innate immune memory studies in other species (27, 28). Here, the response of bovine monocytes to soluble and particulate β-glucans, and to β-glucan-containing particles (Zymosan) was assessed. Our results show that only the particulate β-glucan forms trigger the production of pro-inflammatory cytokines, and implicate boDectin-1 in this effect.

Material and Methods

Isolation of bovine peripheral blood monocytes

Bovine blood from Holstein-Friesian cattle was obtained at a local commercial slaughterhouse (PEC Nordeste – Indústria de Produtos Pecuários do Norte, Penafiel, Portugal) and licensed by National competent authority, Direção Geral de Alimentação e Veterinária, under a by-product handling authorization (N.12.006.UDER). There was no intervention on the animals for research purposes, since blood was collected during bleeding/slaughter of animals for human consumption. Blood was collected from jugular and carotid veins to BD Vacutainer® lithium heparin tubes (BD, Franklin Lakes, NJ, US) and peripheral blood $CD14^+$ monocytes were obtained as previously described, with minor modifications (29). Briefly, whole blood was diluted 1:2 with Dulbecco's phosphate-buffered saline (DPBS) and density gradient centrifuged on Histopaque®-1077 (both from Sigma-Aldrich) at 1200 × g for 15 min in SepMate™ PBMC isolation tubes (Stemcell™ Technologies, Vancouver, BC, Canada). Peripheral blood mononuclear cells (PBMC) were then washed with DPBS by centrifugation at 400 × g for 10 min and $CD14^+$ cells

were isolated with anti-human CD14 MicroBeads, according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Peripheral blood $CD14^+$ monocytes were washed with DPBS by centrifugation at 300 × g for 10 min and resuspended at 2 × 10^6 cells/mL in complete RPMI medium - RPMI-1640 Medium (Sigma-Aldrich) supplemented with 10% FBS (Biowest, Nuaillé, France), 50 µM β-mercaptoethanol (Merck, Darmstadt, Germany), 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mM L-glutamine and 10 mM HEPES (all from Sigma-Aldrich). Purity of $CD14^+$ cells exceeded 90%, as evaluated by flow cytometry using an anti-sheep CD14 mAb (clone VPM65, Bio-Rad, Hercules, CA, US) that cross reacts with bovine, conjugated with DyLight® 405 Conjugation Kit (Abcam, Cambridge, UK).

Cell culture and stimulation with β-glucans

Bovine $CD14^+$ monocytes were plated at 2 × 10^5 cells/well in flat-bottom 96-well culture plates. Stimulation was done with a pure soluble β-glucan preparation (WGP®-Soluble), a purified insoluble preparation of *Saccharomyces cerevisiae* lacking TLR activity, composed mainly of β-1,3-glucans (WGP®-Dispersible), and an insoluble preparation of *S. cerevisiae* cell wall (Zymosan), described to have Dectin-1- and TLR2/6-stimulatory activity (all from InvivoGen, San Diego, CA, US). β-glucans, labelled endotoxin-free (endotoxin level below 0.001 EU/µg), were prepared according to manufacturer's instructions. All assays were performed using sterile, pyrogen-free material. Cells were cultured with 10, 50 and 100 µg/mL of WGP-Soluble, WGP-Dispersible or Zymosan. Cells cultured with 1 µg/mL of *Escherichia coli* lipopolysaccharide (LPS, strain O111:B4; Sigma-Aldrich) or Pam3CSK4 (P3C; InvivoGen) were used as positive controls. A kinetic cytokine mRNA expression analysis was performed in cells cultured for 8, 16, and 24 h at 37 °C and 5% CO_2 to define the time point for sequent analyses. Cytokine production, mRNA expression, and lactate dehydrogenase (LDH) release were assessed in cells cultured for 24 h at 37 °C and 5% CO_2. Cell surface activation markers were assessed by flow cytometry in 8 and 16 h bovine monocyte cultures. Non-stimulated cells were always used as negative controls.

HEK-Blue™ hDectin-1b reporter cell line culture and stimulation with β-glucans

Hek-Blue hDectin-1b cells (InvivoGen) were grown in DMEM medium with 4.5 g/L glucose (Sigma-Aldrich), 10% heat inactivated Fetal Bovine Serum Premium (FBS) (Biowest), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all from Sigma-Aldrich), 100 µg/mL Normocin™ and 1 µg/mL puromycin (both from InvivoGen) in vented T75 flasks. When cells reached 80% confluency, they were re-seeded at 5 ×

10^4 cells/well in flat-bottom 96 well-culture plates and stimulated with 10, 50 and 100 µg/mL of WGP®-Soluble, WGP®-Dispersible, or Zymosan in HEK-Blue™ Detection medium for 16 h. Substrate hydrolysis by secreted alkaline phosphatase (SEAP), upon activation of the receptor, was assessed at 620-655 nm according to manufacturer's instructions in a BioteK™ µQuant Microplate Reader using Biotek™ Gen5™ Data Collection and Analysis Software (Thermo Fisher Scientific, Waltham, MA, US).

Cell viability assays

LDH release was quantified in cell culture supernatants using CyQUANT™ LDH Cytotoxicity Assay kit, according to manufacturer's instructions (Invitrogen, Waltham, MA, US).

Cytokine production

Cytokine levels were assessed in cell culture supernatants by sandwich ELISA. Assessment of bovine Tumor Necrosis Factor Alpha (TNF-α) and bovine Interleukin (IL)-6 was done using Bovine TNF-alpha and Bovine IL-6 DuoSet ELISA kits (R&D Systems, Minneapolis, MN, US) according to the manufacturer's protocol with a minor modification: 1% molecular grade bovine serum albumin (BSA, Albumine Bovine Fraction V, NZYTech, Lisbon, Portugal) in DPBS was used as reagent diluent, instead of 5% Tween 20 in DPBS. Bovine IL-8 (Bovine IL-8 [CXCL8] ELISA development kit, Mabtech AB, Nacka Strand, Sweden) was quantified according to manufacturer's instructions. Detection limits: 8 pg/mL for IL-8 and 125 pg/mL for TNF-α and IL-6. Bovine IL-1β was quantified using the IL-1 beta Bovine Uncoated ELISA Kit (Invitrogen), according to manufacturer's instructions. Detection limit <31.3 pg/mL. Bovine IL-10 was assessed in cell culture supernatants using an in-house ELISA kit, following the standard procedure of Mabtech ELISA Bovine IL-8 kit. Briefly, Nunc Maxisorp™ plates were coated with 1 µg/mL anti-bovine IL-10 mAb (clone CC318; Bio-Rad) in PBS and incubated overnight at 4 ºC. A nine-point standard curve from 2000 to 8 pg/mL was done using Recombinant Bovine Interleukin-10 (Bio-Rad). Cell supernatants and standards were incubated at room temperature for 2 h, followed by incubation for 1 h with the detection antibody at 0.5 µg/mL (mouse anti-Bovine Interleukin-10:Biotin; clone CC320; Bio-Rad) and 1 h with Mabtech's streptavidin-HRP, according to manufacturer's instructions. Detection limit: 8 pg/mL. Only samples above detection limits were used for comparison.

Bovine Dectin-1 knockdown assays (small interference RNA)

The following small interfering RNAs (siRNA) were designed by Custom siRNA Design

Service (Merck) to target both isoforms of bovine Dectin-1 and achieve the knockdown of this receptor: siRNA #1 sense AUG AAG AUG GAU AUA CUC A dTdT, antisense UGA GUA UAU CCA UCU UCA U dTdT; siRNA #2 sense UGA GGA UAG CUG UUA UCU A dTdT, antisense UAG AUA ACA GCU AUC CUC A dTdT; siRNA #3 sense GAG GAU AGC UGU UAU CUA U dTdT, antisense AUA GAU AAC AGC UAU CCU C dTdT (all from Sigma-Aldrich). Transfection procedure was performed for 4 h in serum-free X-VIVO™ 15 haematopoietic medium (Lonza, Basel, Switzerland) with ScreenFect®siRNA transfection reagent, according to manufacturer's instructions (ScreenFect GmbH, Eggenstein-Leopoldshafen, Germany), and 300 nM of siRNA duplexes or siRNA negative control (MISSION® siRNA Universal Negative Control #1, Sigma-Aldrich). After transfection, cells were washed with non-supplemented RPMI-1640 medium and incubated for 24 h at 37 °C and 5% CO2 with WGP®-Soluble, WGP®-Dispersible and Zymosan® at 50 µg/mL in RPMI medium or with medium alone. Supernatants were collected to assess IL-8 production and cells were preserved in NZYol reagent (NZYTech, Lisboa, Portugal) to assess *CLEC7A* mRNA expression.

RNA extraction and cDNA synthesis

Total RNA was obtained using NZYol according to manufacturer's protocol, with minor modifications. Bovine monocytes were lysed with 200 µL NZYol and incubated with 0.1 µg/mL RNA-grade Glycogen (Thermo Fisher Scientific). Each sample was incubated with 80 µL chloroform for phase separation and 200 µL isopropanol was added for RNA precipitation. The precipitated RNA was washed with 70% ethanol and resuspended in 5 µL RNase-free water. Synthesis of first-strand cDNA was done in an Applied Biosystems® 2720 Thermal Cycler (Thermo Fisher Scientific) at 25 °C for 10 min, 50 °C for 30 min, and 85 °C for 5 min using NZY First-Strand cDNA Synthesis Kit, according to manufacturer's instructions (NZYtech). Samples were kept at -20 °C. Negative controls using RNA samples for cDNA synthesis without reverse transcriptase (no RT control), and with no added template (no template control) were also included for all primer pairs.

Real-Time qPCR

Primers for β2 microglobulin (*B2M*), *CLEC7A*, *IL1B*, and *IL6* were designed using Primer-BLAST web tool developed by NCBI (30). Primers for *TNF* and *IL10* were previously designed (31, 32). Sequences of each primer and expected amplicon sizes are detailed in Table 1. Primers targeting *CLEC7A* were designed to both short and long isoforms. Determination of *TNF*, *IL6*, *IL10* and *CLEC7A* mRNA levels was performed in a CFX96™ Real-Time PCR Detection System (Bio-Rad), using NZYSpeedy qPCR Green Master

Mix (2×) ROX plus (NZYTech). *B2M* and MARVEL domain containing 1 (*MARVELD1*), already used as reference genes in bovine gene expression studies, were used for mRNA normalization (33-35). Reaction was performed in low profile, non-skirted, 96-well PCR plates (Thermo Fisher Scientific) containing 5 µL Master Mix, 1 µL cDNA, 3.6 µL H2O and 0.2 mM of specific forward and reverse primers (all from Sigma-Aldrich). PCR program was as follows: denaturation for 5 min at 95 °C followed by 40 cycles at 95 °C for 5 s and 62 °C for 20 s for amplification. Gene expression values were analyzed by the comparative threshold cycle method using the formula 2-(CT gene of interest-CT housekeeping gene) (36). *CLEC7A* PCR products were run in 1.5% (w/v) Tris-acetate-EDTA (TAE) agarose gel electrophoresis to confirm amplicon size. Bands were visualized in a Syngene™ NuGenius Gel Documentation System, excised from the gel and purified using NZYGelpure columns (NZYTech) following manufacturer's instructions. PCR products and DNA purified from excised gel bands were Sanger sequenced to confirm primer specificity.

Table 1 – List of primers used for quantitative real-time PCR.

Gene	**Primer[b] Sequence 5'-3'**	**Amplicon Size (bp)**	**GeneBank Accession Number or Reference**
CLEC7A[a]	F: TGCTGTGACTCTGGGCATTT R: CCAGTTAGGGGGACAAGAGC	235 Long 97 Short	AY937383.1 AY937382.1
TNF	F: CCAGAGGGAAGAGCAGTCCC R: TCGGCTACAACGTGGGCTAC	114	(33)
IL10	F: AGAACCACGGGCCTGACAT R: AGCTCACTGAAGACTCTCTTCACCTT	151	(34)
IL6	F: CCTGAAGCAAAAGATCGCAGA R: ATGCCCAGGAACTACCACAA	204	NM_173923.2
MARVELD1	F: GGCCAGCTGTAAGATCATCACA R: TCTGATCACAGACAGAGCACCAT	100	(36)
B2M	F: AAGTGGGATCGAGACCTGTAA R: GGACATGTAGCACCCAAGGTAA	191	NM_173893.3
IL1B	F: AAACTCCAGGACAGAGAGCAAAA R: CTCTCCTTGCACAAAGCTCATG	126	NM_174093.1

aAmplicon Size (bp) of short and long isoforms.
bPrimer direction: F, Forward, R, Reverse.

Flow Cytometry

Since the commercially existing labelled antibodies for the bovine species are available in limited fluorophore diversity, we conjugated mouse monoclonal anti-bovine MHC class II DR (clone CC108, Bio-Rad) antibody with peridinin-chlorophyll protein-cychrome 5.5 (PerCP-Cy5.5) with LYNX Rapid PerCP-Cy5.5 Antibody Conjugation Kit (Bio-Rad),

according to manufacturers' instructions, and used it at 1:200 to allow multiparametric simultaneous analysis with the monoclonal antibodies mouse anti-bovine CD80 conjugated with R-Phycoerythrin (RPE) (clone IL-A159, Bio-Rad) and mouse anti-bovine CD86 conjugated with Fluorescein isothiocyanate (FITC) (clone IL-A190, Bio-Rad), both used at 1:50. All the antibodies were previously titrated to determine the optimal concentration for bovine monocyte staining. A fixable viability dye (FVD) was included before surface antibody staining to exclude dead cells from the analysis. For that, cells were incubated with eFluor® 506 Fixable Viability Dye (eBioscience, San Diego, CA, US) diluted at 1:1000 in DPBS for 15 min at 4 °C. After washing cells with DPBS, cells were incubated in 2% mouse serum in FACS Buffer (1% BSA in DPBS) for 15 min at 4 °C in the dark before antibody staining to minimize nonspecific binding. A mix containing all antibodies was added to samples that were incubated for 25 min at 4 °C in the dark. Cells were washed with FACS Buffer and analyzed by flow cytometry. Single stainings using UltraComp beads (eBioscience) or/and cells were used for compensation. Fluorescence minus one controls (FMO) of each antibody and FVD were used for gating purposes. Data were acquired in a BD CantoII™ equipment (BD Biosciences) and analyzed with FlowJo version 10.6.2. (FlowJo LLC, Ashland, OR, US).

Bovine Dectin-1 staining

Bovine monocytes and Hek-Blue hDectin-1b cells were incubated with monoclonal mouse anti-Human Dectin-1/CLEC7A antibody (Clone 259931, R&D Systems) at 10 µg/mL for 1 h at 4 °C followed by incubation with the anti-mouse IgG (H+L) F(ab')2 Fragment conjugated with Alexa Fluor® 488 (Cell Signaling Technology, Danvers, MA, US) at 1:200 for 30 min at 4 °C in the dark. Cell staining was evaluated by flow cytometry in a BD CantoII™ cytometer (BD Biosciences) and analyzed with FlowJo version 10.6.2. Imaging of Dectin-1 on the surface of bovine monocytes and HEK-Blue™ hDectin-1b cells was done in cells stained as before. Nuclei were stained with DAPI and samples were plated in 8 well microscopy chamber plates (Ibidi, Gräfelfing, Germany) and observed in a laser scanning confocal microscope Leica TCS SP5 II system (Leica DMI6000-CS microscope with LAS AF Software, Leica Microsystems, Wetzlar, Germany). Images were obtained with a HC PL APO CS 40x /1.10 CORR Water objective. Cells were observed using 405 nm and 488 nm lasers, in the xy plane.

Statistical Analysis

Log transformations were applied to cytotoxicity, cytokine production, mRNA expression, siRNA assays, and flow cytometry data following a lognormal distribution. All data were

analyzed using the MIXED Procedure of the SAS software (Version 9.1, SAS Institute Inc., Carry, NC, US). The model included the fixed effect of treatment (Medium, WGP-Soluble, WGP-Dispersible, and Zymosan), the random effect of animal blood donor and the random residual error. The Tukey-Kramer's post-hoc test was used to compare means of cytotoxicity, cytokine production, cytokine mRNA expression, siRNA assays, and flow cytometry data, whereas for hDectin-1b activity in HEK-Blue™ hDectin-1b cells data were used the Dunnett's multiple comparisons test (SAS software). Cytokine levels or mRNA expression were expressed as Log fold changes to the respective values of control (Medium) samples. The Pearson correlations between bovine Log *CLEC7A* mRNA expression of non-stimulated samples and Log Fold change cytokine production or cytokine mRNA expression, for each stimulus, were estimated using the CORR procedure of the SAS software. Results were considered statistically significant if $P<0.05$ and a tendency if $0.05 \geq P<0.1$. Graphs were constructed with the GraphPad software (Version 9.0.2, San Diego, CA, US).

Results

HEK-Blue™ cell stimulation

The stimulatory effect of different commercial β-glucans or β-glucan-containing particles were tested in the HEK-Blue™ hDectin-1b reporter assay as a control prior to stimulation of bovine monocytes (Supplementary Figure 1). Soluble β-glucans did not activate hDectin-1b at any of the concentrations used, while dispersible β-glucans activated hDectin-1b at 50 and 100 µg/mL ($P<0.01$) comparatively to unstimulated cells. Zymosan significantly stimulated HEK-Blue™ hDectin-1b cells at all concentrations tested, 10 µg/mL ($P<0.001$), 50 µg/mL ($P<0.0001$) and 100 µg/mL ($P<0.01$).

Bovine monocyte stimulation

Viability assays were performed to assure none of the stimuli were cytotoxic to bovine monocytes at the working concentrations. None of the β-glucan sources, LPS, or P3C induced statistically significant cell death when compared to unstimulated cells (Supplementary Figure 2). The production/expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α, and of the anti-inflammatory cytokine IL-10 by bovine monocytes was evaluated after stimulation with different β-glucans, LPS or P3C. These cytokines are the most commonly assessed in β-glucan-stimulated cell studies (16, 28, 37, 38). Cells were stimulated for 8, 16, and 24 h to evaluate the kinetics of cytokine expression (Supplementary Figure 3). The 24 h time point was selected for further studies since it allows simultaneous cytokine protein and mRNA analysis. Cytokine

production and mRNA expression were affected by treatment (P<0.0001 for IL-8, IL-6, TNF-α levels, and *IL1B*, *IL6* and *TNF* mRNA expression; P=0.0013 for IL-1β and P=0.0002 for IL-10 levels; P=0.0005 for *IL10* mRNA expression) and a dose-response effect was observed for IL-8 levels (Figure 1), and *IL1B*, *IL6* and *TNF* expression (Figure 2). WGP-Soluble treatment did not significantly affect the production or gene expression of any cytokine compared with unstimulated cells (P>0.05). WGP-Dispersible, Zymosan, LPS and P3C induced the production of IL-8 (Figure 1A). IL-6 levels (Figure 1B) were increased in bovine monocyte cell cultures stimulated with Zymosan, LPS and P3C.

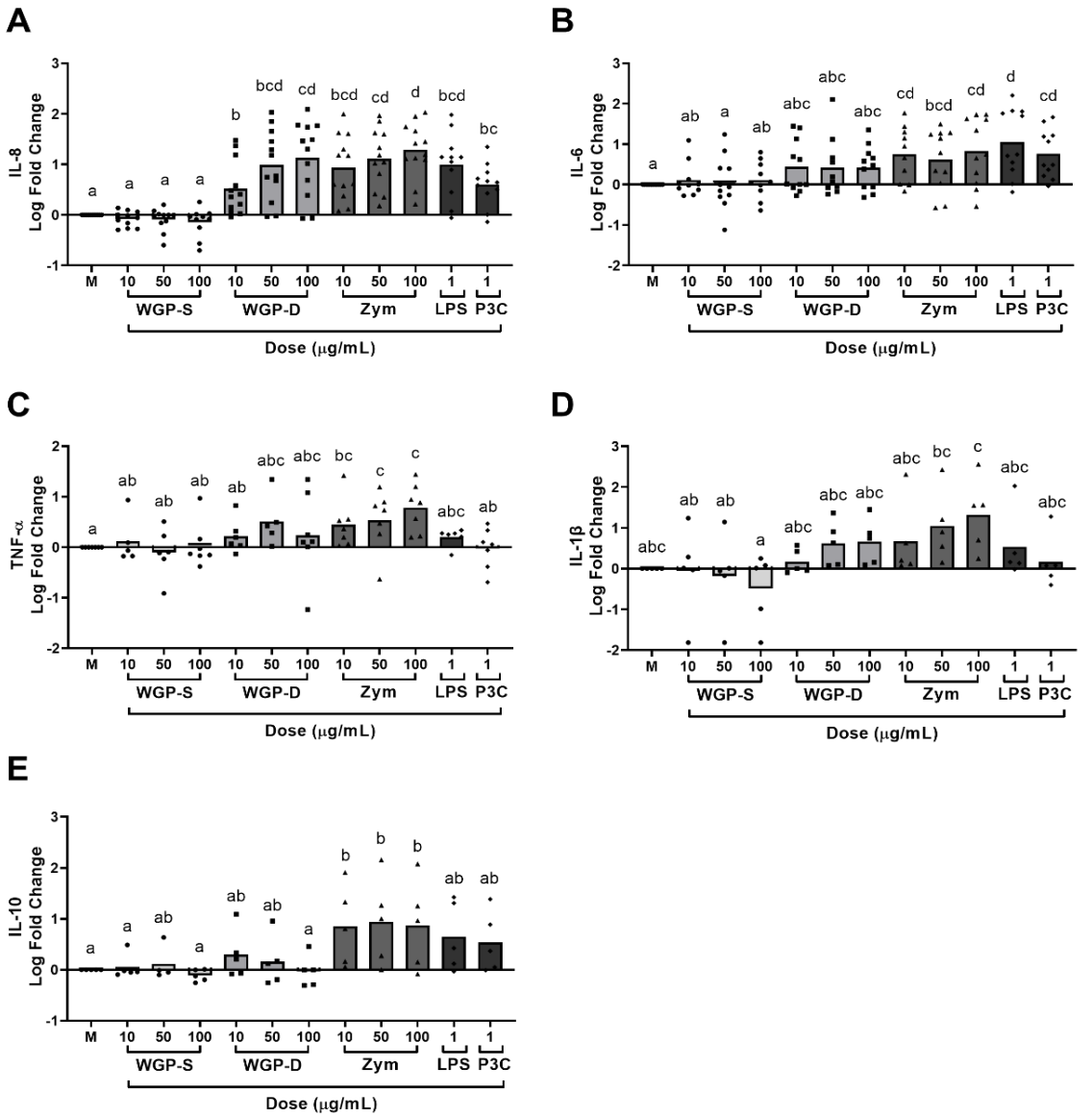

Figure 1 – Cytokine production evaluated by ELISA in the supernatants of bovine monocytes cultured for 24 h with WGP Soluble (WGP-S), WGP Dispersible (WGP-D), Zymosan (Zym), LPS, and Pam3csk4 (P3C). Data are presented as Log fold change relative to medium (M) and represent means of 12 animals for IL-8 (A), 11 animals for IL-6 (B), 7 animals for TNF-α (C), and 5 animals for IL-1β (D) and IL-10 (E). Each symbol corresponds to a different animal. ^{a,b,c,d} Means with different superscript letters are significantly different (P<0.05).

TNF-α (Figure 1C) and IL-10 (Figure 1E) levels were only increased in bovine monocytes cultured with Zymosan. A tendency for increased IL-1β production was observed in cells stimulated with Zymosan (Figure 1D).

Expression of *TNF* (Figure 2A) was significantly increased in cells stimulated with WGP-Dispersible, while *IL6* (Figure 2B) and *IL1B* (Figure 2C) were overexpressed in cells stimulated with WGP-Dispersible, Zymosan, and LPS. Although there was an effect of treatment on *IL10* mRNA transcript levels (Figure 2D), the expression of this cytokine gene was only upregulated in cells stimulated with Zymosan at 100 µg/mL, when compared to unstimulated cells. The cytokine mRNA expression results were similar when normalization was done to *B2M* mRNA expression (Supplementary Figure 4).

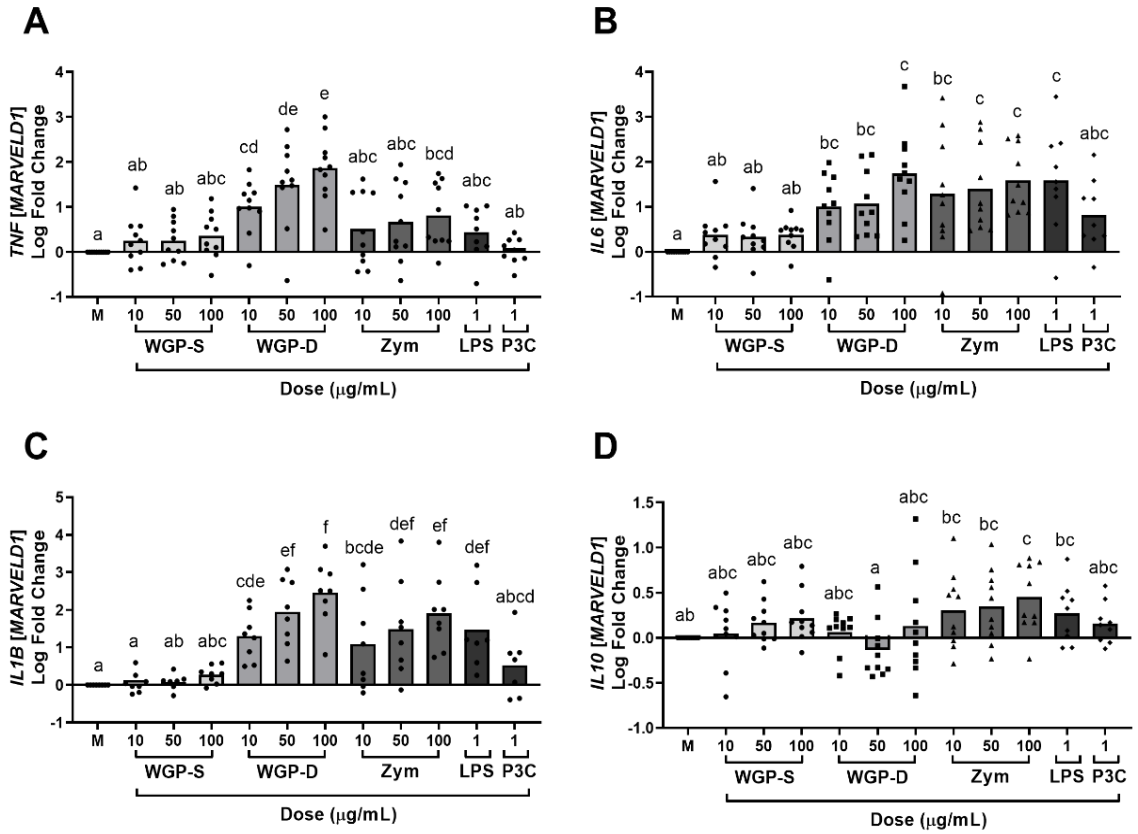

Figure 2 – Cytokine relative mRNA expression, evaluated by RT-PCR and normalized to the mRNA expression of the reference gene *MARVELD1*, in bovine monocytes cultured for 24 h with WGP Soluble (WGP-S), WGP Dispersible (WGP-D), Zymosan (Zym), LPS, and Pam3csk4 (P3C). Data are presented as Log fold change relative to medium (M) and represent means of ten animals for *TNF* (A), *IL6* (B), and *IL10* (D), and eight animals for *IL1B* (C). Each symbol corresponds to a different animal. ^{a,b,c,d,e,f} Means with different superscript letters are significantly different (P<0.05).

Since WGP-Dispersible and Zymosan stimulated cytokine production by bovine monocytes, we next examined the correlation between bovine *CLEC7A* expression of non-stimulated cells and cytokine levels or mRNA expression in response to stimulation

with 10, 50 and 100 µg/mL of WGP-Dispersible and Zymosan. A positive correlation was found between *CLEC7A* mRNA expression and IL-8 concentration in the supernatants of cells stimulated with 10 µg/mL (r = 0.7702) and 50 µg/mL (r = 0.6629) (Figures 3A and 3B, respectively), but not with 100 µg/mL (Figures 3C) of WGP-Dispersible. *TNF* mRNA expression was positively correlated with *CLEC7A* mRNA expression at 10 µg/mL (r = 0.7067) and 50 µg/mL (r = 0.6429) (Figures 3D and 3E), but not at 100 µg/mL (Figure 3F). *IL6* mRNA expression was also correlated with *CLEC7A* expression in cells stimulated with WGP-Dispersible at 10 µg/mL (r = 0.7093, Figure 3G) and a tendency was observed in cells stimulated with 50 µg/mL (r = 0.5939, Figure 3H).

No such correlation was observed when cells were stimulated with 100 µg/mL WGP-Dispersible (Figure 3I). A tendency was observed between *CLEC7A* mRNA expression and *IL1B* mRNA expression in cells stimulated with 10 µg/mL (r = 0.6672, Figure 3J), but not when cells were stimulated with 50 µg/mL or 100 µg/mL WGP-Dispersible (Figures 3K and 3L, respectively). Interestingly, a negative correlation was observed between *CLEC7A* and *IL10* mRNA expression in cells stimulated with 10 µg/mL (r = -0.6978, Figure 3M) and 100 µg/mL (r = -0.7718, Figure 3O) of WGP-Dispersible, and a tendency to a negative correlation in cells stimulated with 50 µg/mL (r = -0.5926, Figure 3N) of this β-glucan form. No correlation was found between *CLEC7A* mRNA expression and cytokine mRNA expression or production in cells stimulated with Zymosan (Supplementary Figure 5).

Sequencing of PCR products was done to confirm primers' specificity (Supplementary Figure 6). Since more than one CLEC7A amplicon was amplified in each sample, PCR products were also visualized in agarose gels to confirm the molecular size of the amplicons (Supplementary Figure 6A). According to our data, different CLEC7A isoforms were expressed simultaneously in monocyte samples, since two different bands, matching the expected molecular size distribution for the two different isoform amplicons (97 bp for the short or 235 bp for the long) appeared on the electrophoresis gel. This is in line with what was previously observed and described by Willcocks et al. [26] for short and long isoforms of boDectin-1. The expression of CLEC7A was decreased upon treatment with WGP-Dispersible and Zymosan, but not with WGP-Soluble (Supplementary Figure 7). Previous reports have shown a downregulation of Dectin-1 expression on the surface of human monocyte- and mouse bone marrow-derived dendritic cells and mouse bone marrow-derived macrophages in response to particulate β-glucan stimulation (39-41). However, no mention was made therein whether CLEC7A gene expression was also reduced.

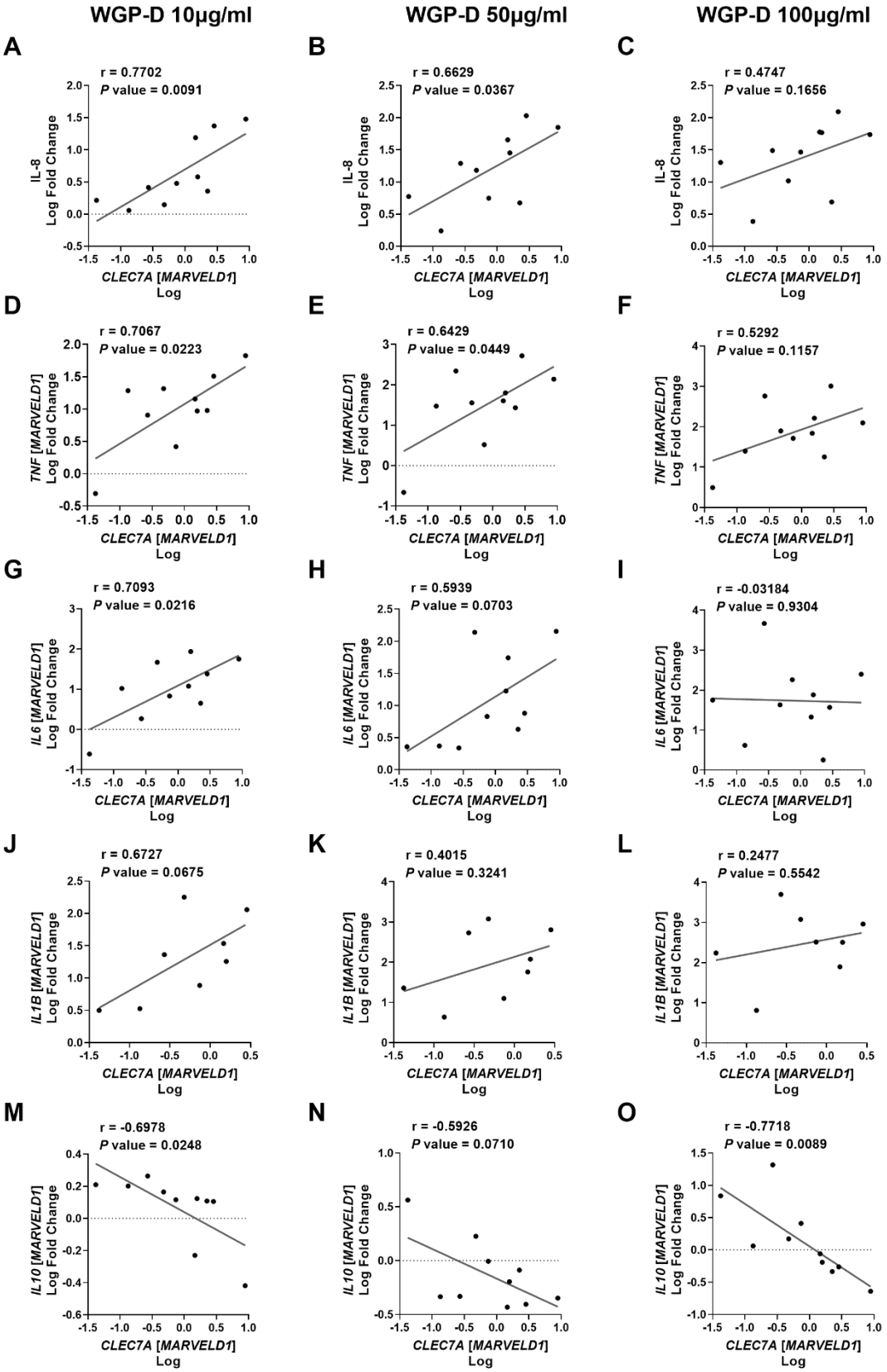

Figure 3 – Correlations between *CLEC7A* mRNA expression and (A, B, C) IL-8 cytokine production, (D, E, F) *TNF*, (G, H, I) *IL6*, (J, K, L) *IL1B*, and (M, N, O) *IL10* mRNA expression upon stimulation with 10, 50 and 100 µg/mL of WGP Dispersible, as indicated. Results are presented as Log fold changes of each cytokine relative to medium vs Log *CLEC7A* mRNA. Data represent simple linear regressions, with Pearson correlation coefficients (r) and P values.

Expression of MHC class II and costimulatory molecules

The expression of costimulatory (CD80 and CD86) and MHC class II molecules on the cell surface of monocytes upon stimulation with the different β-glucans or β-glucan-containing particles was evaluated by flow cytometry, as indicated in Supplementary Figure 8. WGP-Soluble treatment did not alter the expression of MHC class II or costimulatory molecules at any assessed time point (Figure 4).

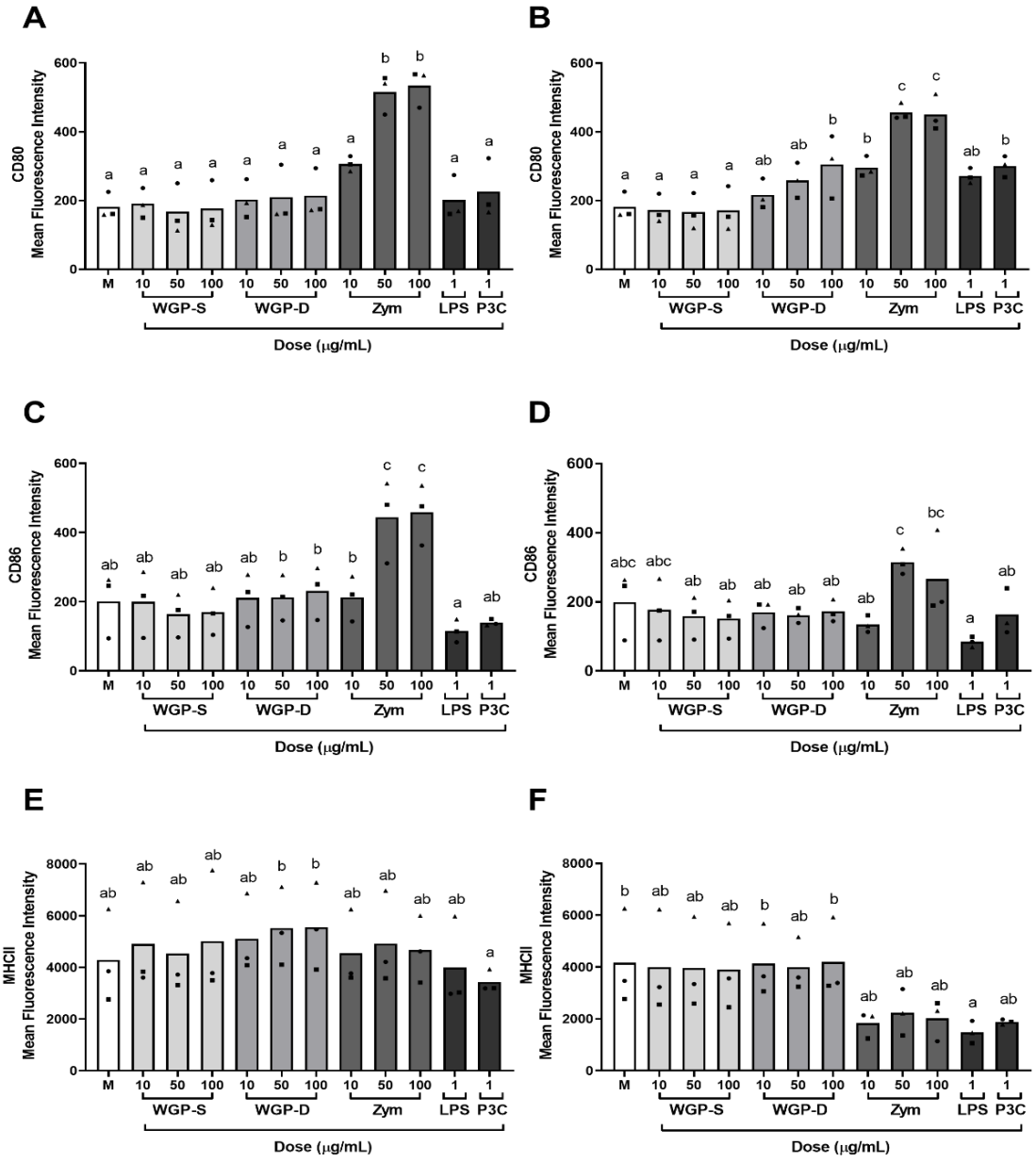

Figure 4 – Expression of (A, B) CD80, (C, D) CD86, and (E, F) MHC class II molecule expression on the cell surface of bovine monocytes stimulated with WGP Soluble (WGP-S), WGP Dispersible (WGP-D), Zymosan (Zym), LPS, and Pam3csk4 (P3C) for 8 h (A, C, E) or 16 h (B, D, F), as evaluated by flow cytometry. Results correspond to means of the mean fluorescence intensities for each analyzed molecule of three independent biological samples (represented by squares, triangles or circles). [a,b,c] Means with different superscript letters are significantly different ($P<0.05$).

Zymosan treatment upregulated the expression of CD80 and CD86 at 8 h (Figure 4A and 4C respectively) at 50 and 100 µg/mL, but only the expression of CD80 remained upregulated at 16 h (Figure 4B and 4D). Cells stimulated for 16 h with 100 µg/mL WGP-Dispersible and P3C increased the expression of CD80 (Figure 4B). No differences in the expression of MHC class II were observed at any of these timepoints (Figures 4E and 4F), besides a decrease in LPS-treated-cells at 16 h post stimulation.

Small interference mRNA and bovine Dectin-1 knockdown

IL-8 levels and *TNF* and *IL6* expression were significantly increased in monocytes stimulated with either WGP-Dispersible or Zymosan, and their increase was found to be correlated with CLEC7A expression in WGP-Dispersible-treated cells. Therefore, we further investigated the role of boDectin-1 in IL-8 production and *TNF* and *IL6* expression by silencing the receptor using a siRNA approach. Three pairs of siRNA duplexes were designed to target and silence the two bovine Dectin-1 isoforms. Transfection did not affect cell viability (Supplementary Figure 9).

Bovine *CLEC7A* was successfully knocked down by siRNA duplex #3 (85.5%, $P<0.01$) and, to a lesser extent, by siRNA #2 (66.6%, $P<0.05$) at 300 nM (Figure 5A). The knockdown efficiency of this siRNA design was particularly evident in two of the samples tested (Biological samples #2 and #3, Supplementary Table 1). siRNA design #1 was not able to successfully knock down bovine *CLEC7A* in any of the samples tested (Figure 5A). Lower IL-8 levels were found in the supernatants of cells transfected with siRNA pairs #2 and #3 upon stimulation with WGP-Dispersible at 50 µg/mL, comparatively to cells treated with medium alone (X-Vivo), ScreenFect®siRNA transfection reagent (Screenfect), and MISSION® siRNA Universal Negative Control #1 (UNC). The percentual change of cytokine production was then calculated considering IL-8 production of Negative Control transfected cells. IL-8 production was significantly decreased ($P<0.05$) in WGP-Dispersible stimulated cells (Figure 5B) when transfection was performed with siRNA duplex #2 (57.6% decrease) and siRNA duplex #3 (54.1% decrease). Only siRNA duplex #2 was able to successfully reduce IL-8 production (56.4% decrease, $P<0.05$) in cells stimulated with Zymosan at 50 µg/mL (Figure 5C). siRNA duplex #2 led to a clear decrease in IL-8 production in two of the samples stimulated with WGP-Dispersible at 50 µg/mL (Figure 5B). A smaller reduction was observed in one of the samples used. The observed effect matches the low *CLEC7A* knockdown efficiency observed in this particular sample (Figures 5A and Supplementary Table 1). The expression of *TNF* in cells stimulated with WGP-Dispersible at 50 µg/mL was also significantly affected by *CLEC7A* knockdown (Figure 5D). No such effect was

observed in Zymosan-treated cells (Figure 5E). Although not statistically different, the expression of IL6 and IL1B was decreased upon siRNA#2 treatment in response to WGP-Dispersible (Supplementary Figure 10). Given the high homology of bovine and human Dectin-1, and since no bovine-specific anti-Dectin-1 antibody is available, neutralization of this receptor was attempted using an anti-human Dectin-1 mAb. The used antibody did not recognize boDectin-1 as evaluated by flow cytometry and confocal microscopy (Supplementary Figures 11 and 12, respectively).

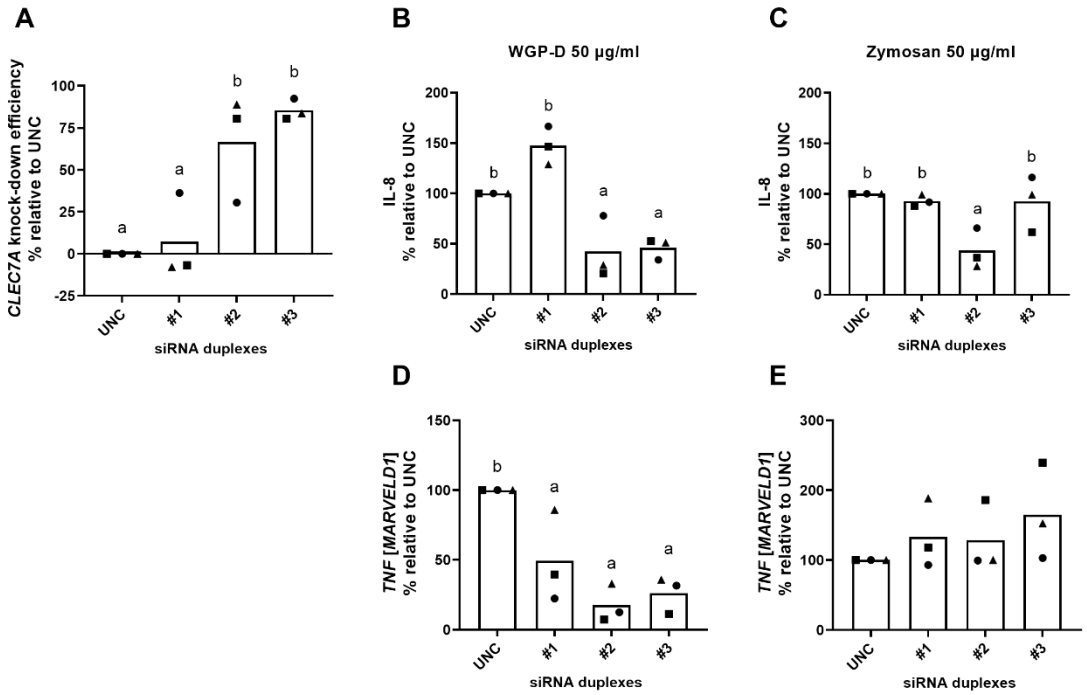

Figure 5 – Bovine *CLEC7A* knockdown efficiency (A), calculated relative to MISSION® siRNA Universal Negative Control #1 (UNC) treated cells. Cells were cultured with RPMI-1640 after transfection procedure with three different siRNA duplexes (#1, #2 and #3). IL-8 production (B, C) and *TNF* expression (D, E) of cells transfected with duplexes #1, #2 and #3 and MISSION® siRNA Universal Negative Control #1 (UNC) and stimulated with WGP-Dispersible (B, D) or Zymosan (C, E) at 50 µg/mL, calculated in percentual change relative to UNC transfected cells. Results correspond to means from three different animals (represented by squares, triangles or circles). [a,b] Means with different superscript letters are significantly different (P<0.05).

Discussion

The effects of β-glucans or β-glucan-containing products have been explored in ruminants, either *in vitro* (42-44) or *in vivo*, by oral administration (23, 24, 44-47), with the purpose of increasing immunity or response to stressors. However, the immunostimulatory effect of β-glucan-containing products, such as Zymosan, on bovine cells mostly involved the analysis of reactive oxygen and nitrogen species production by

neutrophils and monocyte-derived macrophages (48-50). BoDectin-1 has been previously identified and *CLEC7A* gene expression was detected in several bovine immune cell populations, such as monocytes, monocyte-derived dendritic cells, CD4+ T cells, CD21+ B cells, and NK cells (26). Contrary to human and mouse (10, 51) neutrophils, bovine neutrophils do not seem to express CLEC7A (52). Thus, although bovine neutrophils respond to Zymosan by increasing ROS production, this effect was dependent on Ca^{+} influx and mediated, at least in part, by CD11b (52), a component of CR3, an important β-glucan receptor in human neutrophils (53). Bovine-derived macrophages also increased the production of ROS in response to Zymosan, although the receptor involved was unravelled (54). In that line, a bovine macrophage cell line (BOMAC) challenged with *S. cerevisiae* cell wall components consistently expressed higher levels of IL-6, regardless of the yeast strain used, but no confirmation of the receptor responsible for cell activation nor evaluation of putative β-glucan-receptors' expression was done (55). Nevertheless, a human fibroblast cell line (HEK293) transfected with boDectin-1 responded to Zymosan by increasing the production of IL-8, indicating that this bovine receptor could directly recognize β-glucan-containing particles (37).

Here we demonstrated that bovine monocytes respond to particulate β-glucans, through Dectin-1 triggering, resulting in increased expression of pro-inflammatory cytokines. Incubation of bovine monocytes with soluble β-glucans did not induce the production and mRNA expression of any of the cytokines assessed, nor the expression of MHC class II and costimulatory molecules CD80 and CD86 on the surface of monocytes, suggesting that soluble β-glucans do not activate bovine CLEC7A *in vitro*. Soluble β-glucans, despite being ligands of human and murine Dectin-1, are not able to cluster and activate *in vitro* the receptor (16), thus not inducing downstream cell signaling and activation (56). Pro-inflammatory cytokine production and cytokine gene expression were significantly increased in cells stimulated with dispersible β-glucans and Zymosan in a dose-dependent manner. These results are consistent with *in vitro* data obtained with murine bone marrow-derived macrophages and dendritic cells (57), murine resident macrophages (56), human whole blood (58), and porcine innate immune cells, namely peripheral-blood mononuclear cells and neutrophils (59). Cytokine response was in accordance with the increased costimulatory molecule expression observed on the surface of WGP-Dispersible and Zymosan-treated monocytes. Dispersible β-glucans and Zymosan, induced the upregulation of costimulatory molecules, which might contribute to improve T cell stimulation. Other authors (12, 14) have previously highlighted the importance of a crosstalk between different receptors, such as Dectin-1 and TLR-2. When several PRRs are activated simultaneously by particulate β-glucans,

a complex cascade of cell signaling is usually amplified by this collaboration (60). Both Dectin-1 and TLR-2 recognize Zymosan, thus Zymosan is likely able to induce a more sustained and marked cell stimulation with a concomitant higher cytokine production.

The positive correlation found here between *CLEC7A* expression and *TNF* and *IL6* expression and IL-8 production in cells stimulated with WGP-Dispersible indicates that BoDectin-1 mediates β-glucan recognition in bovine monocytes. We found, however, no correlation between *CLEC7A* expression and cytokine production or gene expression in Zymosan stimulated cells. Since Zymosan contains other pathogen-associated molecular patterns besides β-glucans, other receptors being triggered by those compounds could be contributing to cytokine production and hamper a direct association. Although a combined recognition of Zymosan by multiple PRRs, was reported in human and mouse cells, namely by Dectin-1 plus TLR-2 (61), Willcocks et al. (37) have reported that HEK293 cell line transfected with both boDectin-1 and boTLR-2 did not increase the production of IL-8 in response to Zymosan comparatively to HEK293 cells expressing boDectin-1 alone. Indeed, in that particular study, HEK293-boTLR-2 did not respond to Zymosan (37).

We found a negative correlation between *CLEC7A* and *IL10* gene expression, reinforcing the ability of this highly pure β-glucan in inducing the production of pro-inflammatory cytokines, rather than anti-inflammatory cytokines. In contrast, Zymosan at 100 µg/mL induced the expression of the anti-inflammatory cytokine *IL-10*, comparatively to control cells. This is in accordance with previous reports describing an increased production of IL-10 in human and murine dendritic cells in response to these β-glucan-containing particles, which may confer immunological cell tolerance (62). Since WGP-Dispersible, which does not trigger TLR-signaling, did not induce *IL10* gene expression, we hypothesize that TLR-2, a receptor already associated with high IL-10 production (63), is being activated upon recognizing other Zymosan components.

These results indicate that boDectin-1 on bovine monocytes is effectively being triggered by particulate β-glucans similarly to mouse and human monocytes. In contrast, in porcine macrophages, Dectin-1 silencing did not affect cytokine production (38), reinforcing the species-specific nature of the PRR response to several agonists (37, 54). We have attempted Dectin-1 blockade with an anti-human Dectin-1 neutralizing mAb without success. The availability of a bovine anti-Dectin-1 mAb, with neutralizing functions, would be of most importance to both assess cell surface Dectin-1 expression and perform further functional assays. This would more directly allow to uncover the signaling pathways elicited by particulate β-glucans. Since Dectin-1 knockdown using siRNAs did not completely resume IL-8 production and *TNF*, *IL6*, and *IL1B* expression to negative control levels, it is possible that other receptors could additionally be involved in the

recognition of WGP-Dispersible. It thus remains to be elucidated whether bovine CR3 is also playing a role on the recognition of particulate β-glucans by bovine monocytes, as in human monocytes (64) and in mouse (65) and swine macrophages (38).

Recognition of β-glucans by Dectin-1 has been shown to induce epigenetic modifications in immune cells, that render them more efficient in responding to infection (66), a phenomenon referred to as trained immunity (66, 67). It is therefore conceivable that bovine monocytes, such as those of mice (68), humans (28), dogs (69), and chicken (70) may be prone to induction of trained immunity. That would provide a plausible explanation for the beneficial effects of β-glucan-containing dietary supplements observed *in vivo* in cattle (23, 45-47). The *in vitro* results obtained in this study may help clarify β-glucan recognition by bovine monocytes and lend support to further studies addressing trained immunity events in this species.

Data Availability Statement

The datasets generated and analysed in the current study are available from the corresponding author on request. PCR CLEC7A long and short product sequences are available at NCBI nucleotide database under the accession numbers MZ146868 and MZ146869.

Author Contributions

ARVP conducted the experiments, performed data acquisition and analysis, and wrote the manuscript. AC, TL, RF, and BL participated in the experiments. EGM and ICR assisted in the design of the experiments. ARJC and AJMF assisted in the interpretation of data. AC, MRGM, and MV conceived and designed the experiments, supervised the experimental work, assisted in data acquisition and analysis, and assisted in manuscript writing. All authors read and approved the final manuscript.

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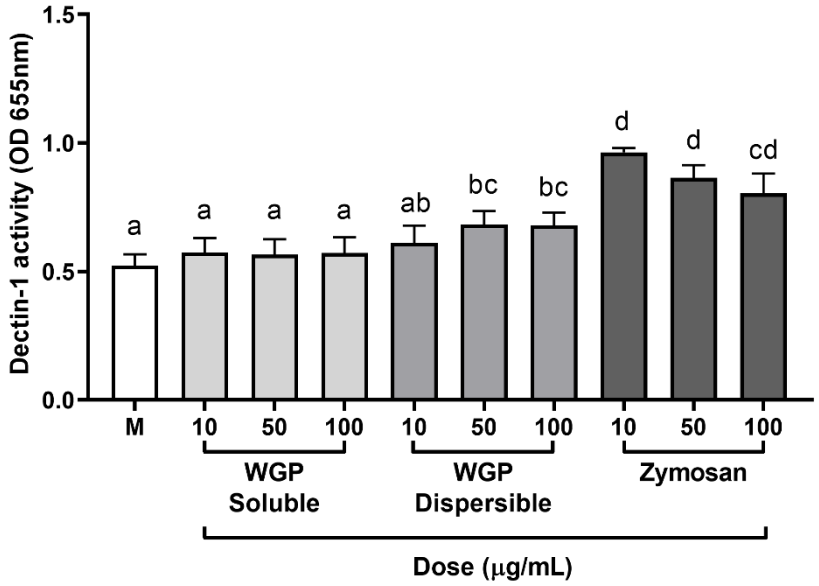
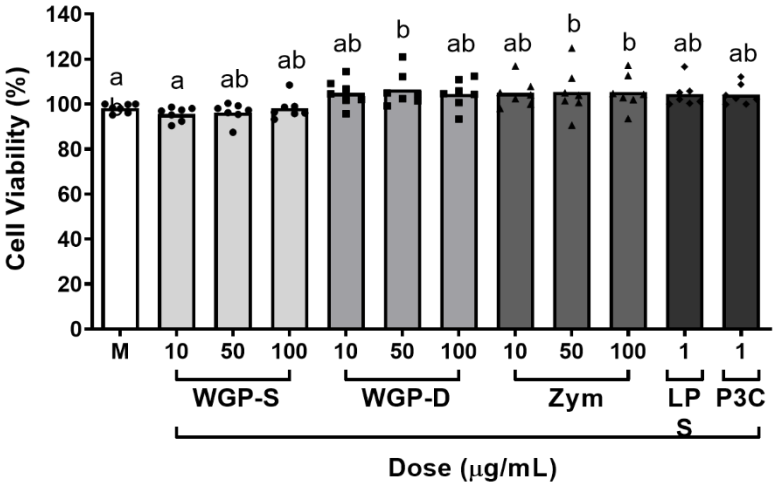
Conflict of Interest

The authors declare no conflicts of interest.

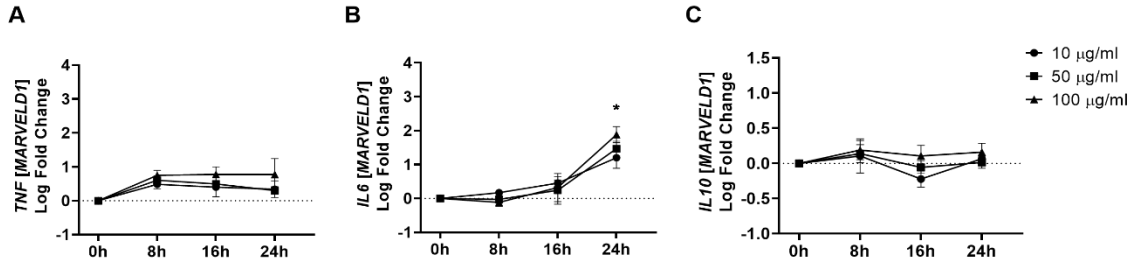
Suplementary Material



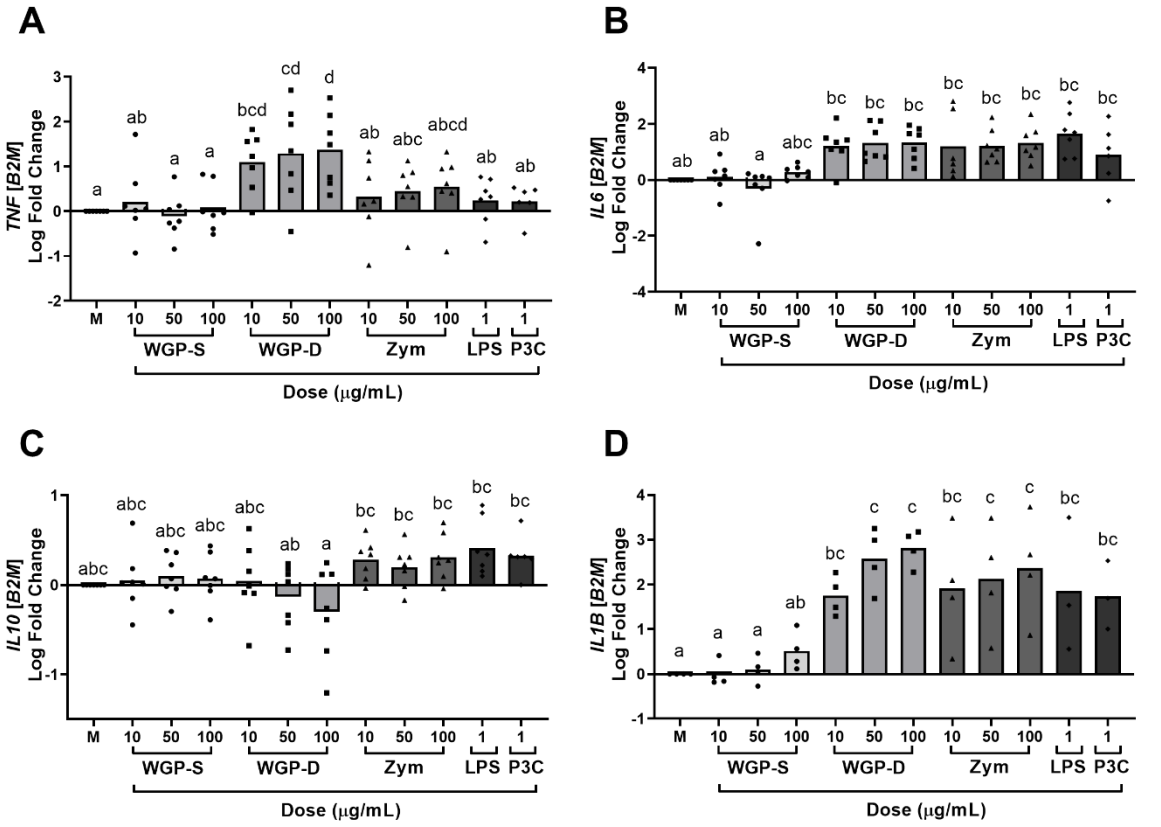
Supplementary Figure 1 – Human Dectin-1b activity measured through substract hydrolysis by secreted embryonic alkaline phosphatase (SEAP), produced upon activation of NF-κB in HEK-Blue™ hDectin-1b cells cultured for 16 h without stimulus (M) or stimulated with WGP Soluble (WGP-S), WGP Dispersible (WGP-D) or Zymosan (Zym). Data from three independent experiments performed in triplicate, displayed as means plus SEM. [a,b,c,d]Means with different superscript letters are significantly different (P<0.05).



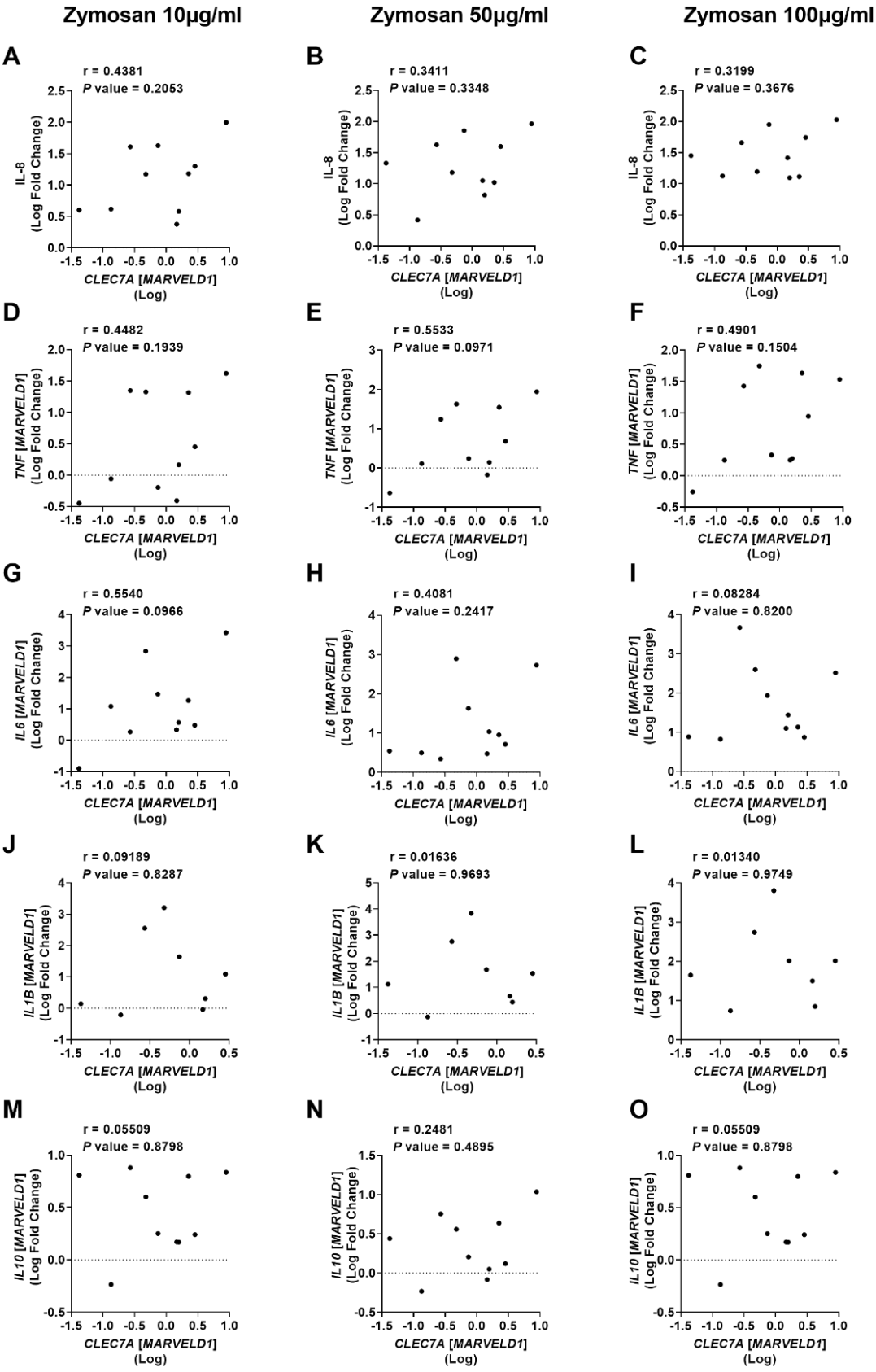
Supplementary Figure 2 – LDH released by bovine monocytes cultured for 24 h without stimulus (M) or stimulated with WGP Soluble (WGP-S), WGP Dispersible (WGP-D), Zymosan (Zym), LPS, and pam3csk4 (P3C). Bars represent means plus SEM of data from seven independent animals. [a,b]Means with different superscript letters are significantly different (P<0.05).



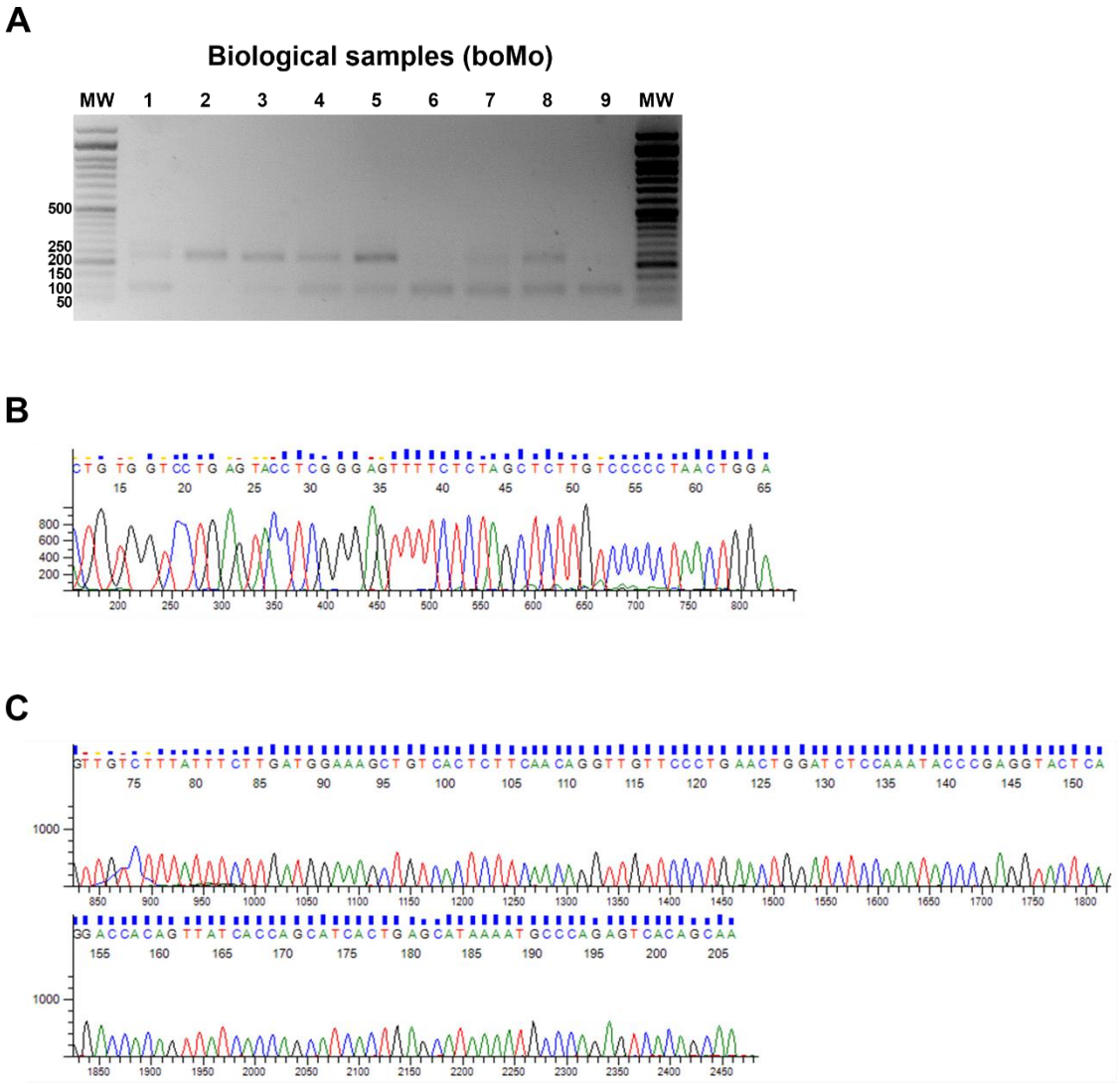
Supplementary Figure 3 – Cytokine relative mRNA expression evaluated by RT-PCR in bovine monocytes and normalized to the mRNA expression of the reference gene *MARVELD1*. Cells cultured for 8, 16, and 24 h with WGP Dispersible. Data are presented as Log fold change relative to medium (M) and represent means plus SEM of data from four animals. * P<0.05, relative to 0 h condition



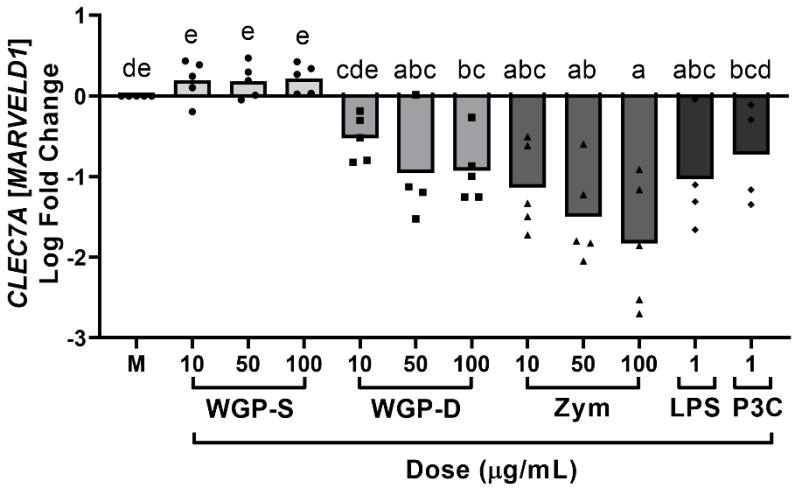
Supplementary Figure 4 – Cytokine relative mRNA expression (A, B, C) evaluated by RT-PCR in bovine monocytes and normalized to the mRNA expression of the reference gene *beta-2-microglobulin* (*B2M*). Cells were cultured for 24 h with WGP Soluble (WGP-S), WGP Dispersible (WGP-D), Zymosan (Zym), LPS, and pam3csk4 (P3C). Data are presented as Log fold change relative to medium (M) and represent means plus SEM of data from seven animals for *TNF*, *IL6*, and *IL10*, and four animals for *IL1B*. Each symbol corresponds to an independent biological sample. [a,b,c,d]Means with different superscript letters are significantly different (P<0.05).



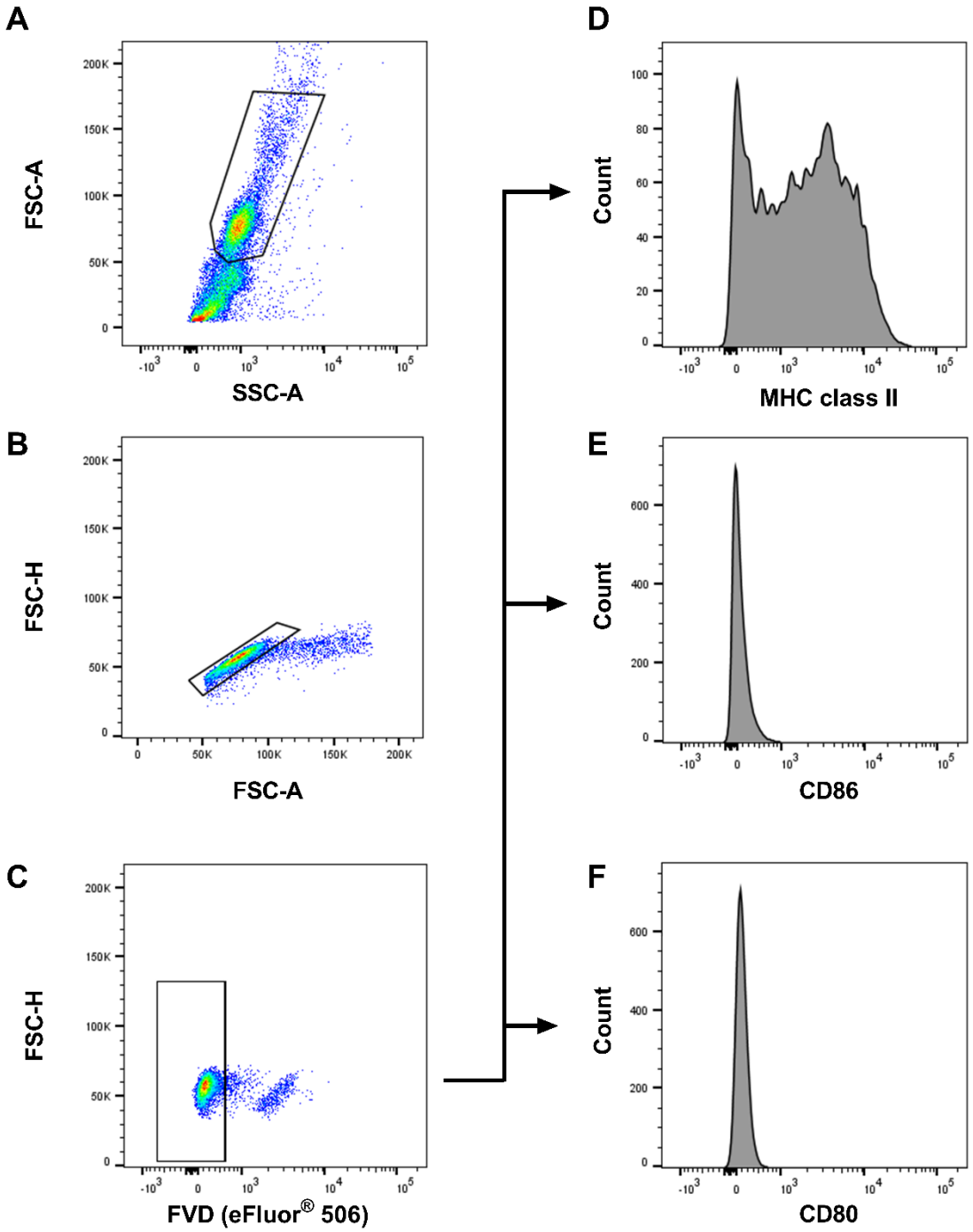
Supplementary Figure 5 – Correlations between *CLEC7A* mRNA expression and (A, B, C) IL-8 cytokine production, (D, E, F) *TNF*, (G, H, I) *IL6*, (J, K, L) *IL1B*, and (M, N, O) *IL10* mRNA expression upon stimulation with 10, 50, and 100 µg/mL of Zymosan, as indicated. Results are presented as Log fold changes of each cytokine relative to medium vs Log *CLEC7A* mRNA. Data represent simple linear regressions, with Pearson correlation coefficients (r) and P values.



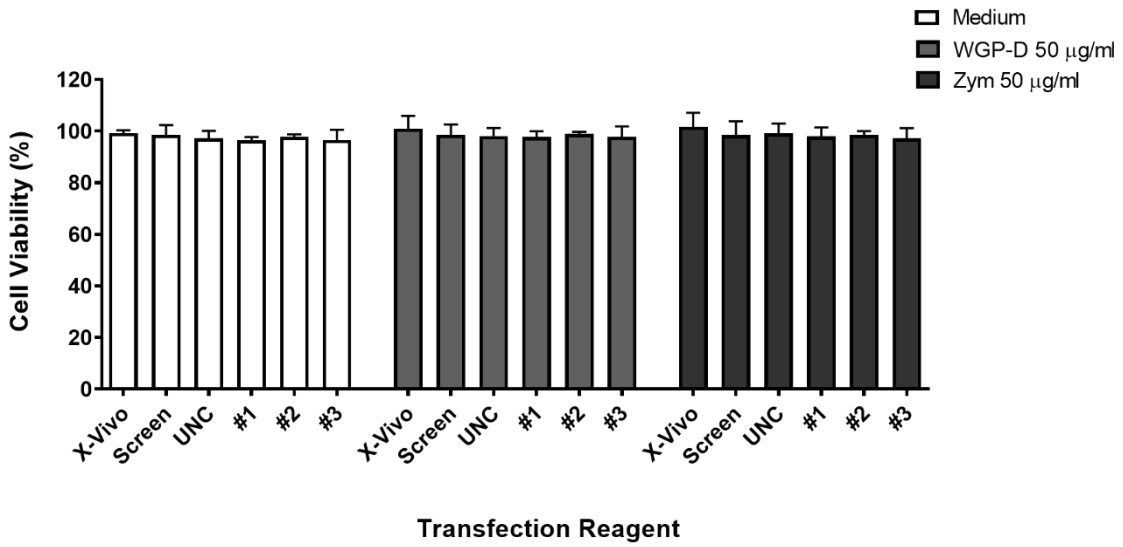
Supplementary Figure 6 – Electrophoretic profile of CLEC7A PCR amplification products from bovine monocytes cDNA, visualized in a 1.5% TAE agarose gel stained with ethidium bromide (A). MW - Molecular weight marker NZYDNA Ladder VI. Band sizes are shown in base pairs (bp); Representative electropherograms of (B) bovine CLEC7A PCR product sequencing of the smaller PCR product and (C) of the larger PCR product. The PCR products had 100% identity with Bovine CLEC7A mRNA sequences XM_005207062.4, XM_024991882.1, XM_005207061.4, XM_005207064.4, AY937382.1, BC102340.1, and NM_001031852.1, compared using the NCBI database and BLAST algorithm. DNA sequencing of PCR fragments was performed at the Genomics i3S Scientific Platform. PCR products were purified using illustraTMSephadexTMG-50 Fine DNA Grade according to the manufacturer's protocol. Sequencing products were analyzed by capillary electrophoresis on a 3500 Genetic Analyzer (Applied Biosystems). Electropherograms were visualized using Sequence Scanner Software 2 v2.0 (Applied Biosystems).



Supplementary Figure 7 - *CLEC7A* relative mRNA expression evaluated by RT-PCR in bovine monocytes and normalized to the mRNA expression of the reference gene MARVELD1. Cells were cultured for 24 h with WGP Soluble (WGP-S), WGP Dispersible (WGP-D), Zymosan (Zym), LPS, and pam3csk4 (P3C). Data are presented as Log fold change relative to medium (M) and represent means plus SEM of data from five animals. Each symbol corresponds to an independent biological sample. ^{a,b,c,d,e}Means with different superscript letters are significantly different ($P<0.05$).



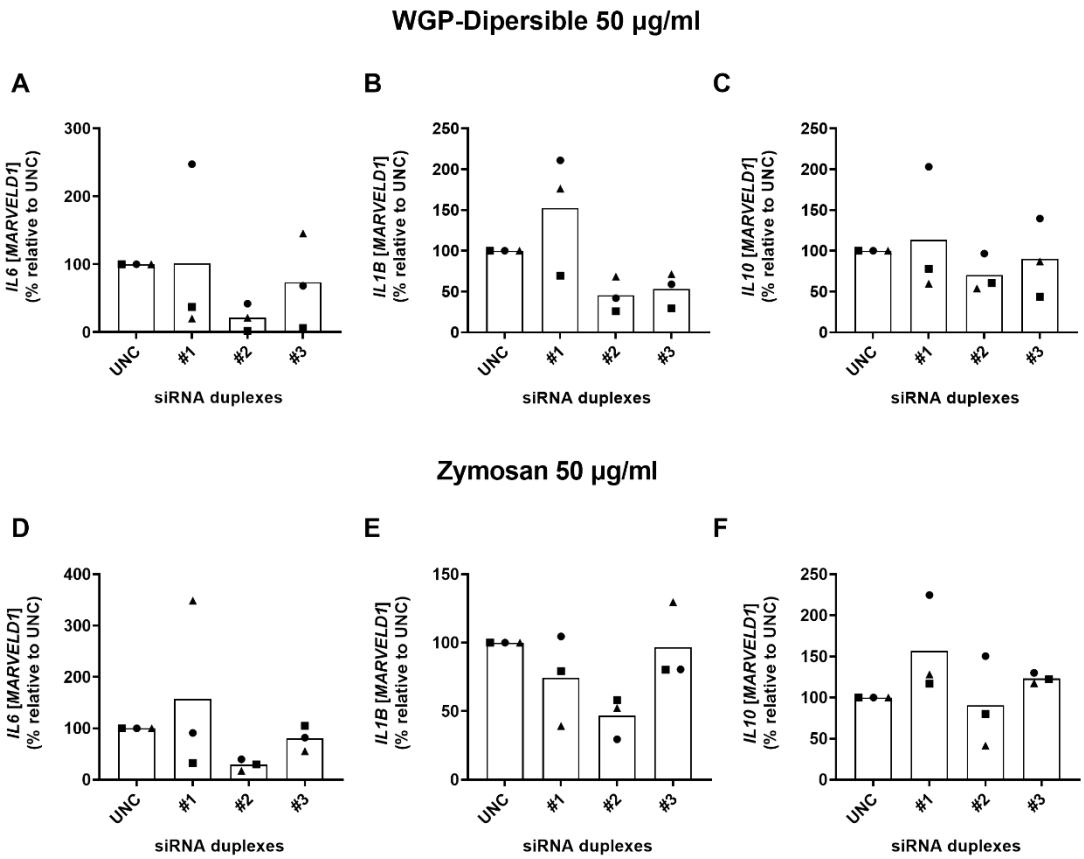
Supplementary Figure 8 – Flow cytometry gating strategy used for evaluation of cell surface co-stimulatory (CD80/CD86) and MHC class II molecule expression on bovine monocytes. Gating strategy was based on (A) exclusion of cell debris, (B) selection of single cells, and (C) exclusion of dead cells using a Fixable Viability Dye (eFluor® 506), followed by analysis of the mean fluorescence intensities due to (D) MHC class II, (E) CD86 and (F) CD80 staining. Dot plots and histograms are representative examples and correspond to bovine monocytes stimulated with for 16 h WGP Dispersible.



Supplementary Figure 9 – LDH released by bovine monocytes treated with serum-free X-Vivo™medium (X-Vivo), transfection reagent medium (Screenfect), MISSION® siRNA Universal Negative Control #1 (UNC) and three different siRNA duplexes targeting bovine Dectin-1 mRNA (siRNA #1, siRNA #2 and siRNA #3). After 4 hours of transfection, cells were stimulated WGP-Dispersible (WGP-D) and Zymosan (Zym) at 50 µg/mL or medium (M), for 24 hours. Results are presented as percentage of live cells and correspond to means plus SEM from three different animals.

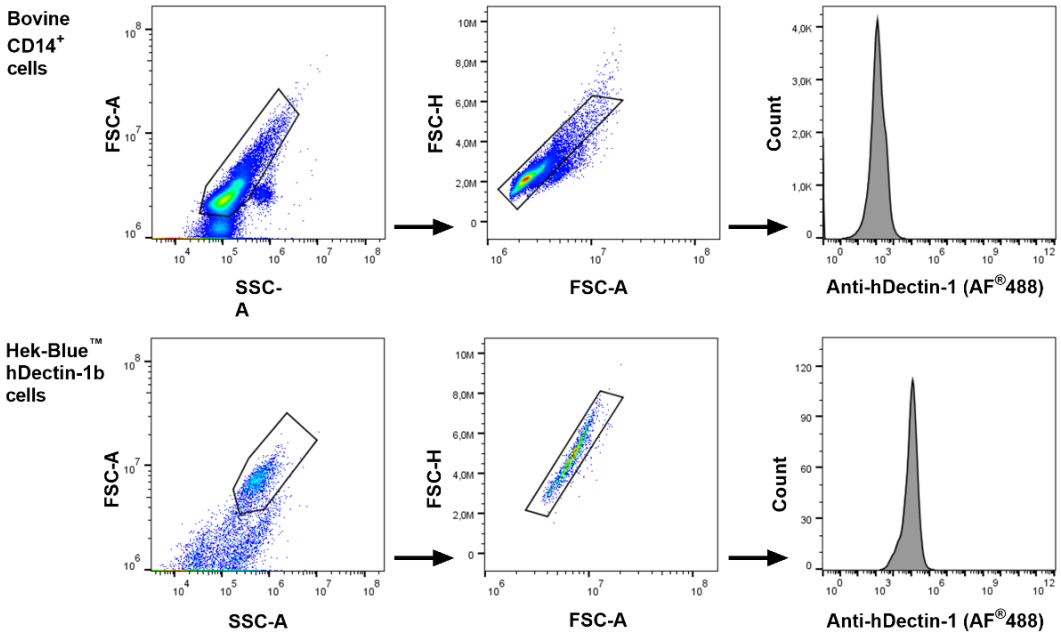
Supplementary Table 1 – Bovine Dectin-1 mRNA expression (E), normalized to MARVELD1, of cells treated with serum-free X-Vivo™medium, transfection reagent medium (ScreenFect® siRNA), MISSION® siRNA Universal Negative Control #1 and three different siRNA duplexes targeting bovine Dectin-1 mRNA (siRNA #1, siRNA #2 and siRNA #3).

Transfection Reagent	Biological Sample #1	Biological Sample #2	Biological Sample #3
X-Vivo™medium	1.067	1.448	1.138
ScreenFect® siRNA	0.179	0.412	0.911
Universal Negative Control	0.127	1.044	1.391
siRNA duplex #1	0.081	1.115	1.501
siRNA duplex #2	0.088	0.204	0.154
siRNA duplex #3	0.010	0.204	0.229

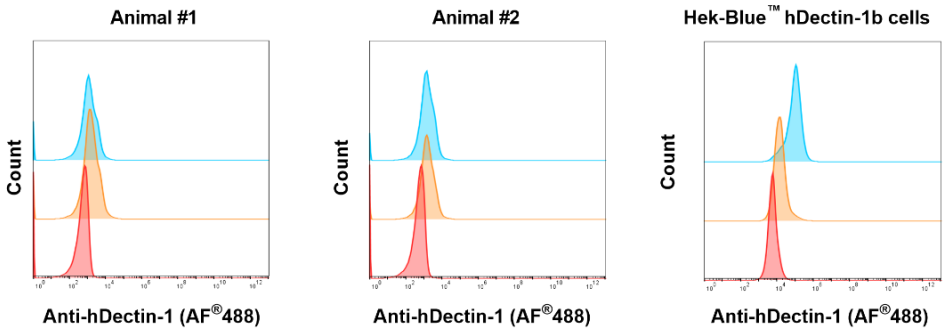


Supplementary Figure 10 – *IL6* (A and D), *IL1B* (B and E), and *IL10* (C and F) mRNA expression of cells transfected with duplexes #1, #2 and #3 and MISSION® siRNA Universal Negative Control #1 (UNC) and stimulated with WGP-Dispersible (A, B and C) or Zymosan (D, E, and F) at 50 µg/mL, calculated in percentual change relative to UNC transfected cells. Results correspond to means from three different animals (each one represented by squares, triangles or circles in A, B, and C).

A



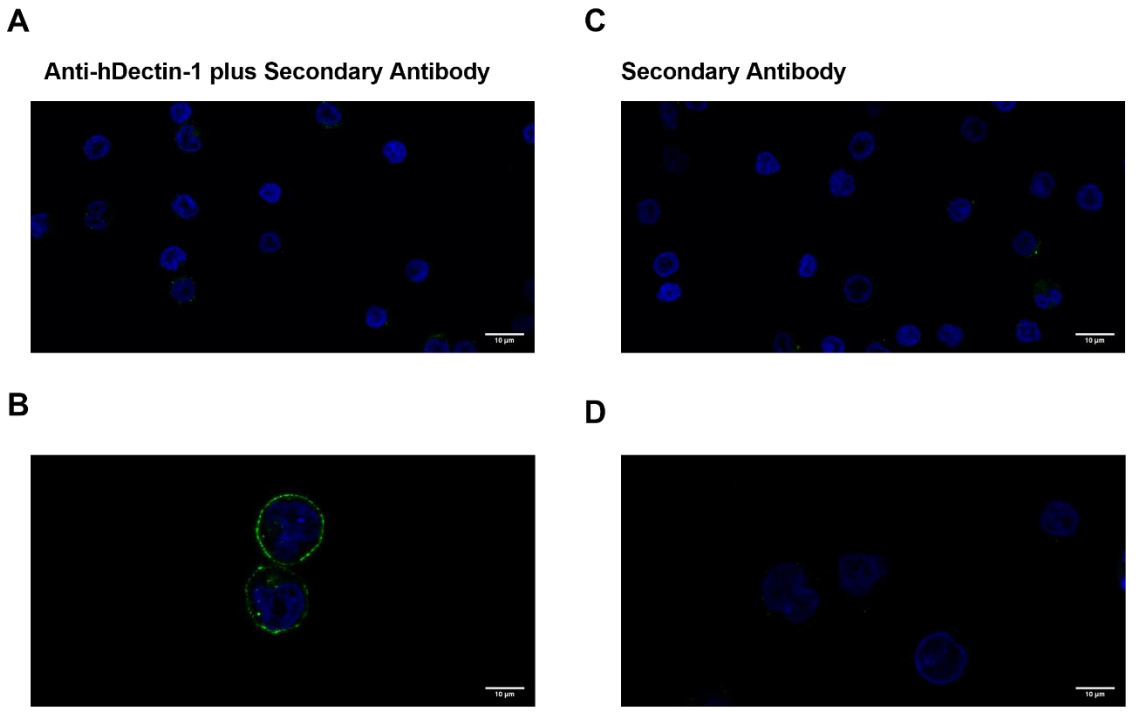
B



C

Samples	Mean Fluoresce Intensities		
	Unstained	Secondary Antibody	Anti-hDectin-1 plus Secondary Antibody
Animal #1	574	3 949	2 186
Animal #2	573	3 346	3 030
Hek-Blue™ hDectin-1b Cells	7 172	27 007	122 260

Supplementary Figure 11 - Flow cytometry gating strategy (A) used for cell surface detection of Dectin-1 on bovine CD14+ cells (monocytes) and on HEK-Blue™ hDectin-1b cells. Gating strategy was based on exclusion of cell debris, followed by selection of single cells and analysis of Dectin-1 expression. (B) Flow cytometry histogram overlays of bovine CD14+ cells from two different animals and HEK-Blue™ hDectin-1b cells stained with anti-human Dectin-1/CLEC7A antibody followed by incubation with a secondary antibody conjugated with Alexa-Fluor® 488. Histograms in red correspond to unstained samples, in orange to samples incubated only with the secondary antibody, and in blue samples incubated with primary and secondary antibodies. (C) Mean Fluorescence Intensities obtained by flow cytometry analysis of bovine CD14+ cells from the two different animals and HEK-Blue™ hDectin-1b cells.



Supplementary Figure 12 – Confocal Microscopy imaging of (A) bovine monocytes (Animal #1) and (B) HEK-Blue™ hDectin-1b cell line labelled with anti-human Dectin-1/CLEC7A antibody and secondary goat anti-mouse IgG conjugated with Alexa-Fluor 488. (C) Bovine monocytes and (D) HEK-Blue™ hDectin-1b cell line labelled with secondary goat anti-mouse IgG conjugated with Alexa-Fluor® 488 only, to detect unspecific binding. Blue: nuclei stained with DAPI; Green: Dectin-1 labelled with anti-human Dectin-1/CLEC7A antibody and secondary goat anti-mouse IgG conjugated with Alexa-Fluor 488.

CHAPTER III

Cytokine and reactive-oxygen species production by bovine peripheral blood monocyte-derived macrophages, stimulated *in vitro* with digested *Chlorella vulgaris*, *Nannochloropsis oceanica* or *Tetraselmis* sp.

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2023

Cytokine and reactive-oxygen species production by bovine peripheral blood monocyte-derived macrophages, stimulated *in vitro* with digested *Chlorella vulgaris*, *Nannochloropsis oceanica* or *Tetraselmis* sp.

Ana R. V. Pedro [1, 2, 3, †], Tânia Lima[1], Catarina Gonçalves[4], Isabel C. Ramos[5], Elisabete G. Martins[6, 7, 8], Ana R. J. Cabrita[3], António J. M. Fonseca[3], Margarida R. G. Maia[3], Manuel Vilanova[1,2], Alexandra Correia[1,2]*

[1] Immunobiology Group, i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal

[2] Laboratório de Imunologia, DIMFF, ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[3] LAQV, REQUIMTE, ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[4] Department of Life Sciences, Food Processing Group, International Iberian Nanotechnology Laboratory, Braga 4715-330, Portugal

[5] Cooperativa Agrícola de Vila do Conde, Vila do Conde, Portugal

[6] ADM Portugal, SA, Murtede Portugal

[7] EPIUnit, Instituto de Saúde Pública, Universidade do Porto, Porto, Portugal

[8] Escola Universitária Vasco da Gama, Coimbra, Portugal

[†] Present Address: Escola Universitária Vasco da Gama, Coimbra, Portugal

Abstract

Microalgae have long been used as food and feed, but only recently have the immune-stimulating properties of microalgae been thoroughly studied. Several *in vitro* studies dissected the anti-inflammatory, anti-tumoral, antioxidant and immunomodulatory properties of microalgae and their extracts, mostly in human primary cells, human cell lines and mouse cell lines. However, information regarding immune-stimulating effects in bovine cells and underlying mechanisms responsible for those effects it is still missing. Besides, most of the research *in vitro* have used microalgae extracts, with some degree of purification, that did not undergo the metabolic processes that occur during digestion. We thus aimed at evaluating the effects of *in vitro* digested eukaryotic microalgae species (*Chlorella vulgaris*, *Nannochloropsis oceanica* and *Tetraselmis* sp.) on bovine monocyte-derived macrophages. Our results show that all digested microalgae were able to induce the production of TNF-α, IL-1β, IL6, IL-8 and IL10 and mRNA expression of *TNFA*, *IL1B*, *IL6*, *IL10*, *IL12A*, *IL12B* and *IL23A*. These effects were mediated, at least in part, through NF-κB signalling, since production and mRNA expression of those cytokines were abrogated when cells were pre-treated with TPCA-1, an IKK-2 inhibitor, at 10 μM. Reactive-oxygen species production was also increased upon stimulation with all digested microalgae species. We have also observed that digested microalgae presented anti-inflammatory properties *in vitro*: cells pre-incubated with digested *Tetraselmis* sp. and subsequently stimulated with LPS produced less IL-8, while cells pre-incubated with all digested microalgae expressed *TNFA*, *IL1B* and *IL12A* at lower levels than non-treated LPS-stimulated cells. No effect of digestive fluids and enzymes were observed on bovine monocytes. Overall, *in vitro* digested *C. vulgaris*, *N. oceanica*, and *Tetraselmis* sp. present *in vitro* immunostimulatory properties, partially mediated through NF-κB signalling, but when preceding a strong pro-inflammatory stimulus, these microalgae species also have anti-inflammatory effects.

Keywords

Bovine monocyte-derived macrophages, *Chlorella vulgaris*, *Nannochloropsis oceanica*, *Tetraselmis* sp., cytokines, anti-inflammatory, NF-κB, ROS

Introduction

Microalgae are eukaryotic or prokaryotic unicellular microorganisms that usually present autotrophic metabolism but can also be cultivated under heterotrophic or mixotrophic

conditions (1). The interest in these ubiquitous organisms has increased in the last decades, being currently used for diverse applications such as biofuel production, wastewater management, cosmetology, nutritional supplements, and nutraceuticals (2). As dietary supplements, either for food or feed, microalgae are a valuable source of macro and micronutrients, including proteins, polysaccharides, polyunsaturated fatty acids (PUFA), organic minerals, vitamins, minerals and antioxidants (3, 4), which content greatly vary among species and within species according to culture conditions (5). Several bioactive compounds obtained from microalgae have been screened and studied, due to their beneficial effect on health (6). Of these we can highlight β-glucans. These polysaccharides are present in the cell wall of bacteria and fungi and have also been identified in several microalgae species such as *Chlorella vulgaris, Euglena gracillis*, *Tetraselmis suecica* and *Scenedesmus* sp. (7, 8). β-glucans have been widely shown to enhance the production of cytokines, phagocytosis, and production of reactive-oxygen species (ROS) by innate immune cells through Dectin-1 signaling (9), including in bovine cells (10). Long-chain PUFA, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are also bioactive compounds found in marine microalgae that play a pivotal role in numerous physiological functions (11). Health benefits from dietary supplementation with microalgae are reported to be due to their immunomodulatory, antioxidant, anti-inflammatory, anti-tumoral, and anti-microbial properties (12, 13). Nevertheless, the use of microalgae as nutraceuticals needs further research and understanding, particularly at a mechanistic level (14). Moreover, *in vitro* studies usually explore the immunomodulatory effects of microalgae using its extracts (15-18). However, upon ingestion of whole microalgae, these will be exposed to digestive fluids, pH shifts and digestive enzymes, which may affect microalgae cell wall structure and impact their effect on the immune system, as luminal antigens are sampled by gut mucosal or lamina propria macrophages and dendritic cells (19-21). In this study, we used an *in vitro* model that mimics the *in vivo* monogastric digestion (abomasal and upper intestinal) to digest three microalgae species for further interaction with bovine monocyte-derived macrophages (20, 22-24). Thus, static *in vitro* digestion of three eukaryotic microalgae species was performed: *C. vulgaris*, *Nannochloropsis oceanica* and *Tetraselmis* sp. These microalgae are among the most produced species in Europe and most used in animal feeding (25), with potential health benefits (26-28). The response of bovine monocyte-derived macrophages (MDM) to digested microalgae was then assessed. Our results show that all digested microalgae induced the production of pro and anti-inflammatory cytokines and the production of ROS by bovine MDM. Production and mRNA expression of cytokines was mediated, at least in part, by NF-κB, since bovine MDM pre-treated with TPCA-1, an IKK-2 inhibitor, at 10 μM, did not produce

IL-8 nor expressed any of the cytokines assessed when stimulated with LPS or digested *C. vulgaris*, *N. oceanica* or *Tetraselmis* sp.

Material and Methods

Monogastric *in vitro* digestion of *Chlorella vulgaris*, *Nannochloropsis oceanica* and *Tetraselmis* sp.

The microalgae species *C. vulgaris* (CV), *N. oceanica* (NO) and *Tetraselmis* sp. (T) used in the current study are commercially available products, and were kindly provided by Allmicroalgae Natural Products, S.A. (Pataias, Portugal) as spray-dried biomass in sealed bags protected from light. Static *in vitro* digestion of microalgae was adapted from a standardized methodology, proposed by Minekus et al. (29) within COST-Infogest network. Briefly, primary solutions were prepared in advance, with sterile, pyrogen-free Aqua B. Braun™ water (B. Braun, Melsungen, Germany) according to Table 1.

Table 1 – Primary salts and solutions needed to prepare electrolyte stock solutions and perform static *in vitro* digestion.

Constituent	**Simulated Salivary Fluid** (1.25×)	**Simulated Gastric Fluid** (1.25×)	**Simulated Intestinal Fluid** (1.25×)
KCl, 0.5 M	15.1 mL	6.9 mL	6.8 mL
KH_2PO_4, 0.5 M	3.7 mL	0.9 mL	0.8 mL
$NaHCO_3$, 1 M	6.8 mL	12.5 mL	42.5 mL
$MgCl_2(H_2O)_6$, 0.15 M	0.5 mL	0.4 mL	1.1 mL
$(NH_4)_2CO_3$, 0.5 M	0.06 mL	0.5 mL	-
NaCl, 2 M	-	11.8 mL	9.6 mL
	Volume adjusted to 400 mL	Volume adjusted to 400 mL	Volume adjusted to 400 mL
	pH adjusted to 7	pH adjusted to 3	pH adjusted to 7

All reagents were standard analytical grade and sodium bicarbonate (0.5 M) was filtered with a polyethersulfone (PES) 0.22 µm syringe filter (Filtropur S, Sarstedt, Nümbrecht, Germany). Simulated salivary, gastric, and intestinal fluids (SSF, SGF and SIF, respectively) were prepared on the day before the experiment, according to Table 2, inside a class II biosafety cabinet and filtered with 0.22 µm PES syringe filters (Filtropur S, Sarstedt). Simulated fluids were then kept at 4 ºC in sterile Schott Duran glass bottles (Duran®, Mainz, Germany) until use. All digestion procedures were performed in aseptic conditions.

For the *in vitro* digestion, 500 mg of *C. vulgaris*, *N. oceanica* or *Tetraselmis* sp. were resuspended in 5 mL sterile, pyrogen-free water (Aqua B. Braun™, B. Braun), in Falcon®

50 mL centrifuge tubes (Corning Inc., Corning, New York). A tube with 5 mL sterile, pyrogen-free water, without microalgae, was used throughout *in vitro* digestions as a control of the digestion procedure (herein referred as "digested blank"). For the oral phase, 4 mL of SSF, 25 µL of 0.3 M $CaCl_2(H_2O)_2$ and 975 µL of H_2O were added to the tubes containing resuspended microalgae or water. Salivary α-amylase was not included since newborn ruminants do not produce significant amounts of salivary α-amylase (30).

Table 2 – Volumes and adjustments needed to prepare electrolyte stock solutions at 1.25× (Simulated Salivary Fluid, Simulated Gastric Fluid and Simulated Intestinal Fluid)

Constituent	Molarity	Weight	Final Volume
KCl	0.5 M	1.865 g	50 mL
KH_2PO_4	0.5 M	0.680 g	10 mL
$NaHCO_3$	1 M	8.40 g	100 mL
NaCl	2 M	5.85 g	50 mL
$MgCl_2(H_2O)_6$	0.15 M	0.305 g	10 mL
$(NH_4)_2CO_3$	0.5 M	0.480 g	10 mL
$CaCl_2(H_2O)_2$	0.3 M	0.441 g	10 mL
NaOH (for pH adjustments)	1 M	2 g	50 mL
HCl (for pH adjustments)	1 M	4.14 mL of HCl 37%	50 mL

For the gastric phase, 6.4 mL of SGF, 5 µL $CaCl_2(H_2O)_2$, and 1.6 mL of porcine pepsin (1:10 000 U; VWR, Radnor, US), previously prepared in SGF at 2.5 mg/mL (to achieve a concentration of 2000 U/mL in the total volume), were added to each tube. The pH was adjusted to 3 with HCl 1 M and H_2O was added to make up to 10 mL gastric mixture (20 mL total volume). The tubes were then incubated in a water bath for 2 h, at 39 ºC, to mimic mean body temperature of calves. Meanwhile, porcine bile extract at 40 mg/mL and porcine pancreatin 4 × USP at 8 mg/mL (both from Sigma-Aldrich, Missouri, US) were prepared in SIF. Calculations were made considering characteristics of both products, to obtain 100 U/mL of trypsin activity and 10 mM of bile salts in the final mixture (final volume of 40 mL). After the 2 h-incubation, 11 mL of SIF, 5 mL of pancreatin, 2.5 mL of porcine bile (both previously prepared in SIF) and 40 µL of $CaCl_2(H_2O)_2$ were added to the tubes. NaOH was used to adjust pH to 7 and H_2O added to make up to 20 mL intestinal mixture (40 mL total and final volume). Intestinal digestion was also performed in a water bath for 2 h, at 39 ºC. The digested microalgae and blanks were then aliquoted and stored at -20 ºC until use.

Isolation of bovine peripheral blood monocytes and differentiation of monocyte-derived macrophages

Bovine blood samples were obtained from Holstein-Friesian heifers aged 12 to 13 months old, at a commercial slaughterhouse (PEC Nordeste – Indústria de Produtos Pecuários do Norte, Penafiel, Portugal). Blood collection was performed during slaughter of the animals for human consumption, to avoid intervention for research purposes. This procedure was licensed by Direção Geral de Alimentação e Veterinária (national competent authority) under a by-product handling authorization (N.12.006.UDER). Blood processing and monocyte isolation was performed exactly as previously described (31). Briefly, blood was collected into BD Vacutainer® lithium heparin tubes and clot activating tubes (BD, Franklin Lakes, NJ, US) from jugular and carotid veins. Serum was obtained upon centrifugation of clotted blood at 20 817 × *g* for 10 min at 4 ºC. Whole heparinized blood was then diluted 1:2 with Dulbecco's phosphate-buffered saline (DPBS) and centrifuged for 15 min on Histopaque®-1077 (both from Sigma-Aldrich) at 1200 × *g* in SepMate™ PBMC isolation tubes (Stemcell™ Technologies, Vancouver, BC, Canada), to obtain peripheral blood mononuclear cells (PBMC). These were then collected and washed at 400 × *g* for 10 min. $CD14^+$ cells were selected by magnetic sorting using an anti-human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's instructions. Isolated cells were centrifuged at 300 × *g* for 10 min and resuspended at 2×10^6 cells/mL in RPMI-1640 Medium (Sigma-Aldrich) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mM L-glutamine, 10 mM HEPES (all from Sigma-Aldrich), 50 µM β-mercaptoethanol (Merck, Darmstadt, Germany) and 10% autologous serum. To differentiate bovine $CD14^+$ monocytes into macrophages, cells were seeded at 2×10^5 per well, in flat-bottom 96-well culture plates for 7 days. One third of the culture medium (67 µL) was removed on the 3rd day, and 100 µL of fresh supplemented RPMI-1640 Medium were added to the wells. On the 7th day bovine monocyte-derived macrophages (MDM) were observed under the microscope (Figure 1) and used for stimulation assays with digested microalgae.

Cell culture and stimulation with *in vitro* digested microalgae

Bovine MDM were cultured with digested microalgae at 10-fold and 100-fold dilution, which correspond to approximate concentrations of 1.25 mg/mL and 125 µg/mL of microalgae, respectively. Digested blank samples were also used at 10-fold and 100-fold dilution, as controls.

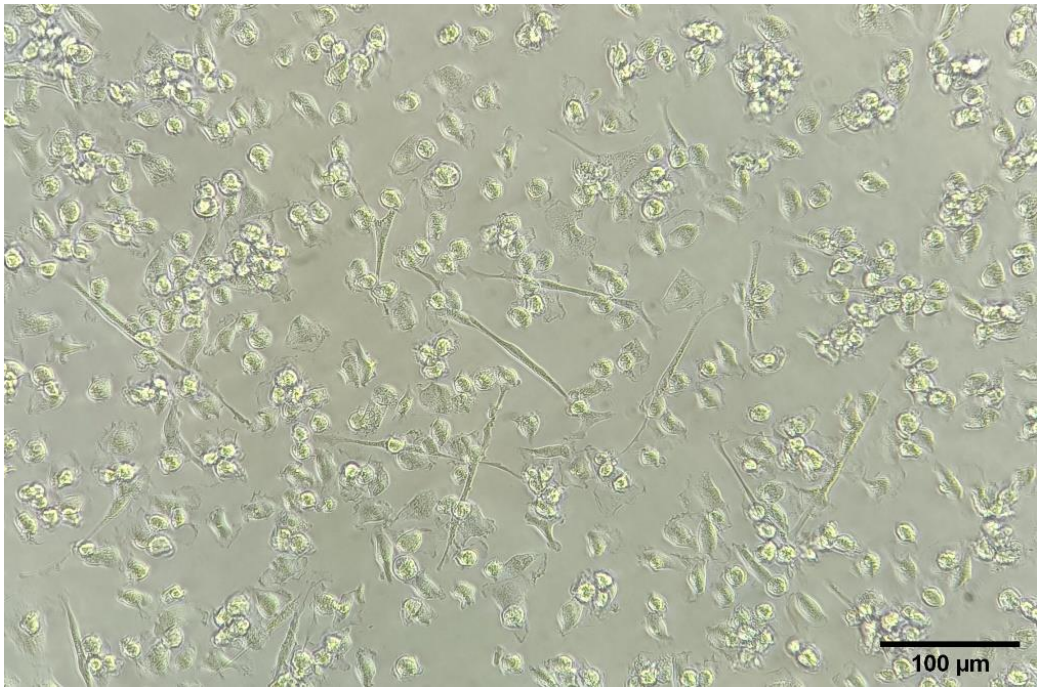

Figure 1 – Bovine peripheral blood monocyte-derived macrophages, at the 7th day of differentiation period with autologous serum. Amplification of 200x (objective 20x; ocular 10x).

Cells cultured in medium without any stimuli were used as negative controls, while cells cultured with 2 µg/mL of *Escherichia coli* lipopolysaccharide (LPS, strain O111:B4; Sigma-Aldrich) were used as positive controls.

Lactate dehydrogenase (LDH) release, cytokine mRNA expression and cytokine production were evaluated in cells cultured for 24 h at 37 °C and 5% CO_2, as performed previously (31). LDH was determined in cell culture supernatants using CyQUANT™ LDH Cytotoxicity Assay kit, according to manufacturer's protocol (Invitrogen, Waltham, MA, US) using BioTek™ Gen5™ Data Collection and Analysis Software in a BioTek™ µQuant Microplate Reader (BioTek Instruments, Vermont, US).

Inhibition of NF-κB pathway

Inhibition of NF-κB pathway was performed using 2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1), an IκB kinase inhibitor which inhibits nuclear localization of NF-κB (Abcam, UK). TPCA-1 was resuspended in DMSO (Dimethyl sulfoxide Hybri-Max™, Sigma-Aldrich) at 100 mM, aliquoted and stored in tightly sealed cryotubes at -80 ºC until further use, according to the manufacturer's instructions. On the day of the assay, TPCA-1 was diluted in DMSO to obtain a 1 mM solution. The final dilutions (10 µM and 1 µM) were performed in complete medium to avoid cytotoxicity from DMSO. To perform the inhibition assay, bovine MDM were pre-treated with 10 or 1 µM of TPCA-1 for 1 h before being stimulated with LPS at 2 µg/mL,

digested blank at 100-fold dilution or digested microalgae at 100-fold dilution for 24 h. Non-treated stimulated cells and treated non-stimulated cells were used as controls. Supernatants were then collected and stored at -80ºC. Cells were preserved in Trizol® at -80 ºC until further analysis.

Anti-inflammatory assays

To assess anti-inflammatory properties of digested microalgae, bovine MDM were pre-incubated with digested blank, digested *C. vulgaris*, *N. oceanica* or *Tetraselmis* sp. at 1000-fold dilution for 1 h. A higher dilution was chosen since digested microalgae at 100-fold dilution induced identical mRNA expression and cytokine production comparatively to LPS-stimulated cells. Non treated cells were cultured as controls. LPS was then added to the wells, to achieve a final concentration of 2 µg/mL for 24 h. Supernatants were then collected, and cells were kept in Trizol® at -80ºC.

Cytokine production

Bovine Tumor Necrosis Factor Alpha (TNF-α) and bovine Interleukin (IL)-6 were assessed using Bovine TNF-alpha and Bovine IL-6 DuoSet ELISA kits (R&D Systems, Minneapolis, MN, US) according to the manufacturer's instructions (detection limit: 125 pg/mL). However, a small modification was performed to improve signal-to-noise ratio: instead of using 5% Tween 20 in DPBS as reagent diluent, a 1% molecular grade bovine serum albumin (BSA, Albumine Bovine Fraction V, NZYTech, Lisbon, Portugal) in DPBS solution was used. Bovine IL-8 was quantified using Bovine IL-8 (CXCL8) ELISA development kit (Mabtech AB, Nacka Strand, Sweden) according to manufacturer's protocol (detection limit of 8 pg/mL). Bovine IL-10 was measured accordingly to the standard protocol of Mabtech ELISA Bovine IL-8 kit, using an in-house ELISA procedure. Briefly, ELISA plates (Nunc Maxisorp™) were coated and incubated overnight at 4 ºC with 1 µg/mL anti-bovine IL-10 mAb (clone CC318; Bio-Rad, Hercules, CA, US) in PBS. A standard curve was performed with Recombinant Bovine Interleukin-10 (Bio-Rad), from 1000 to 8 pg/mL. Standards and supernatants were incubated for 2 h at room temperature before washing and incubation with 0.5 µg/mL anti-bovine IL-10 mAb for 1 h at room temperature (mouse anti-Bovine Interleukin-10:Biotin; clone CC320; Bio-Rad). Mabtech's streptavidin-HRP, was used similarly as used for bovine IL-8 quantification. Detection limit for bovine IL-10 was 8 pg/mL. IL-1 beta Bovine Uncoated ELISA Kit (Invitrogen) was used to determine bovine IL-1β concentration in cell culture supernatants, according to manufacturer's protocol (detection limit <31.3 pg/mL). Readings were performed at 450 nm and 570 nm in a BioTek™ µQuant Microplate

Reader using BioTek™ Gen5™ Data Collection and Analysis Software. Quantification limits were used when sample readings were below background values.

RNA extraction, cDNA synthesis and Real-Time qPCR

Total RNA extraction and cDNA synthesis were performed as described previously, using NZYol protocol and NZY First-Strand cDNA Synthesis Kit (both from NZYtech) (31). Synthesis of first-strand cDNA was performed in a BioRad T100^{TM} Thermal Cycler at 25 °C for 10 min, 50 °C for 30 min, and 85 °C for 5 min. After synthesis of cDNA, samples were kept at -20 °C. The best combination of reference genes was chosen using Normfinder software.

Five different housekeeping genes (Table 3) were tested: MARVEL domain containing 1 [*MARVELD1*] and β2 microglobulin [*B2M*] (10, 32), and Peptidylprolyl isomerase A [*PPIA*], Ubiquitously Expressed Prefoldin Like Chaperone [*UXT*] and Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta [*YWHAZ*]), for which primers were designed in this study with Primer-BLAST web tool developed by NCBI (30). Stability values for each candidate reference genes, assessed by Normfinder, are presented in Table 4. Although *UXT* was the most stable individually, the combination of *MARVELD1* and *PPIA* presented the best stability value (0.079; Table 5) and were chosen for gene expression analysis. Primers for *IL12A*, *IL12B* and *IL23A* were designed as above, while primers for *TNF, IL10, IL1B and IL6* were previously developed (Table 3).

Measurement of bovine *TNF*, *IL1B*, *IL6*, *IL10, IL12A, IL12B and IL23A* mRNA levels was performed in a CFX384 Touch Real-Time PCR Detection System using NZYSpeedy qPCR Green Master Mix (2×) ROX plus (NZYTech) in thin wall, skirted, clear Hard-Shell® 384-Well PCR Plates (Bio-Rad). Reactions were performed with 5 µL Master Mix, 0.2 mM of specific forward and reverse primers (all from Sigma-Aldrich), 3.6 µL H_2O and 1 µL cDNA. RT-PCR run 5 min at 95 °C for denaturation, followed by a 40 cycles' amplification at 95 °C for 5 s and 62 °C for 20 s. Gene expression analysis was performed using the formula $2^{-(\text{CT gene of interest-CT housekeeping gene})}$ according to the comparative threshold cycle method (36). The CT housekeeping gene corresponded to the CT geometric mean of *MARVELD1* and *PPIA*.

Table 3 – List of primers used for quantitative real-time PCR.

Gene	Primer[a] Sequence 5'-3'	Amplicon Size (bp)	GeneBank Accession Number or Reference
MARVELD1	F: GGCCAGCTGTAAGATCATCACA R: TCTGATCACAGACAGAGCACCAT	100	(32)
B2M	F: AAGTGGGATCGAGACCTGTAA R: GGACATGTAGCACCCAAGGTAA	191	(31)
UXT	F: CACGGATCTATGTGGCCCTT R: TAGCTCTCTAAGCCCCTCTAGC	176	NM_001037471.2
PPIA	F: GTGGCAAGTCCATCTATGGCG R: CCTCTTTCACCTTGCCAAAGTACC	184	NM_178320.2
YWHAZ	F: GCAAAAGACGGAAGGTGCTG R: ACTGGTCCACAATCCCTTTCT	236	NM_174814.2
TNF	F: CCAGAGGGAAGAGCAGTCCC R: TCGGCTACAACGTGGGCTAC	114	(33)
IL10	F: AGAACCACGGGCCTGACAT R: AGCTCACTGAAGACTCTCTTCACCTT	151	(34)
IL6	F: CCTGAAGCAAAAGATCGCAGA R: ATGCCCAGGAACTACCACAA	204	(31)
IL1B	F: AAACTCCAGGACAGAGAGCAAAA R: CTCTCCTTGCACAAAGCTCATG	126	(31)
IL12A	F: ACGCTACAGAAGGCCAGACAA R: ACTCTCATTCGTGGCTAATTCCA	135	NM_174355.2
IL12B	F: CCCGCATTCCTACTTCTCCC R: TCCTGAAGATGGGCTGTAC	208	NM_174356.1
IL23A	F: TGCACACCTACCAATGGGACA R: ATTCTTTGCAAGCAGGACTGAC	144	NM_001205688.1

[a]Primer direction: F- Forward, R- Reverse.

Table 4 – Expression stability of candidate reference genes, evaluated using NormFinder, in bovine monocyte-derived macrophages.

Gene name	Stability value	Rank
UXT	0.084	1
PPIA	0.110	2
MARVELD1	0.113	3
YWHAZ	0.118	4
B2M	0.179	5

Table 5 – Expression stability of the best pair of candidate reference genes, evaluated using NormFinder, in bovine monocyte-derived macrophages.

Best combination of two genes	MarvelD1 and PPIA
Stability value for best combination of two genes	0.079

Reactive-oxygen species production

To evaluate intracellular ROS-production, a cell-permeable fluorogenic probe (2',7'-dichlorodihydrofluorescein diacetate [H_2DCFDA], Invitrogen) was used in bovine MDM cultured in a separate plate, since timepoint used for ROS was much shorter than for cytokine mRNA expression and cytokine production. The procedure was performed according to manufacturer's instructions with minor modifications. Firstly, a concentrated stock solution of 40 mM H_2DCFDA was prepared and stored at -20 °C. On the experiment day, cell culture medium was removed, and cells were washed twice with warm, serum-free X-VIVO™ 15 haematopoietic medium (Lonza, Basel, Switzerland). Then, 100 µL of H_2DCFDA 25µM, prepared in X-VIVO™ medium, were added to the wells and cells were incubated for 30 min at 37 °C and 5% CO_2. Cells were washed twice to remove H_2DCFDA that was not internalized, and digested CV, NO, T or digested blanks, at 1:10 or 1:100-fold dilutions, were added to the wells. Non-stimulated cells were used as controls. A kinetic of ROS production was obtained in a BioTek Synergy™ 2 Plate Reader (BioTek Instruments, Vermont, US) for 60 min at 37 ºC. Readout was performed at 0, 15, 30, 45 and 60 min, with excitation and emission wavelengths of 485 and 535 nm, respectively.

Statistical Analysis

Data distribution was analyzed, and log transformations applied for a lognormal distribution. For the anti-inflammatory assays, all cytokine production and mRNA expression data were normalized to LPS-stimulated cells. Data were analyzed using the MIXED Procedure of the SAS software (SAS® OnDemand for Academics, SAS Institute Inc., Carry, NC, US). The model included the fixed effect of treatment, the random effect of animal blood donor and the random residual error. Results were considered statistically significant if $P<0.05$ and a tendency if $0.05 \geq P<0.1$. The Tukey-Kramer's post-hoc test was used to compare means of cytotoxicity, cytokine mRNA expression, cytokine production and ROS production. GraphPad software was used to construct the graphs (Version 9.4.0, San Diego, CA, US).

Results

Cytotoxicity

The effect of different dilutions of digested microalgae on bovine MDM viability was evaluated prior to the assessment of immune parameters. Cytotoxicity was significantly affected by treatment ($P<0.001$). None of the digested microalgae at 100-fold dilution,

blanks or LPS affected cell viability. All digested microalgae at 10-fold dilution, but not blank samples, induced an increased LDH release (P<0.001) by MDM comparatively to medium-cultured cells (Figure 2). This suggests the effects on cell viability are due to digested microalgae and not to the salts and enzymes present in the digestive simulated fluids.

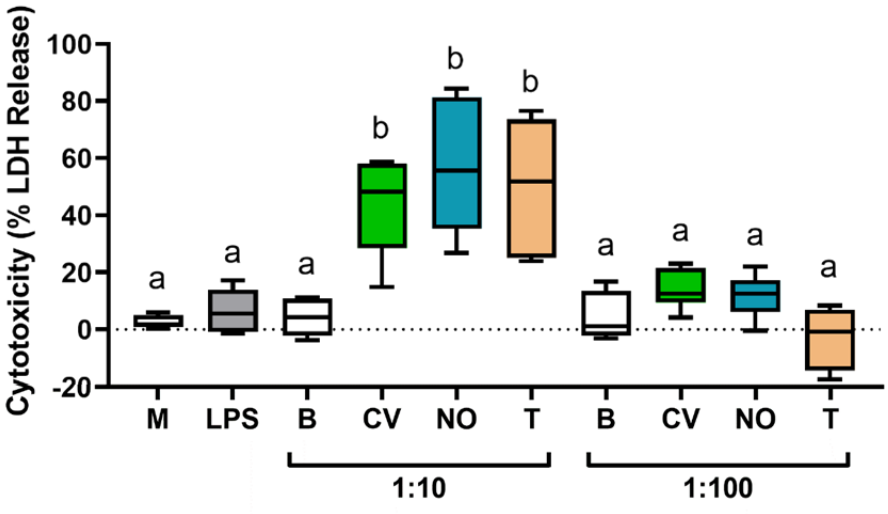

Figure 2 – Cytotoxicity assessed by LDH release by bovine monocyte-derived macrophages cultured for 24 h without stimulus (M) or stimulated with *E. coli* lipopolysaccharide (LPS) at 2 µg/mL, simulated digestive fluids and enzymes (blank – B), *in vitro* digested *Chlorella vulgaris* (CV), *Nannochloropsis oceanica* (NO) or *Tetraselmis* sp. (T) at 10- and 100-fold dilutions. Boxes represent the interquartile range between the 10th and 90th quartiles, and the horizontal line inside the box defines the median from six different biological samples. Whiskers represent the lowest and highest values. [a,b] Means with different superscript letters are significantly different (P<0.05).

Cytokine production and mRNA expression

Cytokine production and gene expression were evaluated 24 h upon stimulation of bovine MDM with digested CV, NO, T and blank at 100-fold dilution. Unstimulated cells and cells stimulated with LPS at 2 µg/mL were used as negative and positive controls, respectively. The 24 h timepoint for cytokine production and gene expression was selected according to previously studied time kinetics (31), since it allows the simultaneous determination of cytokine gene expression and protein production by bovine peripheral-blood monocytes. Production and mRNA expression of all cytokines assessed, using the 100-fold dilution of digested microalgae, were significantly affected by treatment (P<0.001). Bovine MDM increased the production of the pro-inflammatory cytokines TNF-α (Figure 3A), IL-6 (Figure 3B), IL-8 (Figure 3C), IL-1β (Figure 3D), and the anti-inflammatory cytokine IL-10 (Figure 3E) in response to 2 µg/mL LPS or digested CV, NO or T at 100-fold dilution. No significant production of any of those cytokines was observed in cells cultured with blank samples, which indicates that cytokines produced

are due to the recognition of microalgae digestion specific products and not due to the salts or enzymes present in simulated digestive fluids.

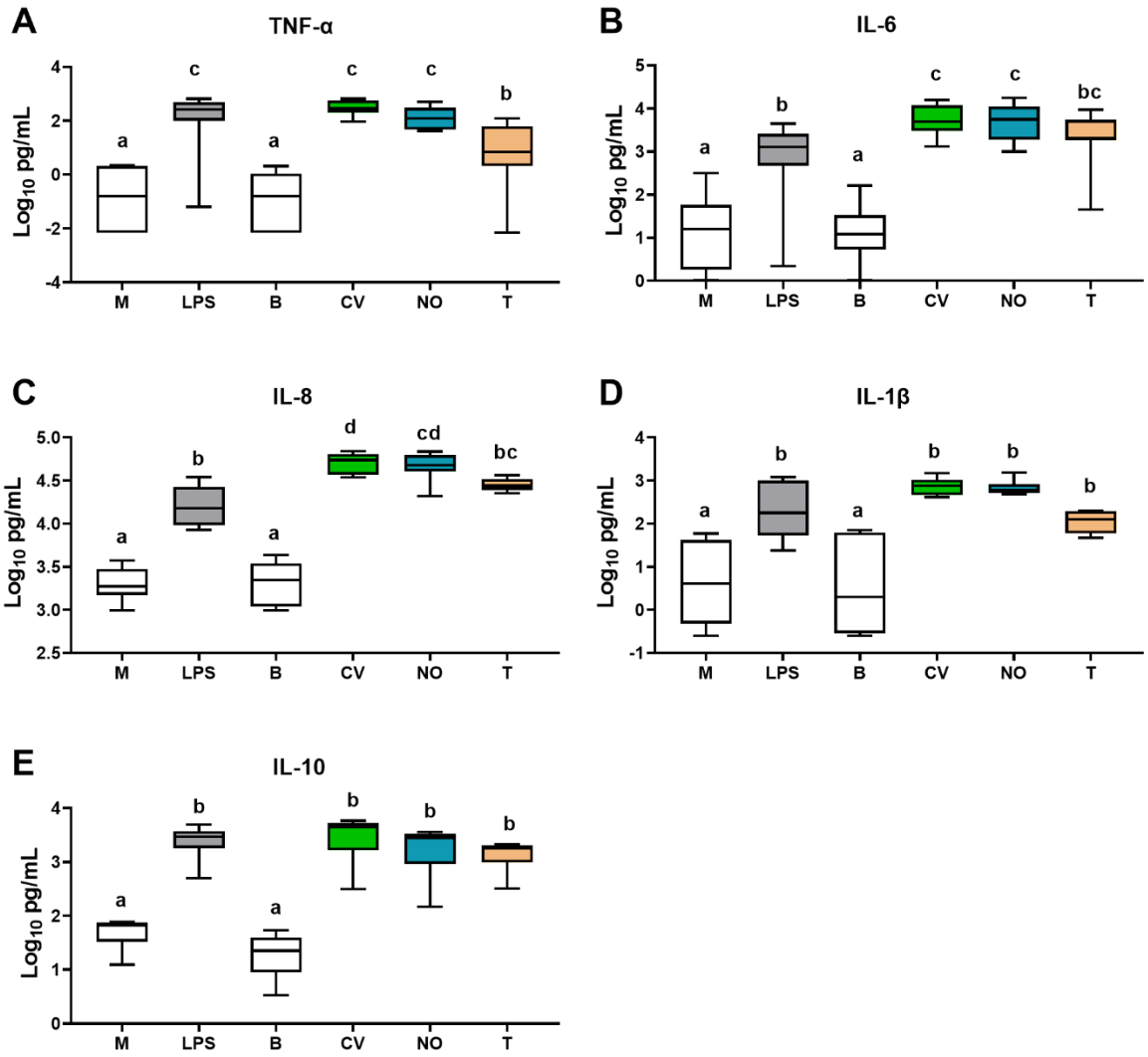

Figure 3 - Cytokine production, evaluated by ELISA in the supernatants of bovine monocyte-derived macrophages cultured for 24 h without stimulus (M) or stimulated with *E. coli* lipopolysaccharide (LPS) at 2 µg/mL, simulated digestive fluids and enzymes (blank – B), *in vitro* digested *Chlorella vulgaris* (CV), *Nannochloropsis oceanica* (NO) or *Tetraselmis* sp. (T) at 100 fold dilution. Boxes represent the interquartile range between the 10th and 90th quartiles, and the horizontal line inside the box defines the median of eight different biological samples for TNF-α, IL-6 and IL-8 and six different biological samples for IL-1β and IL-10. Whiskers represent the lowest and highest values. Data are presented as Log pg/mL of each analysed cytokine. [a,b,c,d] Means with different superscript letters are significantly different (P<0.05).

Production of IL-10 and IL-1β by bovine MDM was identical among microalgae-stimulated cells. All digested microalgae at 100-fold dilution and LPS induced the mRNA expression of *TNFA* (Figure 4A), *IL1B* (Figure 4B), *IL6* (Figure 4C), *IL10* (Figure 4D), *IL12A* (*IL12p35*; Figure 4E), *IL12B* (*IL12p40*; Figure 4F), and *IL23A* (*IL23p19*; Figure 4G), in bovine MDM comparatively to medium and blank-stimulated cells (*P*<0.001).

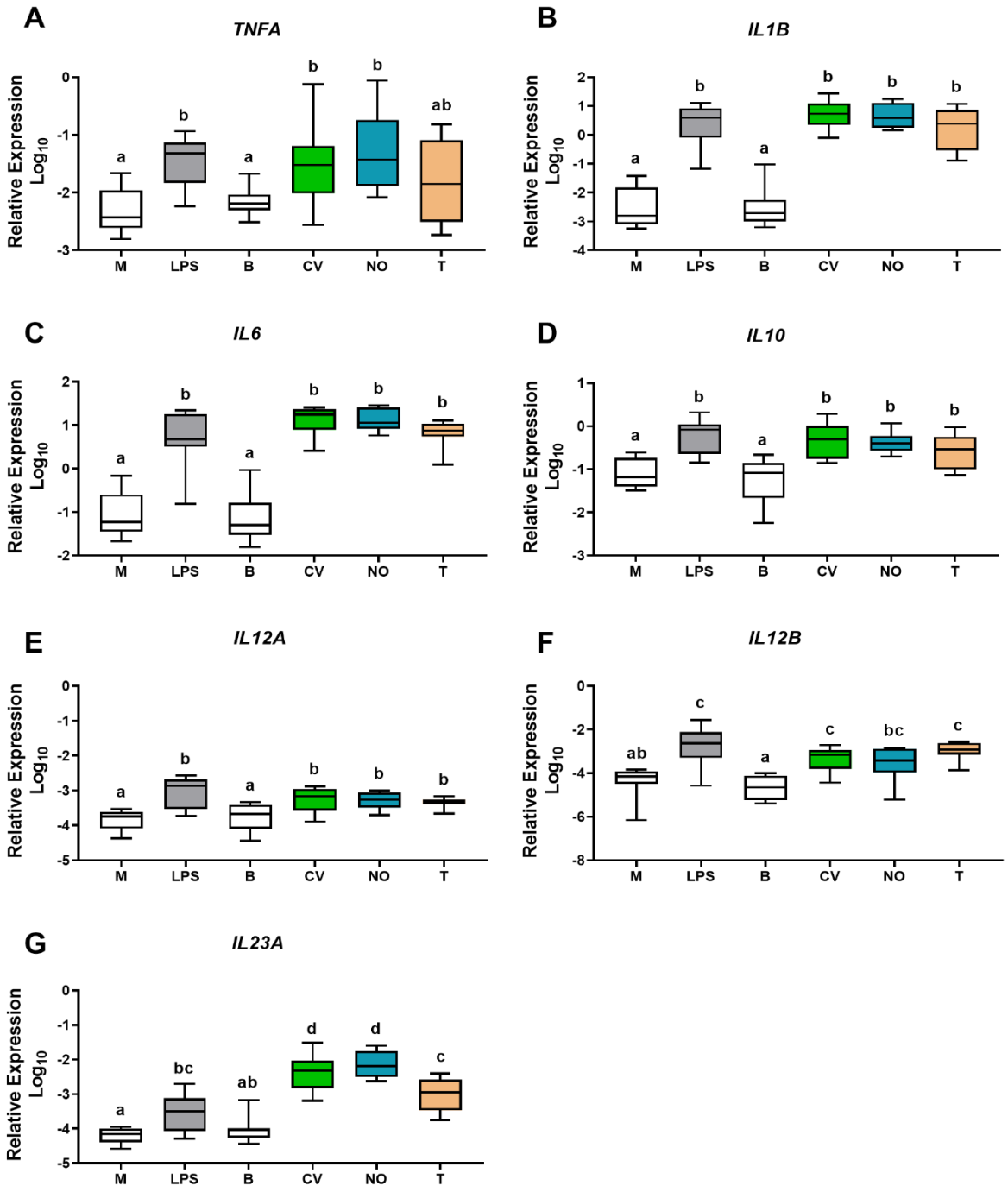

Figure 4 - Cytokine relative mRNA expression evaluated by RT-PCR in bovine monocyte-derived macrophages and normalized to the geometric mean of the mRNA expression of the reference genes *MARVELD1* and *PPIA*. Cells were cultured for 24 h without stimulus (M) or stimulated with E. coli LPS at 2 µg/mL, simulated digestive fluids and enzymes (blank – B), *in vitro* digested *Chlorella vulgaris* (CV), *Nannochloropsis oceanica* (NO) or *Tetraselmis* sp. (T) at 100-fold dilution. Boxes represent the interquartile range between the 10th and 90th quartiles, and the horizontal line inside the box defines the median of eight different biological samples. Whiskers represent the lowest and highest values. Data are presented as Log relative expression. ^{a,b,c,d} Means with different superscript letters are significantly different ($P<0.05$).

As observed for cytokine production, no effect of blank samples on cytokine gene expression (*P*>0.05) was observed, which reinforces the assumption that salts and enzymes from digestive fluids did not induce cytokine production nor gene expression. Most cytokine mRNA expression was identical among microalgae-stimulated cells, namely *IL1B*, *IL6*, *IL10*, *IL12A*, and *IL12B*. *TNFA* was increased in digested *C. vulgaris* and *N. oceanica*-stimulated cells, comparatively to blank cells, which is in accordance with the TNF-α levels measured in cell culture supernatants. *Tetraselmis* sp. stimulated cells did not differ from blank cells and *C. vulgaris* and *N. oceanica*-stimulated cells. *IL23A* was significantly increased in cells stimulated with *C. vulgaris* and *N. oceanica*, comparatively to *Tetraselmis* sp., although all microalgae samples induced overexpression of *IL23A* comparatively to medium and blank-stimulated cells.

Inhibition of NF-κB signalling pathway

NF-κB pathway was reported to have an important role in the maturation of dendritic cells and the activation of T-cells by microalgae extracts (35). As such, we intended to assess if production and mRNA expression of cytokines were also mediated through NF-κB pathway stimulation. We used TPCA-1, an IKK inhibitor, to block nuclear translocation of NF-κB and assessed the production and expression of cytokines upon stimulation with digested microalgae. Non-stimulated cells (medium cultured cells) and cells treated with TPCA-1 at 10 µM and further stimulated with LPS or digested microalgae produced comparable amounts of IL-8 (Figure 5A). TPCA-1 at 10 µM was therefore able to abrogate the production of IL-8 in stimulated cells. At 1 µM the production of IL-8 was slightly decreased, but not completely blocked (Figure 5A). Cytokine mRNA expression was also decreased by pre-treatment with TPCA-1 at 10 µM, namely *TNFA* (Figure 5B), *IL1B* (Figure 5C), *IL6* (Figure 5D), *IL10* (Figure 6A), *IL12B* (Figure 6C) and *IL23A* (Fig. 6D). Although there was a small numerical increase of TPCA-1 treatment on *IL12A* expression on LPS and microalgae-stimulated cells, it was not statistically significant (*P*>0.05; Figure 6B).

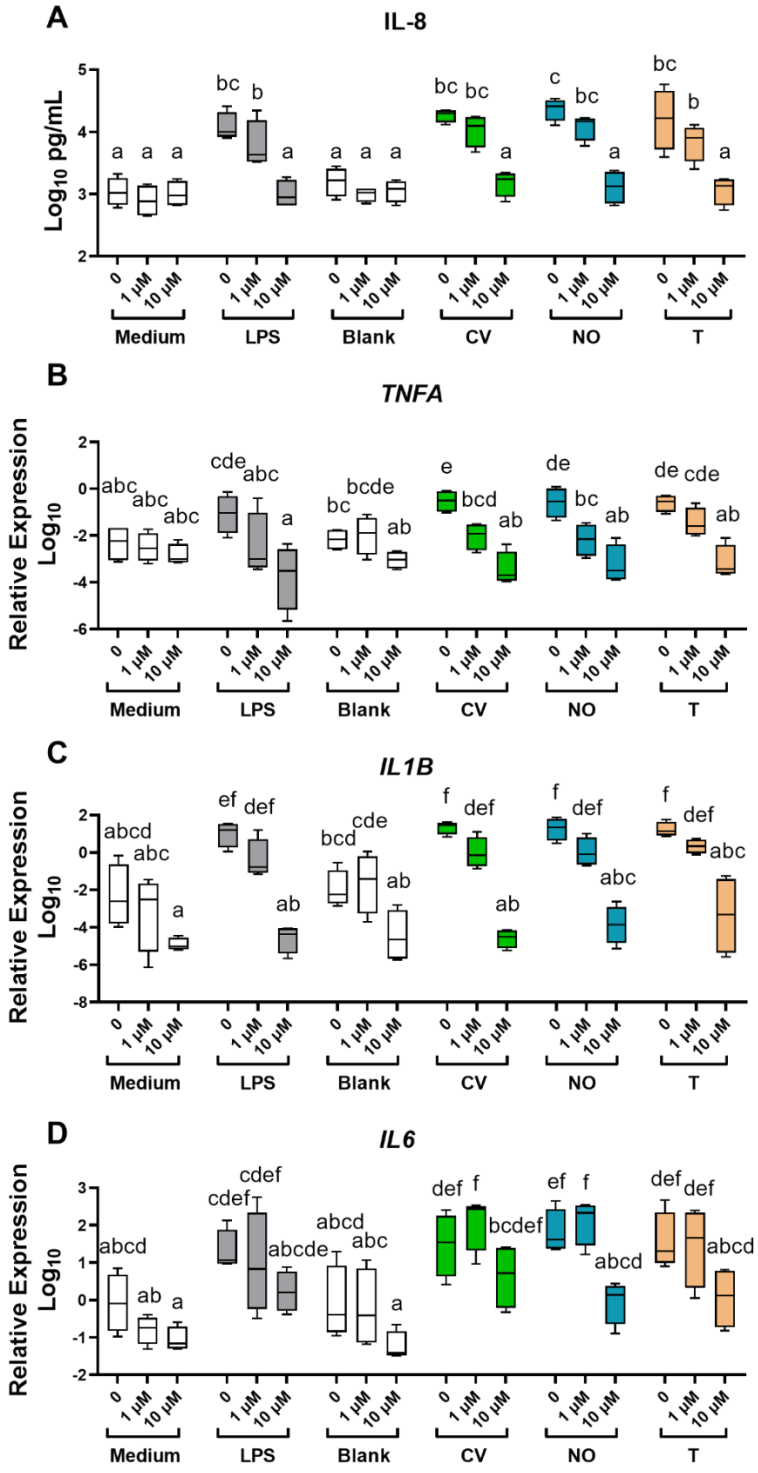

Figure 5 - Cytokine (IL-8) production (A) and relative mRNA expression (B-D) in bovine MDM. Cells were cultured with the IKK-2 inhibitor TPCA-1 at 1 or 10 µM for 1h prior to the 24 h stimulation with LPS at 2 µg/mL, simulated digestive fluids and enzymes (blank), *in vitro* digested *Chlorella vulgaris* (CV), *Nannochloropsis oceanica* (NO) or *Tetraselmis* sp. (T) at 100-fold dilution. Non-stimulated cells (Medium) were used as controls. Boxes represent the interquartile range between the 10th and 90th quartiles, and the horizontal line inside the box defines the median of four different biological samples. Whiskers represent the lowest and highest values. Data are presented as Log relative expression. ^{a,b,c,d,e,f} Means with different superscript letters are significantly different ($P<0.05$).

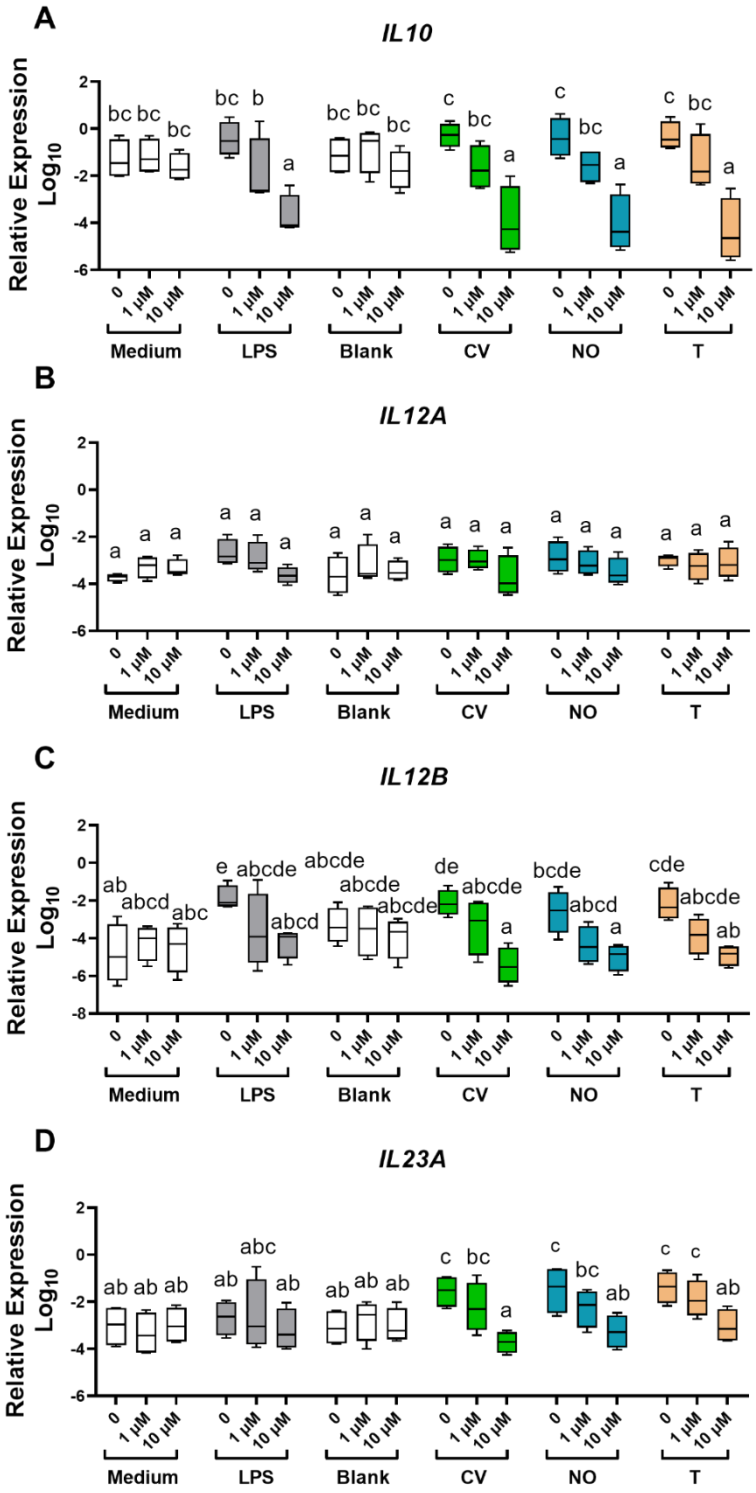

Figure 6 - Cytokine mRNA expression in bovine MDM. Cells were cultured with the IKK-2 inhibitor TPCA-1 at 1 or 10 µM for 1h prior to the 24 h stimulation with LPS at 2 µg/mL, simulated digestive fluids and enzymes (blank), *in vitro* digested *Chlorella vulgaris* (CV), *Nannochloropsis oceanica* (NO) or *Tetraselmis* sp. (T) at 100-fold dilution. Non-stimulated cells (Medium) were used as controls. Boxes represent the interquartile range between the 10th and 90th quartiles, and the horizontal line inside the box defines the median from four different biological samples. Whiskers represent the lowest and highest values. Data are presented as Log relative expression. [a,b,c,d,e,f] Means with different superscript letters are significantly different (P<0.05).

Anti-inflammatory assays

Anti-inflammatory effects of microalgae extracts have been widely acknowledged (36-38), namely through decreasing the production of pro-inflammatory cytokines in LPS-stimulated macrophages (39-41). In that line, we investigated whether pre-incubation of bovine MDM with digested microalgae could decrease pro-inflammatory cytokine production induced by LPS stimulation.

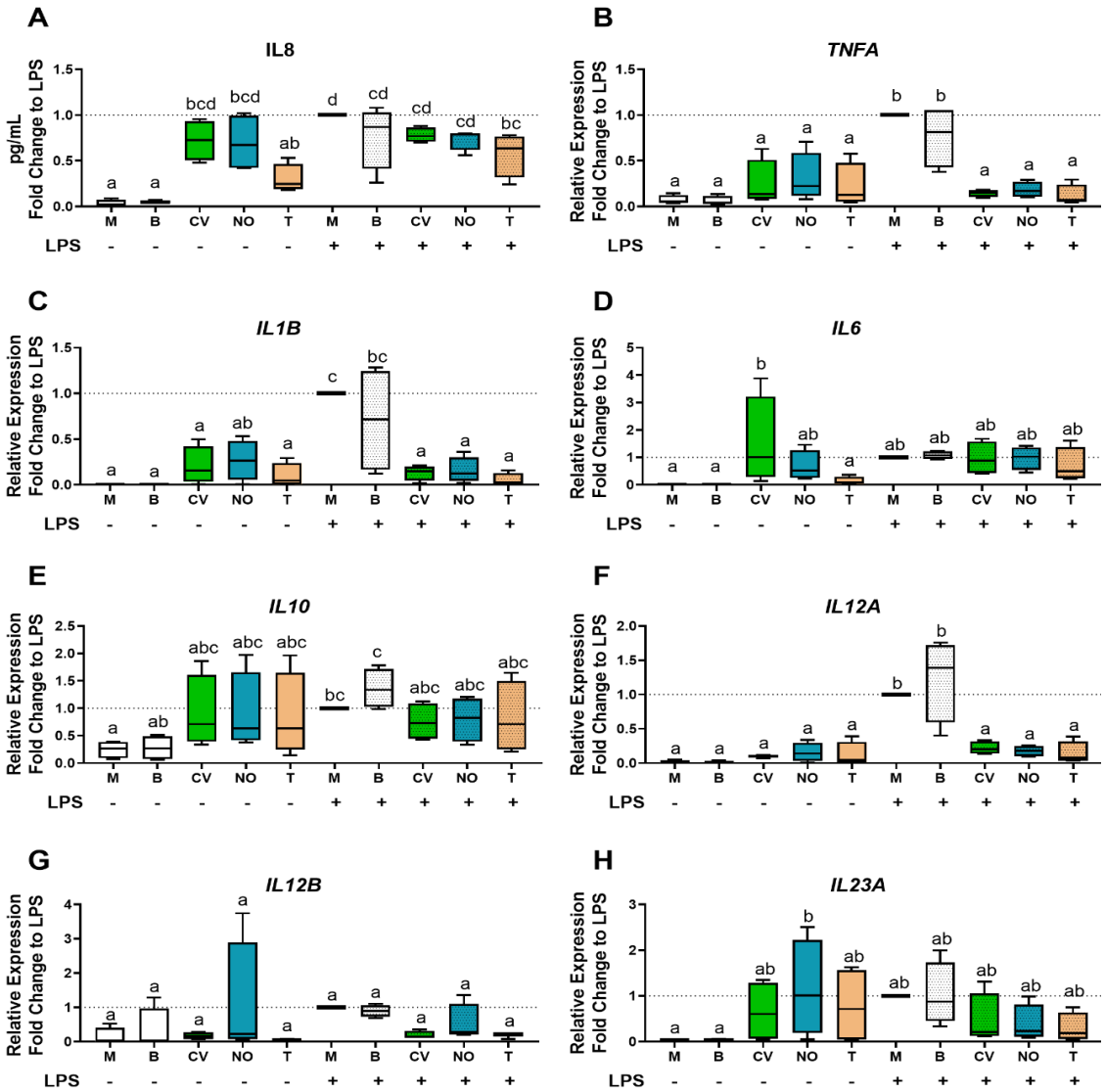

Figure 7 - Cytokine production (IL-8 – A), evaluated by ELISA in the supernatants of bovine monocyte-derived macrophages, and cytokine relative mRNA expression (B, C, D, E, F, G, H) evaluated by RT-PCR in bovine monocyte-derived macrophages and normalized to the geometric mean of the mRNA expression of the reference genes *MARVELD1* and *PPIA*. Cells were cultured with simulated digestive fluids and enzymes (blank – B), *in vitro* digested *Chlorella vulgaris* (CV), *Nannochloropsis oceanica* (NO) or *Tetraselmis* sp. (T) at 1000-fold dilution, and subsequently stimulated for 24 h with E. coli lipopolysaccharide (LPS) at 2 µg/mL. Non-stimulated cells were used as controls. Boxes represent the interquartile range between the 10th and 90th quartiles, and the horizontal line inside the box defines the median from four different biological samples. Whiskers represent the lowest and highest values. Data are presented as Log. ^{a,b,c,d} Means with different superscript letters are significantly different (P<0.05).

Indeed, cells stimulated with LPS at 2 µg/mL produced less IL-8 (Figure 7A) and expressed less *TNFA* (Figure 7B), *IL1B* (Figure 7C) and *IL12A* (Figure 7F) when pre-treated with digested microalgae at 1000-fold dilution (*P*<0.001). IL-8 production decreased significantly in cells pre-treated with *Tetraselmis* sp. comparatively to non-treated cells (P=0.043) and there was a particularly marked and significant decrease in *TNFA*, *IL1B* and *IL12A* mRNA expression in microalgae-treated LPS-stimulated cells (*P*<0.001). There was also a significant interaction effect between microalgae treatment × LPS stimuli concerning *IL6* (P=0.037; Figure 7D), *IL10* (0.001; Figure 7E) and *IL23A* (0.001; Figure 7H), but no differences were observed for LPS stimulated pre-treated without (blank cells) and with microalgae species. No effect of pre-treatment nor interaction effect was observed on the expression of *IL12B* (*P*>0.05; Figure 7G).

Reactive-oxygen species production

In addition to cytokine production, we hypothesized that digestion products of microalgae would also be able to induce ROS. Indeed, ROS production was significantly affected by stimuli (*P*<0.001; Figure 8) as all digested microalgae increased ROS production comparatively to medium and blank-stimulated cells at all time points.

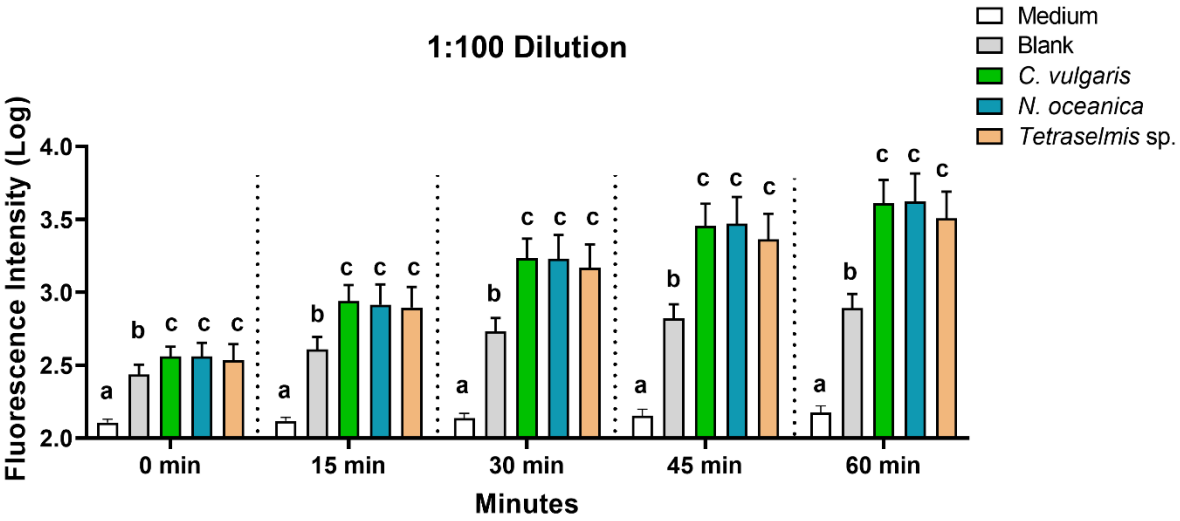

Figure 8 – Reactive-oxygen species (ROS) production by bovine monocyte-derived macrophages stimulated for up to 60 min with simulated digestive fluids and enzymes (black line and open black symbols) and *in vitro* digested *Chlorella vulgaris*, *Nannochloropsis oceanica* or *Tetraselmis* sp. at 100-fold dilution. Data are presented as Log. [a,b,c] Means with different superscript letters are significantly different (P<0.05).

However, differently from cytokine production and mRNA expression, blanks at 100-fold dilution also induced ROS production by bovine MDM (Figure 8).

Discussion

Biochemical composition of microalgae has leverage the interest for its use in human and animal nutrition (4, 6, 42, 43), while microalgal bioactive compounds, namely β-glucans, antioxidants such as carotenoids, tocopherol, flavonoids, and phenolic compounds, and PUFAs, have been reported to exert health-promoting properties (12, 44). As such, dietary microalgae inclusion has been suggested to promote the nutritional and functional value of animal feeding, namely to improve immune function (43). However, *in vivo* studies are scarce and most *in vitro* studies evaluate the effects of microalgae using either non-processed organisms or its extracts (3, 13). Yet, upon digestion, microalgae may suffer enzymatic and pH changes that may modify the cell wall and bioactive compounds' bioavailability (45). Eukaryotic microalgae species are poorly digested by monogastric animals due to their recalcitrant cell walls and high structural polysaccharides composition (46-48), which may limit the bioavailability of intracellular bioactive compounds to the animal. A similar pattern is expected by newborn calf. On the other hand, the complex structural polysaccharides may exert prebiotic effects in the lower gut of animals (49-51), supporting the growth of beneficial bacteria in the gut, which in turn control the proliferation of pathogenic organisms, and promoting gut and animal health (52). Prebiotics are reported to have immunomodulating properties either directly by regulating mucosal signalling and cytokine production, but also through its fermentation products (short-chain fatty acids) that nourish intestinal epithelium and regulate cytokine production and immune cell recruitment (53). Indeed, the prebiotic effects of *in vitro* digested *C. vulgaris*, *Desmodesmus maximus*, *Chlorococcum* sp. cf *hypnosporum*, and *Arthrospira* (formerly *Spirulina) platensis*, using INFOGEST's protocol, similarly to the methodology described in our study, were assessed on human colonic microbiota (54). The authors reported digested microalgae to present higher prebiotic effect than fructooligosaccharides, as they stimulated the growth of beneficial microorganisms and inhibited the development of undesirable bacteria *in vitro* (54). In another study, the effects of dietary *A. platensis*, *Haematococcus pluvialis*, *Phaeodactylum tricornutum* and *C. vulgaris* were evaluated in an *in vitro* canine gut model, which simulates the digestion and colonic fermentation (55). Small changes in fecal fermentation profile towards increased propionate and butyrate and bacterial composition were reported, although these were species-specific.

Even though the digestibility of the microalgae species used in this study was not determined, the cells were observed microscopically after the *in vitro* digestion procedure, and most were intact or slightly disrupted. Thus, we hypothesize that the dietary supplementation of these microalgae species to newborn calves will reach the

small intestine and the intact microalgae or its digestion products to be sampled by microfold (M) cells, located in the follicular-associated epithelium and specialized in sampling and delivering luminal antigens to mononuclear phagocytes such as dendritic cells and resident macrophages (56, 57). Dendritic cells and a particular macrophage population are also able to open tight junctions between epithelial cells, and sample luminal contents (21). It is possible, thus, that diet microalgae or their antigens will get in contact with intestinal immune cells. To the best of our knowledge, our study is the first assessing *in vitro* the effects of digested eukaryotic microalgae species on innate immune cells.

The three microalgae species studied (*C. vulgaris*, *N. oceanica* and *Tetraselmis* sp.) were selected for being among the most produced species in Europe for animal feeding (25) and for presenting different nutritional composition. *Chlorella vulgaris*, one of the most used microalgae in food, is particularly rich in protein (c.a. 50% DM; 49, 58, 59, 60), with well-balanced essential and non-essential amino acids, and a source of bioactive compounds, such as photosynthetic pigments (chlorophyll *a* and *b*), antioxidants (lutein, α-tocopherol, and carotenoids), B-complex vitamins, β-glucans, and Chlorella Growth Factor (CGF) (61-65). *Nannochloropsis* genus has been gaining increased interest as a source of long-chain ω-3 PUFA for food and feed industries (66, 67), but also due to its potential for biofuel production (68). The species *N. oceanica* is a particularly interesting source of EPA, since it may accumulate 5 to 18% of its dry weight (66, 67), which may further be increased by modulation of the cultivation abiotic conditions (69-71). *Tetraselmis* genus is robust, halotolerant, easy to culture, with high biomass productivity (72-74), and moderate protein and lipid content (75, 76) that may be further promoted by culture conditions (75, 77, 78). Biotechnology advances promoted microalgal biomass of quantity and quality in the past few decades, allowing for greater availability and diversity of microalgae species as feedstuff (79). However, current prices are still a limitation of its use on-farm (25, 80).

In this work, we demonstrate that digested *C. vulgaris*, *N. oceanica* and *Tetraselmis* sp. were able to induce the production of pro-inflammatory cytokines, namely TNFα, IL-6, IL-8, IL-1β and the anti-inflammatory cytokine IL-10 by bovine MDM. mRNA expression of *TNFA, IL1B, IL6, IL10, IL12A, IL12B and IL23A* were also increased in these cells upon stimulation with digested microalgae. Fluids and enzymes used for microalgae digestion did not induce the production or mRNA expression of any of the cytokines assessed. This is particularly important since using digested ingredients to screen for immunostimulant properties better mimics the interaction of dietary compound with gut immune cells. We have shown that cytokine production and mRNA expression were partially mediated through NF-κB signalling, since incubation with TPCA-1, an IKK

inhibitor, decreased microalgae-induced MDM activation. Our results are in line with a previous study where a polysaccharide fraction extracted from *Chlorella sorokiniana* was able to induce the maturation of dendritic cells and the activation and proliferation of T-cells through activation of NF-κB, and also PI3K/MAPK pathways (35).

To assess the anti-inflammatory properties of these three microalgae species, accordingly to what has been thoroughly described in other species (37, 38, 81) we assessed cytokine production and mRNA expression in LPS-stimulated bovine MDM previously treated with digested microalgae. Cells stimulated with LPS produced less IL-8 and expressed less *TNFA*, *IL1B*, and *IL12A* when pre-treated with digested microalgae at 1000-fold dilution, confirming the anti-inflammatory potential of all tested microalgae. Recently, Silva and colleagues reported that *Tetraselmis striata* had anti-inflammatory activity, namely by reducing *in vitro* TNF-α production by human LPS-stimulated macrophages (82). In another study, *Euglena gracilis* extracts reduced the production of TNF-α by primary human macrophages and by HT-29 cells (a human intestinal epithelial cell line) upon exposure to LPS (8). Interestingly, microalgae potential anti-inflammatory effects have even been proposed to minimize the cytokine storm syndrome observed in some COVID-19 patients (83). Soontornchaiboon and colleagues aimed at understanding the mechanisms underlying anti-inflammatory effects of violaxanthin from *Chlorella ellipsoidea.* They found a significant reduction of nitric oxide and prostaglandin E_2 production, though inhibition of translocation of NF-κB p65 subunit into the nucleus (84).

Most studies aimed at investigating the antioxidant effect of microalgae (85-87). However, microalgae may also have a ROS-inducing effect, as reported previously regarding antioxidant compounds such as vitamin C, alpha-tocopherol, beta-carotene and phenolic compounds, namely flavonoids (88, 89). This prooxidant effect of antioxidants seems to depend on the environmental conditions, such as the concentration of the antioxidant compound, its redox potential, pH, and the presence of redox ions (88, 89). In our work, we observed that digested microalgae induced the production of ROS in bovine MDM. It remains to be elucidated if digested microalgae could also have an antioxidant effect in bovine immune cells, when used prior to a strong ROS-inducing agent.

Our results suggest that microalgae supplementation exerts an immunomodulatory effect on bovine macrophages. Although simple, this *in vitro* model provides an insight into the response of monocyte-derived macrophages upon contact with digested *C. vulgaris*, *N. oceanica* and *Tetraselmis* sp., namely cytokine production, cytokine mRNA expression and ROS production. A more complex model, combining immune and intestinal cells would thus be helpful to clarify mechanisms of immune modulation with

microalgae, either using gut-on-chip or intestinal organoids, for example. These would allow for a better understanding and characterization of the complex interactions among dietary supplements, bovine intestinal epithelium and bovine immune cells.

Author Contributions

ARVP performed the experiments, data acquisition and analysis, and wrote the manuscript. AC and TL also participated in the experiments. CG, EGM and ICR assisted in the design of the experiments. ARJC and AJMF assisted in the interpretation of data. ARVP, AC, MRGM, and MV conceived and designed the experiments. AC, MRGM, and MV supervised the experimental work, assisted in data acquisition and analysis, and manuscript writing.

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Conflict of Interest

The authors have no conflicts of interest to declare.

CHAPTER IV

Development of healthy and inflamed *in vitro* intestinal models for dietary supplement evaluation

Development of healthy and inflamed *in vitro* intestinal models for diet supplement evaluation

Ana R. V. Pedro [1, 2, 3, †], Patrícia Rodrigues [4], Mafalda Leal [4], Maria Inês Guedes [4], Isabel R. Amado [4], Miguel Xavier [4], Manuel Vilanova[1,2], Margarida R. G. Maia [3], Alexandra Correia[1,2], Catarina Gonçalves[4] *

[1] Immunobiology Group, i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal

[2] Laboratório de Imunologia, DIMFF, ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[3] LAQV, REQUIMTE, ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[4] Department of Life Sciences, Food Processing Group, International Iberian Nanotechnology Laboratory, Braga 4715-330, Portugal

[†] Present Address: Escola Universitária Vasco da Gama, Coimbra, Portugal

Abstract

Co-cultures of intestinal epithelial cells and immune cells are helpful in the initial phase of screening for dietary compounds, namely for evaluating the immunomodulating activity of nutraceuticals. Hence, in this chapter, we describe the preliminary results from work developed with intestinal epithelial cells and immune cells, where we propose a novel *in vitro* model of intestinal epithelium, by combining a triple co-culture model of Caco-2/HT29-MTX/Raji cells, and a model of intestinal inflammation of Caco-2/THP-1 cells, to evaluate the anti-inflammatory properties of dietary supplements. We have also evaluated metabolic activity of non-differentiated Caco-2 cultured with particulate and soluble products of the digested microalgae, and the oxidative and antioxidant potential of *C. vulgaris* and *N. oceanica* on differentiated co-cultures of Caco-2/HT29-MTX. All digested microalgae at 10-fold dilution presented cytotoxic effects to Caco-2 cells. *C. vulgaris* and *Tetraselmis* sp. at 20-fold dilution were apparently safe to use on intestinal epithelial cells, *while N. oceanica* induced a significant decrease on cell metabolic activity. Particulate and soluble products of digested *C. vulgaris* and soluble products of digested *N. oceanica* induced the production of ROS by differentiated co-cultures of Caco-2/HT29-MTX cell lines, but when a stronger inducer of ROS was used, these microalgae presented scavenging properties, since the fold increase in ROS was limited comparatively to controls. It was not possible to draw conclusions on the model of intestinal inflammation and on ROS production by differentiated co-cultures of Caco-2/HT29-MTX, as the sample size of the experiments was too low. However, it appear that inflammation was not successfully induced, and further assays are thus needed to stablish this *in vitro* model.

Keywords

Co-culture; Intestine; Digested-microalgae; Metabolic Activity; Inflammation

Introduction

Caco-2 cell line is probably the most used cell type in *in vitro* models of intestinal epithelium (1). These cells share morphological and functional characteristics with enterocytes such as polarization (the existence of apical and basolateral sites), tight junction expression and a brush border on the apical site (2, 3). However, there are limitations to this model, as these cells are less permeable to paracellular transport due to higher expression of tight junctions, and they have only absorptive functions (4).

Hence, alternatives for *in vitro* models of intestinal barrier have been proposed, to better mimic physiologic conditions, such as Caco-2/HT29-MTX (5) and Caco-2/HT29-

MTX/Raji B co-cultures (6). HT29-MTX have shorter villi than Caco-2 and can differentiate and resemble mucous producing Goblet cells (4, 7). Typically, co-cultures of Caco-2/HT29-MTX are cultured in a 9 to 1 ratio, to simulate physiologic conditions (8). However, when Raji B lymphocytes are added to a Caco-2 culture, some of the latter lose the characteristics of an absorptive cell and acquire an M-type cell phenotype, which has a fundamental role in transporting particles from the gut lumen, which will be further processed by antigen-presenting cells and presented to T cells (6, 9). The intestinal epithelium is a barrier to microorganisms, but epithelial cells influence the activity of underlying immune cells. As complex as it is to mimic interactions between epithelial cells and the immune system, some models have been proposed to study the mechanisms of communication between these different cells, namely co-culture of Caco-2 cells with the monocytic cell line THP-1 (10). By inducing inflammation to any of these models, thereby creating a "leaky gut model" (11), it may also be possible to study the anti-inflammatory activity of dietary compounds (12).

Hence, considering the potential anti-inflammatory activity of digested-microalgae assessed on chapter III, we aimed at developing *an in vitro* model of intestinal epithelial cells, associated with immune cells, to assess the capacity of digested-microalgae to reverse inflammation induced by the addition of TNF-α to the cell culture. Simultaneously, the cytotoxic effects of digested microalgae to undifferentiated Caco-2 cells, as well as the oxidative and antioxidant effects of these microalgae to differentiated co-cultures of Caco2/HT29-MTX cells were evaluated.

Material and Methods

In vitro digestion of *Chlorella vulgaris*, *Nannochloropsis oceanica* and *Tetraselmis* sp.

Chlorella vulgaris (CV), *Nannochloropsis oceanica* (NO) and *Tetraselmis* sp. (T) were kindly supplied as spray-dried biomass, preserved in sealed bags protected from light, by Allmicroalgae Natural Products, S.A. (Pataias, Portugal). Static *in vitro* digestion of microalgae was performed exactly as described in Chapter III. Digestion procedure was adapted from a standardized protocol proposed within COST-Infogest network (13). Digested microalgae were centrifuged at 620 × *g*, for 10 min at 4 ºC, to obtain the supernatant (soluble part; Sol) of the digestion products. Whole digestion products (non-centrifuged) were also used in cell cultures and are herein referred as particulate microalgae (Part). The chosen dilutions, 10 or 20-fold of digested microalgae biomass (0.5 g of microalgae in the beginning of *in vitro* digestion), correspond to the estimated

concentrations to be found in calves' gut lumen after ingesting 2 or 1% (w/w) microalgae in milk replacer, respectively. These calculations were performed considering an average intake of 4 L of milk replacer, at a concentration of 140 g/L and a diluting factor of 10-fold due to digestion fluids produced in calves (approximately 10L in a calf weighting 50 kg) (14).

Cell cultures

Caco-2 cells (American Type Culture Collection, ATCC®, clone HTB-37™) were cultured in T-75 flasks with filter cap (Sarstedt, Nümbrecht, Germany) in Minimal Essential Medium (MEM) supplemented with 20% foetal bovine serum (FBS) (both PAN-Biotech GmbH, Aidenbach, Germany) and 1% Pen/Strep (100 U/mL of penicillin and 100 µg/mL of streptomycin) at 37 °C and 5% CO_2. HT29-MTX cells (European Collection of Authenticated Cell Cultures, clone E12, ECACC 12040401) were cultured at 37 °C and 5% CO_2, in T-75 flasks with filter cap (Sarstedt) using Dulbecco's Modified Eagle Medium (DMEM) (PAN-Biotech) with 10% FBS and 1% Pen/Strep. Both cell lines were split when reaching 70 to 80% of confluence (2 to 3 days). Cells were detached using Trypsin-EDTA (0.25% trypsin; 0.1% EDTA) for 5 min, centrifuged at 150 × *g* for 10 min at room temperature, and culture medium was changed.
Raji and THP-1 cell lines (ATCC®, clones CCL-86™ and TIB-202™, respectively) were cultured in T-75 flasks with filter cap in RPMI-1640 medium supplemented with 10% FBS, 4 mM L-glutamine, 1% Pen/Strep 10 mM HEPES (all from Sigma-Aldrich, St. Louis, MO, US) and 50 µM β-mercaptoethanol (Merck, Darmstadt, Germany).

Metabolic Activity (Resazurin Assay)

For assessing cell metabolic activity 1×10^4 non-differentiated Caco-2 cells were seeded per well, in 96-well plates, and incubated for 24 h at 37 °C and 5% CO_2. Culture medium was completely removed, and controls or samples diluted in supplemented MEM were added to each well for 4 h at 37 °C and 5% CO_2. Samples and controls were then removed and resazurin sodium salt (Sigma-Aldrich), diluted in supplemented MEM at 10 µg/mL, was added to each well. Plate was incubated for another 4 h at 37 °C and 5% CO_2. Cells in MEM and cells in MEM with 10% Phosphate Buffered Saline (PBS) were used as controls. To evaluate metabolic activity of microalgae without cells, non-digested and digested microalgae samples were 10-fold diluted in complete MEM and incubated in 96-well plates for 4 h with 10.0 µg/mL resazurin sodium salt solution. Metabolic activity was measured in a BioTeK® Synergy H1 microplate reader (Winnoski, VT, US7A) by quantifying fluorescence of resorufin (λex = 560 nm; λem = 590 nm). Metabolic activity

was calculated relatively to values obtained in cells cultured in MEM with 10% PBS.

Oxidant and antioxidant assay on differentiated Caco-2 and HT29-MTX co-culture

To form a differentiated monolayer, Caco-2 and HT29-MTX cell lines were cultured at a ratio of 9:1, respectively, at a total density of 4.4×10^4 cells per well, in 96 well-plates, for 21 days at 37 °C and 5% CO_2 in supplemented MEM. Production of reactive oxygen species (ROS) was assessed using 10 µM 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich). Briefly, after the 21-day differentiation period, media was removed and 10 µM DCFH-DA in Hank's Balaced Salt Solution (HBSS) was added to each well for 1 h at 37 °C and 5% CO_2. DCFH-DA was then removed and blanks, digested CV or digested NO, either soluble or particulate, were 20-fold diluted in HBSS and added for 4 h at 37 °C to perform the kinetics of ROS production, in a BioTeK® Synergy H1 microplate reader, by assessing fluorescence of dichlorofluorescein (DCF) (λex= 495 nm, λem= 525 nm). Increased DCF fluorescence intensity was used as indicative of the intracellular accumulation of ROS levels. As positive control of DCF peroxidation, cells were incubated with HBSS with hydrogen peroxide (H_2O_2) at 2.5mM. To evaluate antioxidant capacity of the samples tested, previously conditioned cells with microalgae samples were stressed with 2.5 mM (H_2O_2) and fluorescence was measured for 1 h. An analogue of Vitamin E (Trolox) was used, at 50 µg/mL, as a control of antioxidant ability.

Healthy and inflamed *in vitro* 2D intestinal models using Caco-2, HT29-MTX, Raji and THP-1 cell lines

Caco-2 and HT29-MTX cell lines were seeded (day 0) on 1 µm pore size Transwell™ inserts (Merck Millipore, Burlington, MA, US) in 12-well plates (1.1×10^5 cells per insert, at a ratio of 9:1) using supplemented MEM as described above. Cells were incubated for 21 days at 37 °C and 5% CO_2, with medium renewal every 3 days. A triple cell co-culture (Caco-2, HT29-MTX and Raji) was prepared according to a protocol previously described by Antunes et. al. (6). Briefly, 5×10^5 Raji cells were added to the basolateral side of the wells for the last 7 days of the differentiation period (day 14). A quadruple co-culture was performed based on the previous referred protocol and the methodology proposed by Kampfer et. al. (11). Briefly, on day 19, 1.8×10^5 THP-1 cells were cultured on 12-well plates and differentiated into macrophages with 100 nM (Phorbol Myristate Acetate) PMA (Sigma-Aldrich) for 24 h at 37 °C and 5% CO_2. PMA was then removed and differentiated THP-1 cells were kept overnight at 37 °C and 5% CO_2. On day 21, the inserts with the differentiated Caco-2/HT29-MTX monolayers were removed from the culture plates and inserted in the culture plates where THP-1 were differentiated (Figure 1).

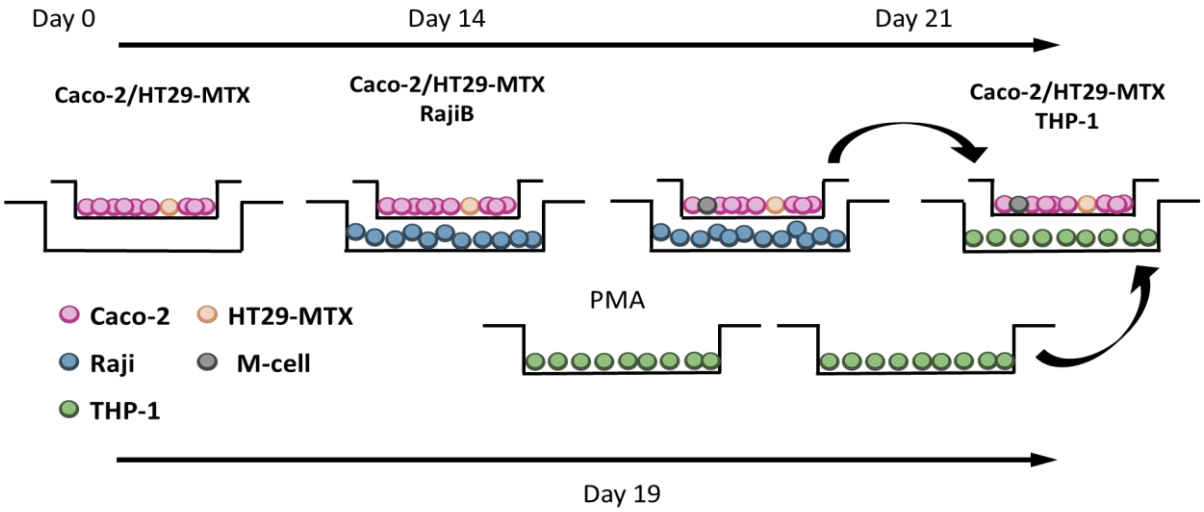

Figure 1 – Schematic representation of the 2D quadruple co-culture. On day 1, Caco-2 and HT29-MTX cells were seeded on Transwell™ inserts. On day 14, Raji cells were added to the basolateral compartment. On day 19, THP-1 cells were differentiated into macrophages in separate plates for 24 h. On day 21, differentiated Caco-2/HT29-MTX monolayers were inserted into THP-1 containing plates.

Inflammation was then attempted on 21-day triple-cultures (Figure 2), using 10 ng/mL recombinant Tumour Necrosis Factor alpha (TNF-α; BioLegend®, San Diego, CA, US) for 4 h, on differentiated monolayers of:

1) Caco-2/HT29-MTX cells;
2) Caco-2/HT29-MTX cells previously cultured with Raji cells;
3) Caco-2/HT29-MTX cells, previously cultured with Raji cells, and incubated with differentiated THP-1 cells on the basolateral compartment.

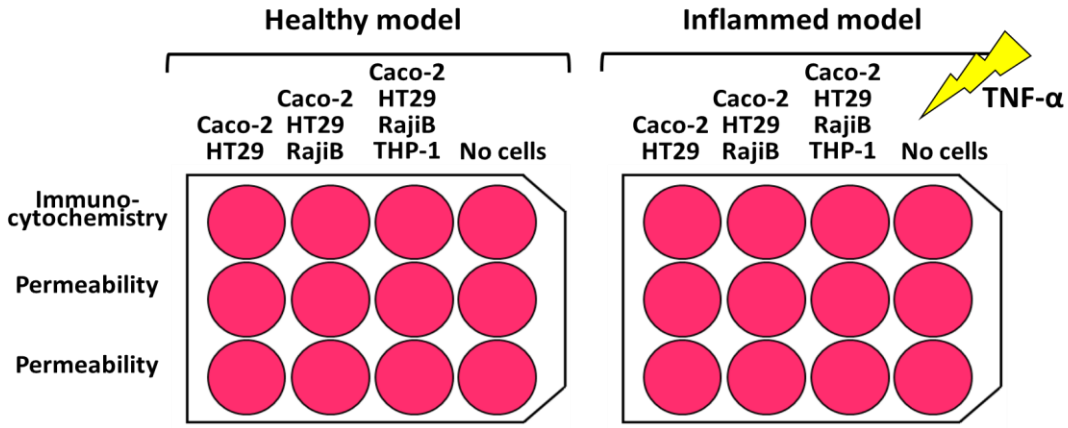

Figure 2 – Schematic representation of the inflammation experiment. On day 21, TNF-α was added to the apical compartment for 4 h, during which transepithelial electrical resistance was evaluated. Samples for cytokine measurement were collected after the 4 h challenge. Thereafter, immunocytochemistry and permeability assays were performed.

The goal of inducing inflammation with TNF-α was to obtain a 20 to 25% transepithelial electrical resistance (TEER) reduction in the stimulated monolayers. Co-cultures in MEM without TNF-α stimulation were used as controls. After the 4 h challenge, a Transwell™ insert were removed, of each condition, for immunostaining and evaluation by immunocytochemistry. In the remaining wells, medium was removed and HBSS-HEPES was added to the wells with the inserts to proceed with the permeability assay. Apical and basolateral medium samples were collected after the 4 h challenge period to measure cytokine production,

Transepithelial electrical resistance (TEER) evaluation

Transepithelial electrical resistance (TEER, Ω × cm^2) was assessed throughout the differentiation periods and also during challenge with TNF-α, and for 6 h after removing medium with TNF-α (permeability assay) to evaluate monolayer integrity of the differentiated co-cultures (Caco2/HT29-MTX, Caco2/HT29-MTX/Raji and Caco2/HT29-MTX/Raji/THP-1) using a Millicell® ERS-2 volt-ohmmeter (Merck Millipore, Billerica, MA, US) and STX electrodes. TEER values presented were obtained by subtracting the readings of cell-free inserts and multiplying by the surface area of the membrane (1.12 cm^2).

Elisa Assay

TNF-α, Interleukin (IL)-8 and IL-10 were quantified in apical and basolateral media of Caco-2/HT29-MTX/Raji/THP-1 model using Human TNF-alpha, Human IL-8/CXCL8 and Human IL-10 DuoSet ELISA kits (R&D Systems, Minneapolis, MN, US) according to the manufacturer's instructions (detection limits: 15.6, 31.2 and 31.2 pg/mL respectively). Readings were performed in a BioTek™ µQuant Microplate Reader, at 450 nm and 570 nm, using BioTek™ Gen5™ Data Collection and Analysis Software.

Immunocytochemistry

Monolayers were washed with PBS and fixed for 20 min at 4 °C with cold methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid). Samples were washed with an aqueous solution of 3% acetic acid and subsequently blocked with 2 % bovine serum albumin (BSA) (w/v) in PBS. For filamentous actin (F-actin) and occludin staining, cells were incubated with 0.1 µg/mL phalloidin-tetramethylrhodamine (phalloidin-TRITC) (Sigma-Aldrich) and 5 µg/mL Alexa Fluor® 488-conjugated anti-occludin monoclonal antibody (clone OC-3F10, Thermo Fisher, Massachusetts, US) at 4 ºC overnight. Nuclei were then stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI,

Invitrogen™) at 0.2 µg/mL for 10 min. Monolayers were kept in Fluoroshield mounting media (Sigma-Aldrich) until use. A Zeiss LSM780 confocal laser scanning microscope (Oberkochen, Germany), using 405-nm and 561-nm diode lasers and a 488-nm argon laser were used to obtain images of the different monolayers. Image analysis was performed in ImageJ 1.53c software (National Institutes of Health, US), using the same brightness/contrast adjustment parameters in all images obtained. Z-stacks were merged to obtain mean fluorescence intensities of every condition. Confocal micrographs are displayed as a composite of four fields per sample

Permeability Assay

Passive transcellular permeability studies were performed using propranolol hydrochloride as a reference standard of high permeability. Propranolol was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and further diluted in HBSS-HEPES (25 mM HEPES) to achieve a final concentration of 100 µM. Five hundred µL of 100 µM propranolol were added to the apical sites of the differentiated co-cultures (Caco2/HT29-MTX, Caco2/HT29-MTX/Raji and Caco2/HT29-MTX/Raji/THP-1) either without stimulus (control) or stimulated with TNF-α. Monolayers were kept at 37 ºC and 5% CO_2 throughout the experiment. After 1 h, 2 h, 3 h and 4h, 100 µL of media were collected from the basolateral sites, to quantify propranolol, and 100 µL HBSS were added each time media was collected. TEER was assessed, as previously described, throughout the assay. Quantification of propranolol in basolateral collected samples was performed in an ultra-high performance liquid chromatography system (UHPLC Agilent 1290 Infinity II, Agilent Technologies, Santa Clara, CA, US) equipped with a Kinetex® LC column (2.6 µm XB-C18 100 Å) from Phenomenex (Phenomenex, Inc., Torrance, CA, US). Column temperature was at 4 °C and mobile phase was a mixture of 3% acetonitrile and 97% 25 mM ammonium acetate (v/v) pH 3.0 under a flow rate of 1 mL/min. Propranolol was eluted and monitored with a Diode Array Detector (DAD, Agilent Technologies) at 230 nm, with 7.7 min retention time. Propranolol concentration was determined by interpolating the area of propranolol peak in the calibration curve (0.5 to 50 µg/mL). Apparent permeability coefficient (Papp) was determined using the following equation: $Papp\ (cm/s) = dQ/dt \times V/(A \times C_0)$, where dQ/dt is the permeability rate, measured in µM/s (corresponds to the slope of the cumulative increase in propranolol concentration in the basolateral chamber, over time), V is the volume of the basolateral chamber measured in cm^3, A is the area of the insert (1.12 cm^2), and C_0 is the initial concentration of propranolol added to the apical compartment (100 µM).

Statistical Analysis

Statistical analysis of metabolic activity was performed using One-Way ANOVA and Dunnett's multiple comparison test. Results from cell metabolic activity of blank and microalgae-conditioned cells were compared to PBS conditioned cells. Results from intrinsic metabolic activity of microalgae (cultured without cells) were compared to medium only (without cells). Statistical analysis of cytokine production and permeability were performed using Two-Way ANOVA with Šídák multiple comparison test. Results were considered statistically significant whenever $P<0.05$. Statistical analysis and graph construction was done using GraphPad Prism 9.4.0 (GraphPad software, San Diego, CA, US). It was not possible to perform statistical analysis of ROS, TEER and immunocytochemistry assays due to the sample size.

Results

Metabolic Activity (Resazurin Assay)

The metabolic activity of Caco-2 cells, evaluated by the resazurin assay, incubated with digestion products of different microalgae was assessed as an indicator of cell viability. Compared to PBS, incubation with 10-fold dilutions of digested microalgae significantly decreased cell metabolic activity ($P<0.0001$, Figure 3A), similarly to particulate blanks. Digestion products of CV and T at 20-fold dilution did not significantly reduce cell metabolic activity compared to PBS (Figures 3B and 3F). Digestion products of NO induced a significant decrease in cell metabolic activity, more meaningful when particulate samples were used (Figure 3D). Particulate non-digested NO also presented some toxicity, even at 20-fold dilution (Figure 3D).

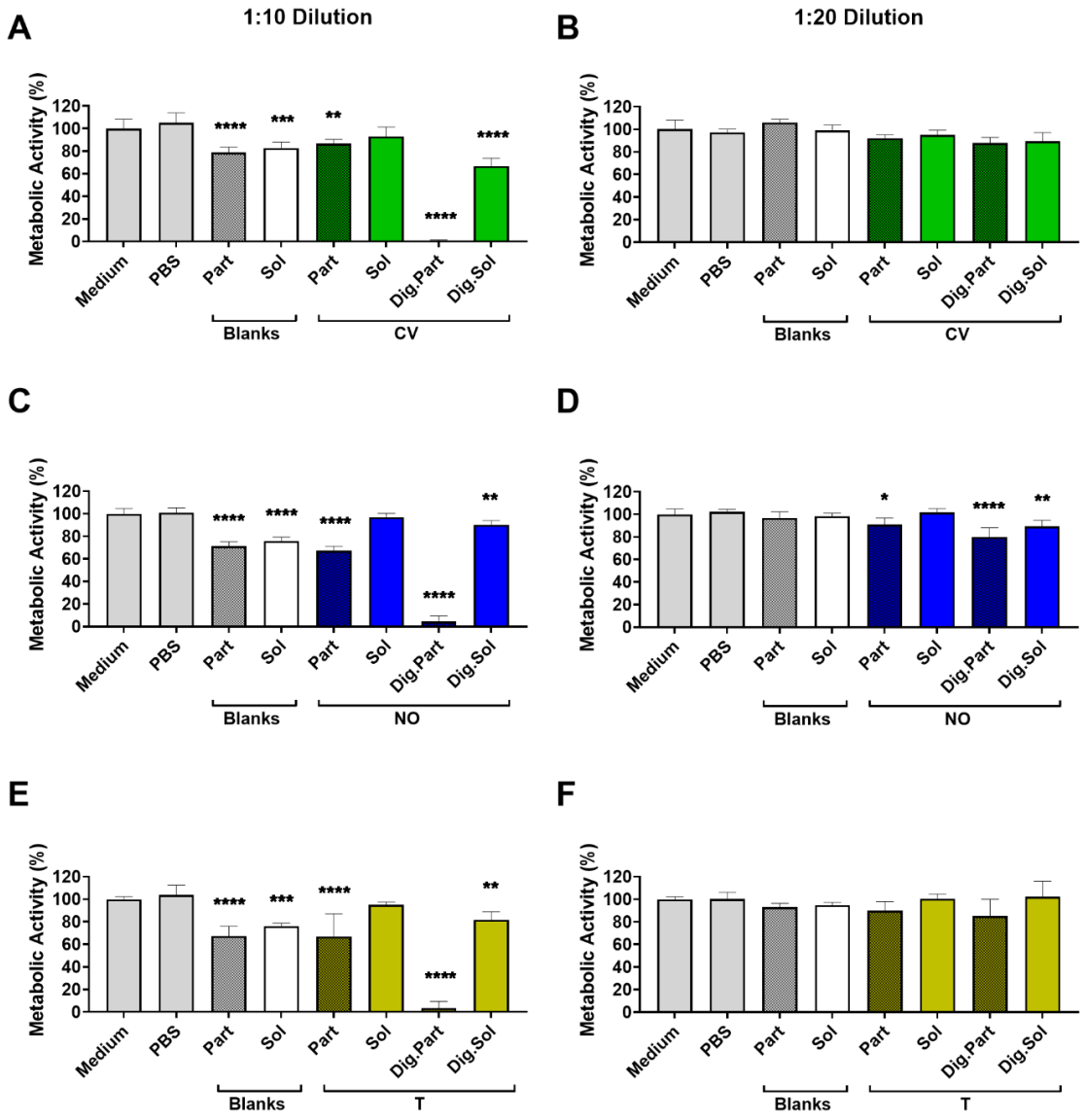

Figure 3 – Metabolic activity of Caco-2 cells cultured for 24 h with medium or PBS, particulate and soluble blanks, non-digested particulate fraction of microalgae (Part), non-digested soluble fraction of microalgae (Sol), digested particulate fraction of microalgae (Dig.Part) and digested soluble fraction of microalgae (Dig.Sol), all at 10 or 20-fold dilutions in complete medium. All conditions were compared to PBS cultured cells. CV- *C. vulgaris*; NO – *N. oceanica*; T – *Tetraselmis* sp. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Metabolic activity of digested and non-digested cultured microalgae was also assessed to ensure the fluorescence obtained was not due to live microalgae nor symbiotic microorganisms eventually present in the microalgae biomass samples. Actually, none of the cultured samples of microalgae presented reading values above those of control (supplemented medium with no Caco-2 cells, Figure 4).

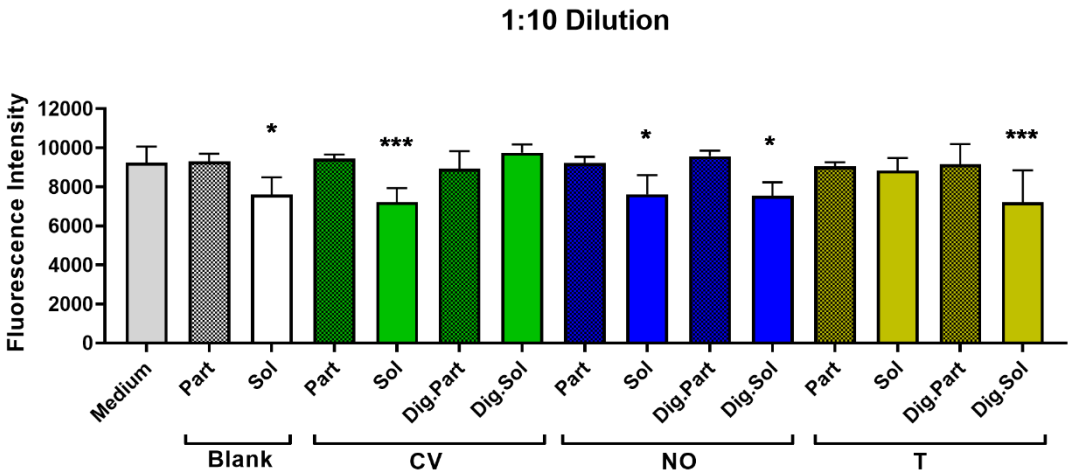

Figure 4 – Fluorescence Intensity of medium or particulate and soluble blanks, non-digested particulate fraction of microalgae (Part), non-digested soluble fraction of microalgae (Sol), digested particulate fraction of microalgae (Dig.Part) and digested soluble fraction of microalgae (Dig.Sol), cultured for 24 h, at a 10-fold dilution in complete medium. All conditions were compared to medium wells with no Caco-2 cells. CV- *C. vulgaris*; NO – *N. oceanica*; T – *Tetraselmis* sp.* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Oxidative and antioxidant assay on Caco-2 and HT29-MTX co-culture

The oxidative effect of digested CV and NO was assessed in differentiated co-cultures of Caco-2 and HT29-MTX cells. Their antioxidant capacity was additionally assessed upon inducing oxidative stress with H_2O_2. Although it was not possible to perform statistical analysis due to the sample size, these data suggest that particulate and soluble fractions of CV and soluble fraction of NO may induce the production of ROS (Figure 5) whereas digestion blanks and particulate fraction of digested NO led to numerical lower and similar ROS production.

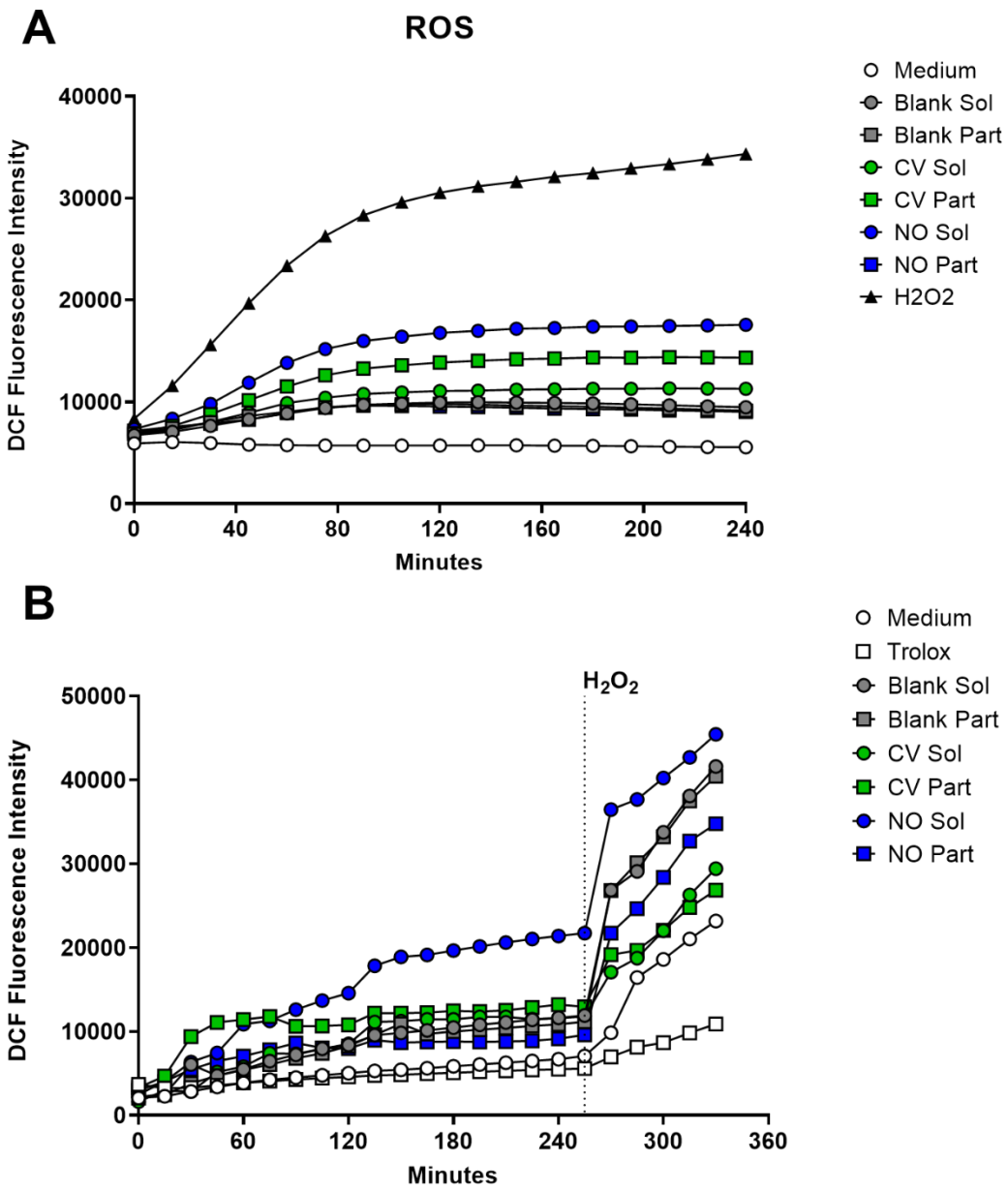

Figure 5 – Reactive-oxygen species (ROS) production evaluated in (A) differentiated co-cultures of Caco-2 and HT29-MTX cells stimulated with medium, H_2O_2, 20-fold diluted digestive fluids and enzymes (Blank; black line and grey symbols), digested *Chlorella vulgaris* (CV; black line and green symbols) or *Nannochloropsis oceanica* (NO; black line and blue symbols). Evaluation of ROS in (B) differentiated co-cultures of Caco-2 and HT29-MTX cells stimulated with medium, Trolox, 20-fold diluted digestive fluids and enzymes (Blank; black line and grey symbols), digested *Chlorella vulgaris* (CV; black line and green symbols) or *Nannochloropsis oceanica* (NO; black line and blue symbols) and subsequent evaluation of the antioxidant activity of these compounds upon stimulation with H_2O_2 (dashed line).

When a potent oxidant (H_2O_2) was added to the co-cultures, 250 min after cell stimulation with the microalgae products, the fold increase in ROS production of cells cultured with both forms of digested CV or with soluble fraction of digested NO was less pronounced

than the increase observed in control cells, blank-conditioned cells and digested particulate NO conditioned cells (Figure 6). The molecule Trolox, used here as a positive control of antioxidant activity, effectively counteracted the production of ROS in response to H_2O_2. This suggest that soluble and particulate products of digested CV and soluble fraction of NO might present antioxidant capacity (Figure 6).

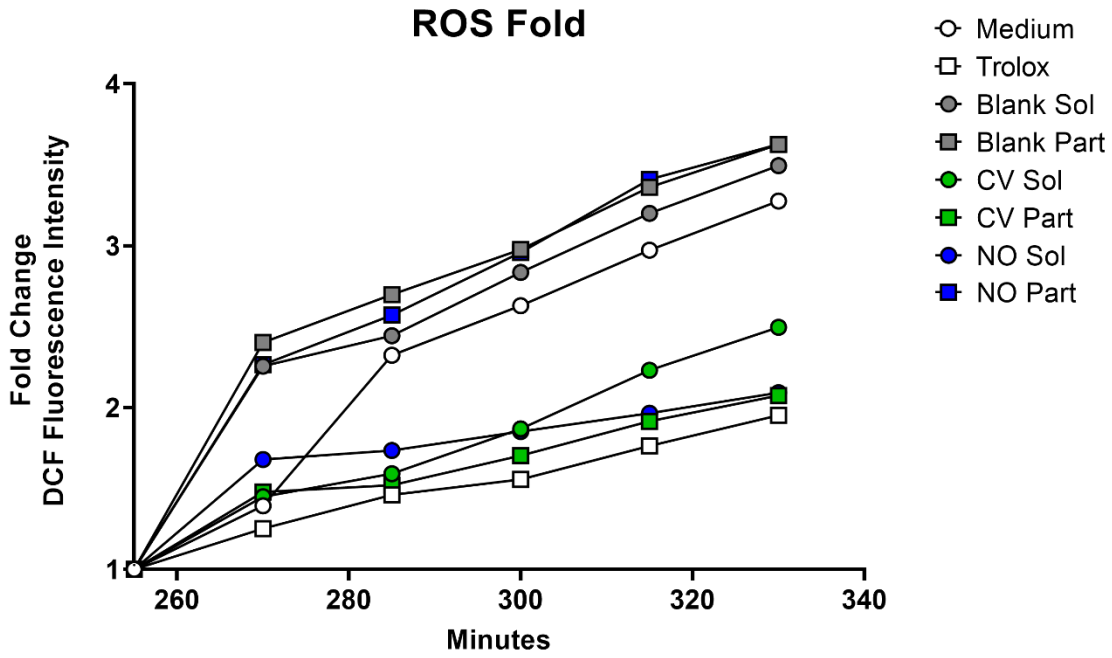

Figure 6 – Fold-change of reactive-oxygen species (ROS) produced after the addition of H_2O_2, illustrated in Figure 7B, produced by differentiated co-cultures of Caco-2 and HT29-MTX cells previously conditioned with medium, Trolox, 20-fold diluted digestive fluids and enzymes (black line and grey symbols), digested *Chlorella vulgaris* (CV, black line and green symbols) or *Nannochloropsis oceanica* (NO, black line and blue symbols).

In vitro model of intestinal inflammation using Caco-2, HT-29MTX, Raji B and THP-1 cell lines

This innovative co-culture proposal was conceptualized to establish a model suitable for the assessment of anti-inflammatory properties of digested microalgae, in both healthy and inflamed intestinal epithelia, using differentiated innate immune cells (macrophages) in the basolateral compartment. In this model, Raji cells are added to the Caco-2/MT29-MTX co-cultures to induce the differentiation of a few Caco-2 cells into M-like cells (15).

Transepithelial Electrical Resistance

TEER progressively decreased in co-cultures of Caco-2 and HT29-MTX cells after the addition of Raji at day 14 in the basolateral compartment (Figure 7A). Even though no

statistical analysis was performed, these values are in broad accordance with values obtained by other authors (6).

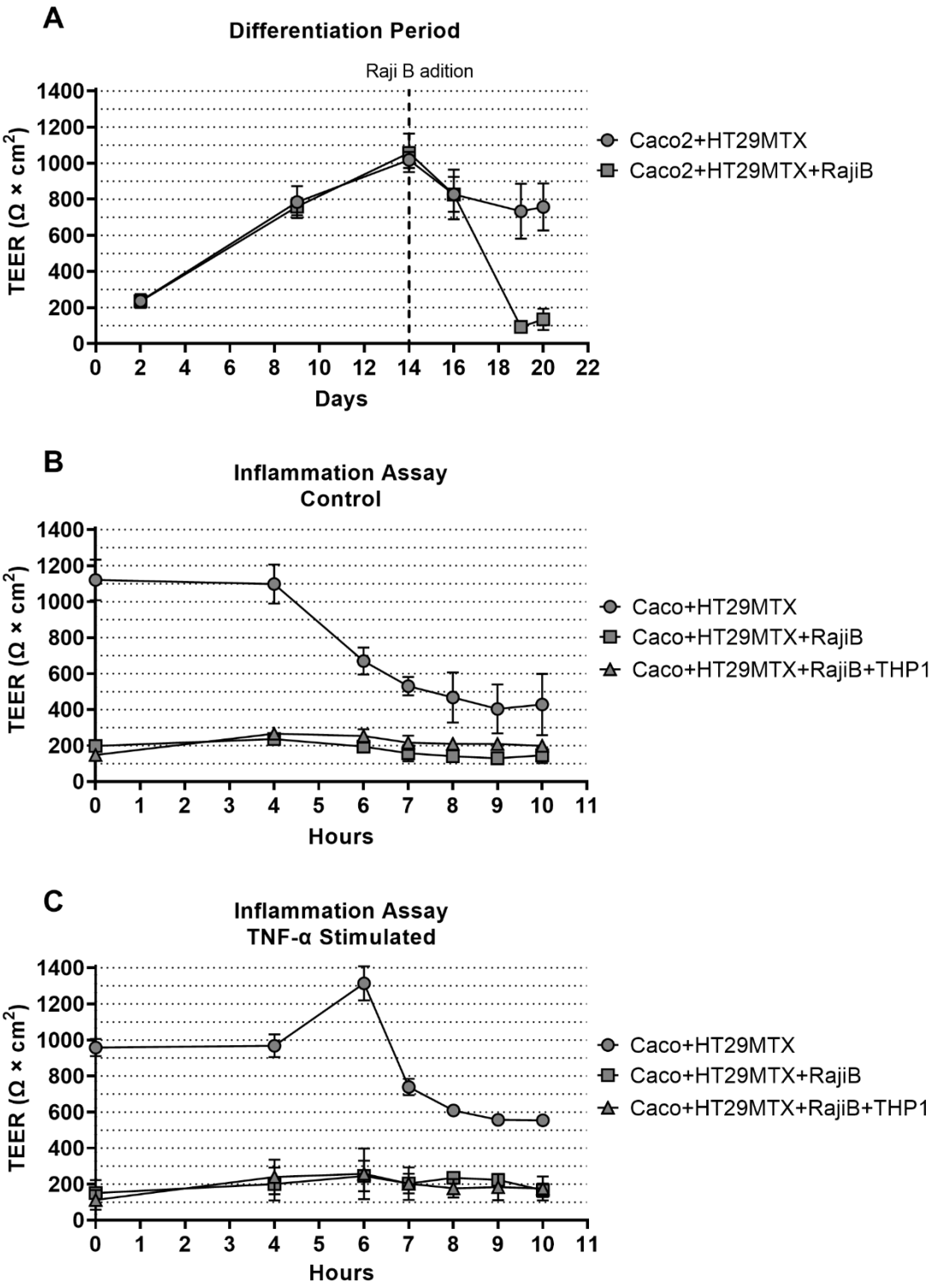

Figure 7 – TEER values obtained throughout differentiating period of Caco-2/HT29-MTX co-cultures and Caco-2/HT29-MTX/Raji co-cultures (A). TEER measured during inflammation assay in control co-cultures (A) and TNF-α-stimulated co-cultures (C).

Caco-2/HT29-MTX co-cultures maintained higher TEER values throughout the differentiating period, suggesting a tighter epithelium than co-cultures of Caco-2/HT29-MTX/Raji, most likely due to higher expression of tight junction proteins. However, a slight decrease was observed in this co-culture from day 14 on, which could be explained by technical issues that have occurred in this assay, namely temperature fluctuations that can negatively TEER measurement (16). During the inflammation assay, although TNF-α seemed to increase the TEER of Caco2/HT29-MTX co-culture at 6 h post stimulus, this effect was transient. TEER values of the remaining co-cultures were very similar between control and TNF-α-stimulated models (Figures 7B and 7C) suggesting that 10 ng/mL of TNF-α for 4 h were not sufficient to affect the intercellular junctions and induce a model of “leaky gut”. A decreased TEER would be expected in the model of inflamed epithelium (after addition of TNF-α), comparatively to that of control healthy epithelium (without TNF-α). No such effect was obtained in this experiment.

Cytokine Production

In that same line, IL-8 and IL-10 concentrations (Figures 8B and 8C) in the media of the apical and basolateral compartments appeared not to be different between control and TNF-α-stimulated models (Figure 9). TNF-α levels were high in the medium collected from the apical compartment of the “inflamed” model and correspond to the recombinant protein used to stress the cells, and not to protein produced by cells from the co-culture (Figure 9A). However, TNF-α levels were not increased in media from the basolateral side, irrespective of the model used (Figure 8).

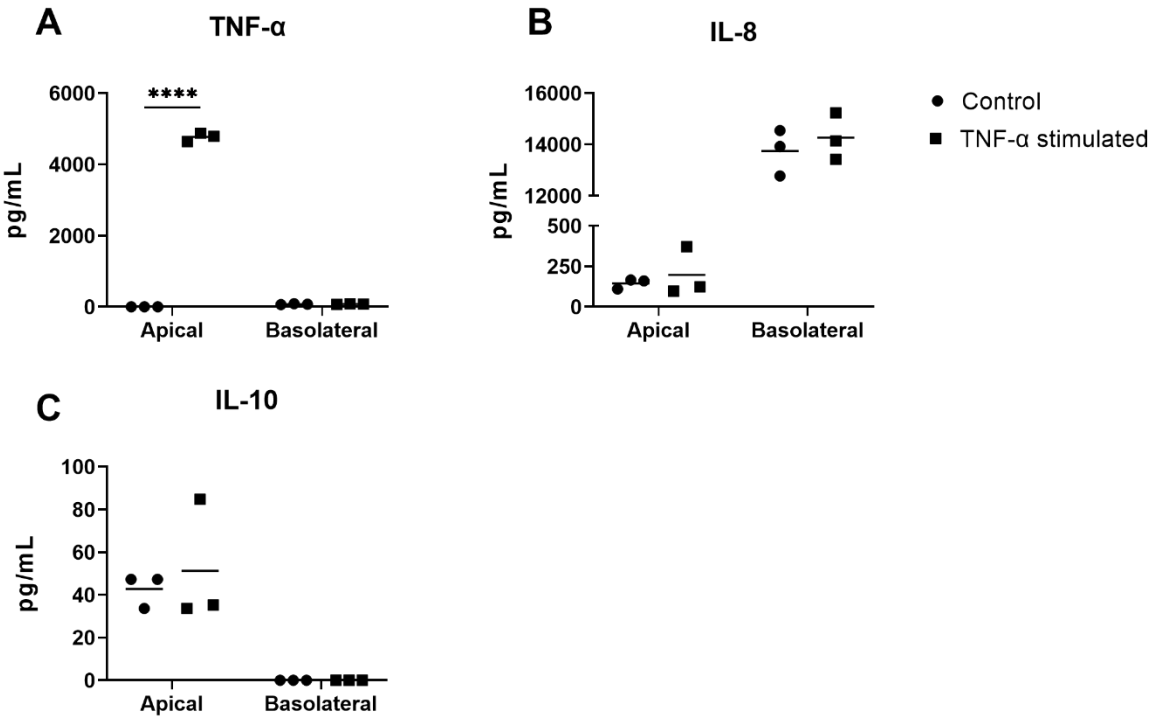

Figure 8 – Cytokine concentration (TNF-α, IL-8 and IL-10) on the apical and basolateral compartments of control (full black circles) and TNF-α-stimulated (full black squares) co-cultures of Caco-2, HT29-MTX, Raji and THP-1 cell lines after 4h challenge with TNF-α. **** *P*<0.0001.

Permeability Assay

Propranolol was added after the 4 h TNF-α challenge and its concentration was determined every hour in the basolateral compartment (Figures 9A and 9B). Apparent permeability coefficient was then calculated from the concentrations obtained over time (Figure 9C). Although preliminary, these results suggest that no differences were observed in propranolol concentrations, nor in apparent permeability coefficients, between control and TNF-α stimulated co-cultures. Propranolol concentrations determined in the basolateral media of the Transwell™ system were identical between co-cultures and were transported at the same rate over time. Passive transcellular transport was thus identical between conditions.

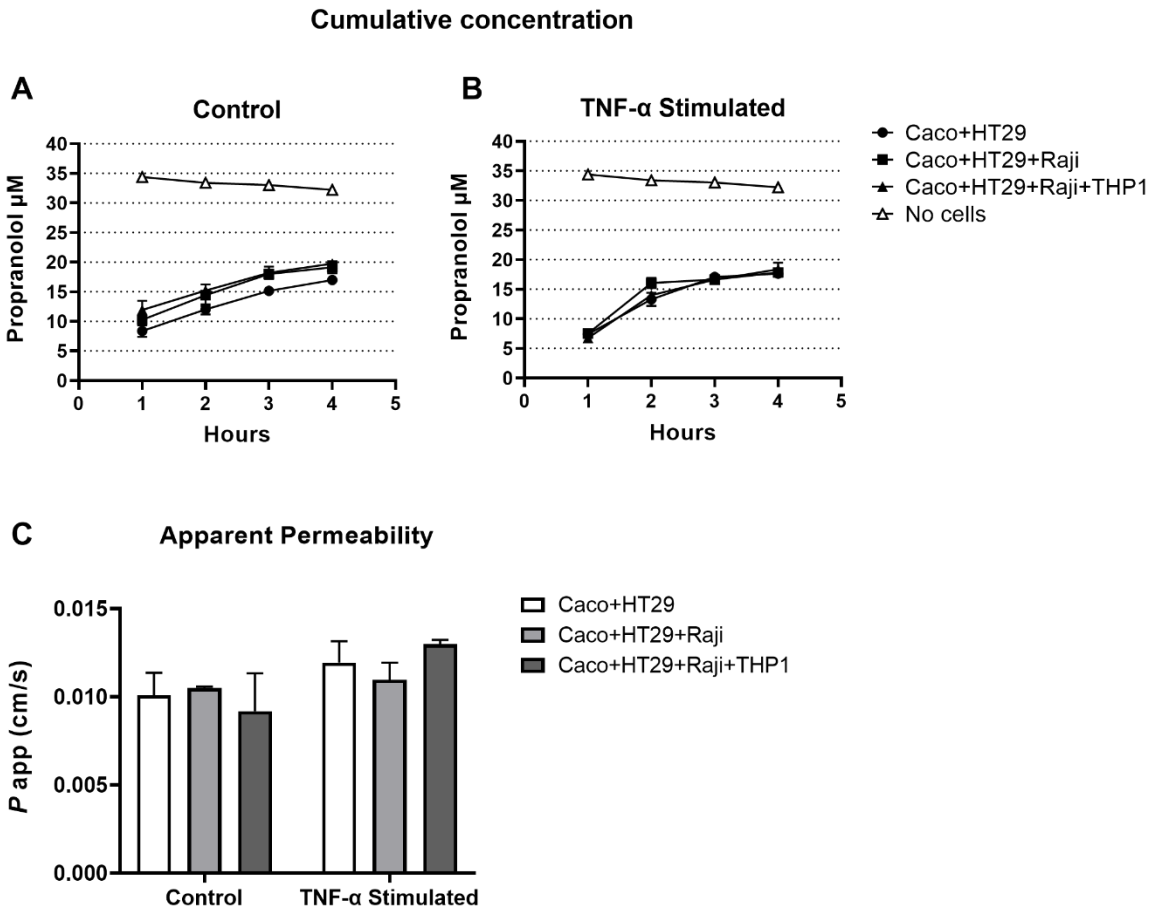

Figure 9 – Cumulative concentration of propranolol transported from the apical to the basolateral compartment of control (A) and TNF-α-stimulated (B) co-cultures. Apparent permeability of propranolol (C) across control and TNF-α-stimulated co-cultures from the apical to the basolateral compartments.

Immunocytochemistry

Confocal microscopy images were obtained to observe and quantify F-actin and occludin protein expression, as well as barrier integrity. There are areas on the confocal images that appear to have no cells. However, when observing different planes, it was possible to confirm the presence of cells, in areas designated as domes, indicative of epithelial cell differentiation (17, 18).

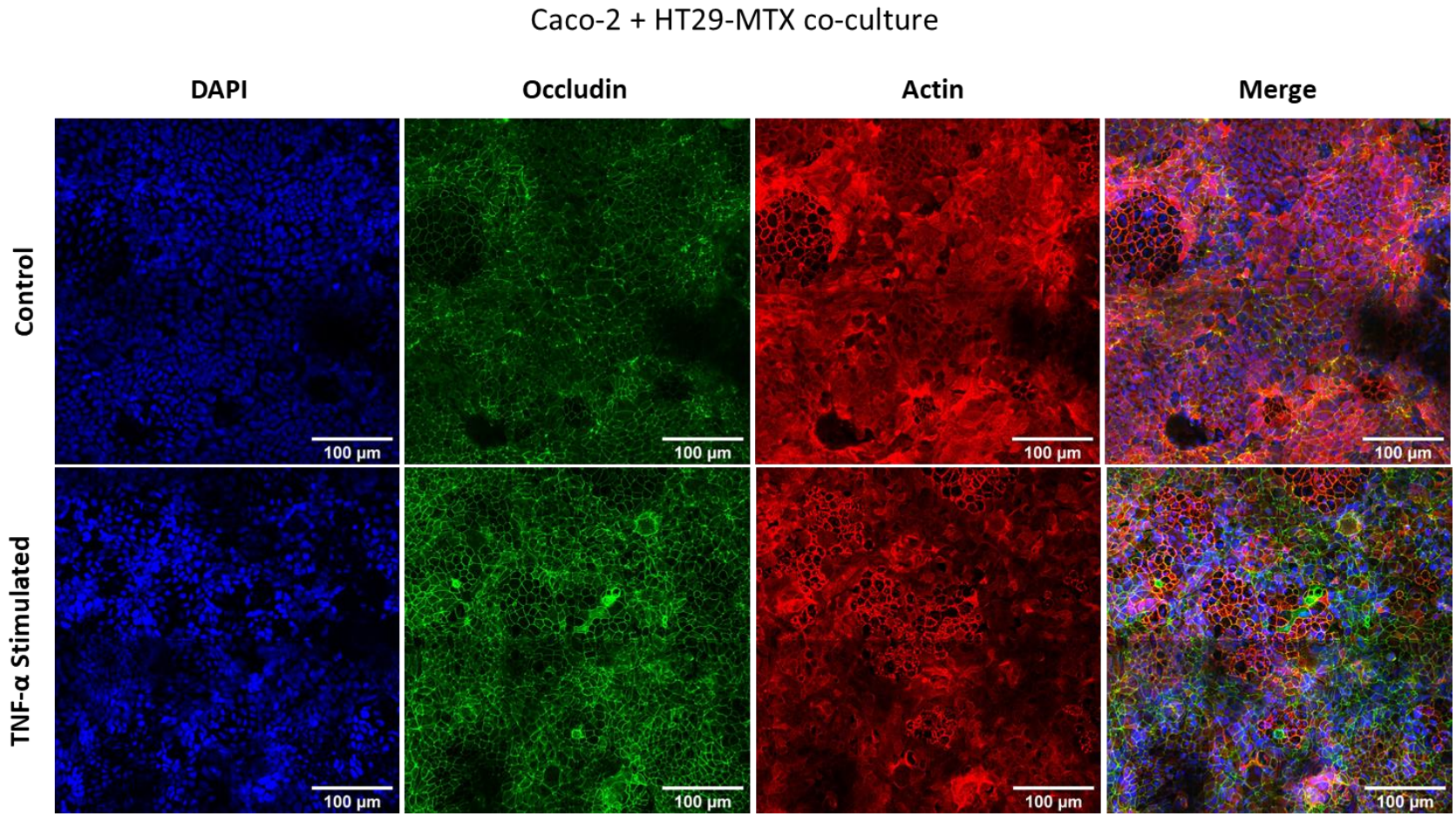

Figure 10 – Confocal microscopy images (40× magnification) of immunocytochemistry of control (A) and TNF-α-stimulated (B) Caco-2/HT29-MTX co-cultures grown in Transwell™ inserts. Nuclei were sained with DAPI (blue), occludin with an Alexa Fluor® 488-conjugated occludin monoclonal antibody (green) and F-actin with phalloidin-TRITC (red).

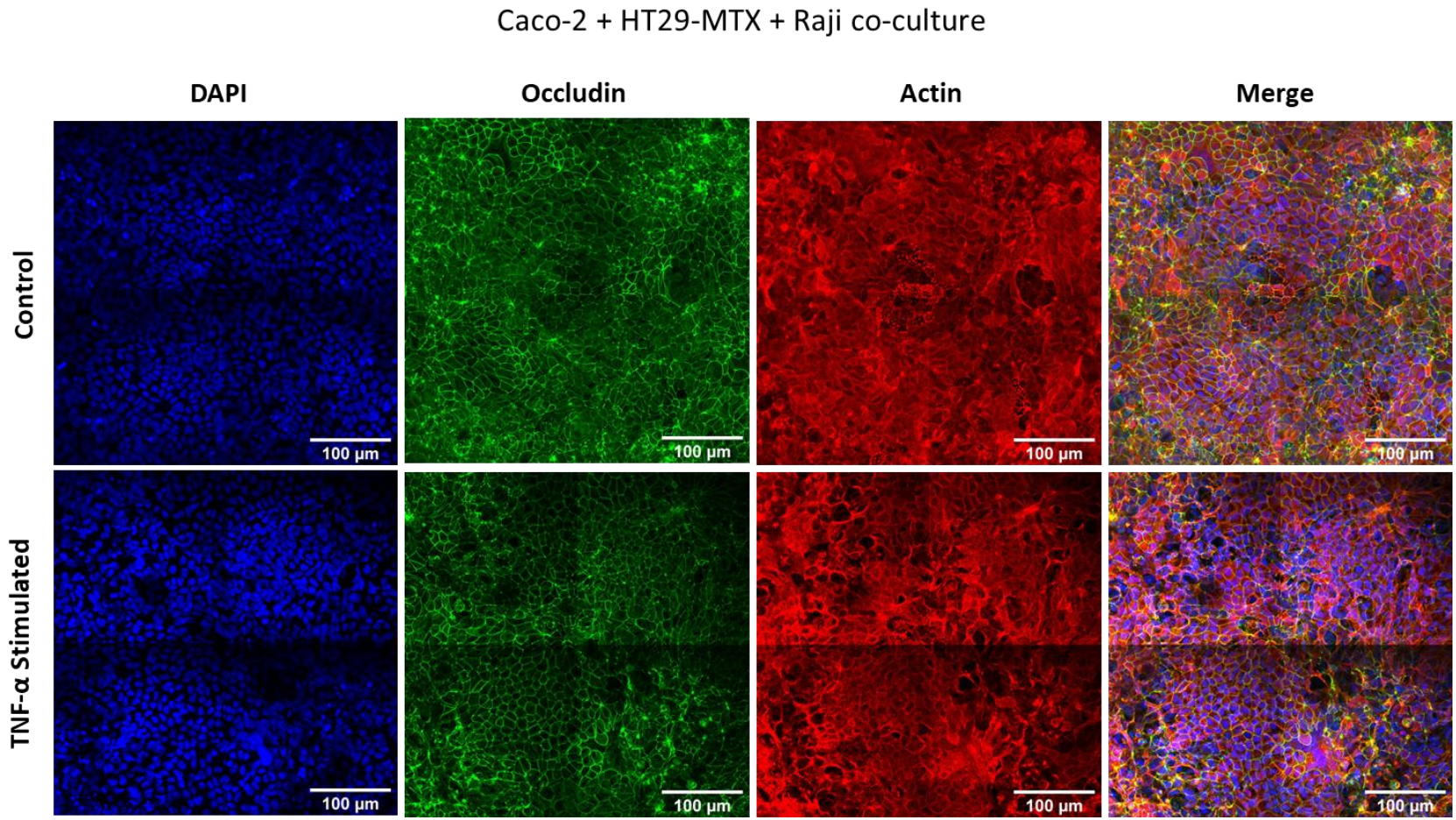

Figure 11 – Confocal microscopy images (40× magnification) of immunocytochemistry of control (A) and TNF-α-stimulated (B) Caco-2/HT29-MTX/Raji co-cultures grown in Transwell™ inserts. Nuclei were sained with DAPI (blue), occludin with an Alexa Fluor® 488-conjugated occludin monoclonal antibody (green) and F-actin with phalloidin-TRITC (red).

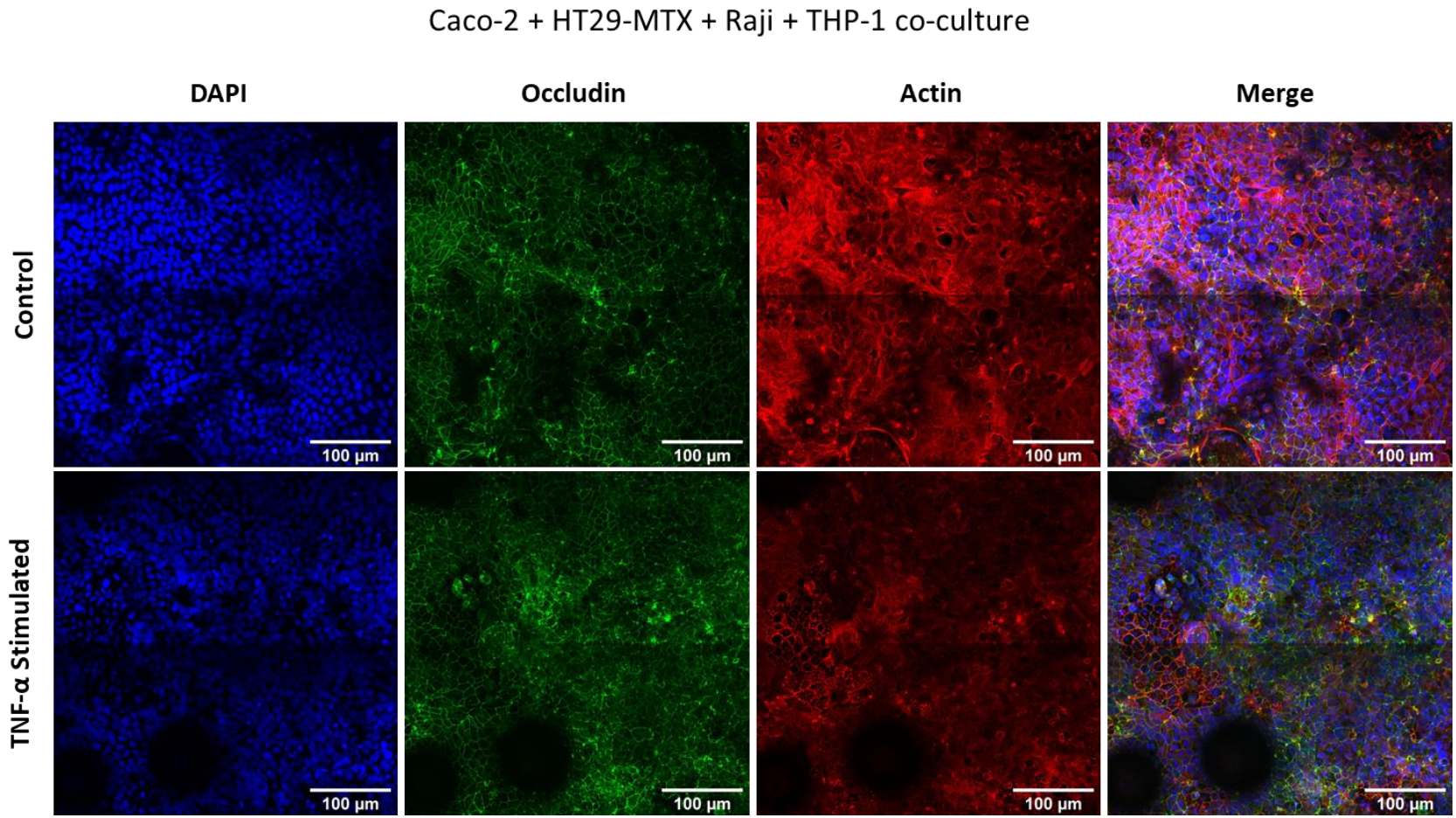

Figure 12 – Confocal microscopy images (40× magnification) of immunocytochemistry of control (A) and TNF-α-stimulated (B) Caco-2/HT29-MTX/Raji/THP-1 co-cultures grown in Transwell™ inserts. Nuclei were sained with DAPI (blue), occludin with an Alexa Fluor® 488-conjugated occludin monoclonal antibody (green) and F-actin with phalloidin-TRITC (red).

Mean fluorescence intensities of the three co-cultures were evaluated in both control and TNF-α-stimulated co-cultures (Figure 13) after performing merge of z-stacks obtained in each channel, for each condition (Figures 10 to 12). Although numerical differences in occludin and F-actin fluorescence intensity could be observed among co-cultures and between control and TNF-α-stimulated co-cultures (Figure 12), this preliminary assay was performed only once and only one sample of each condition was evaluated. Thus, no conclusions can be drawn regarding protein expression.

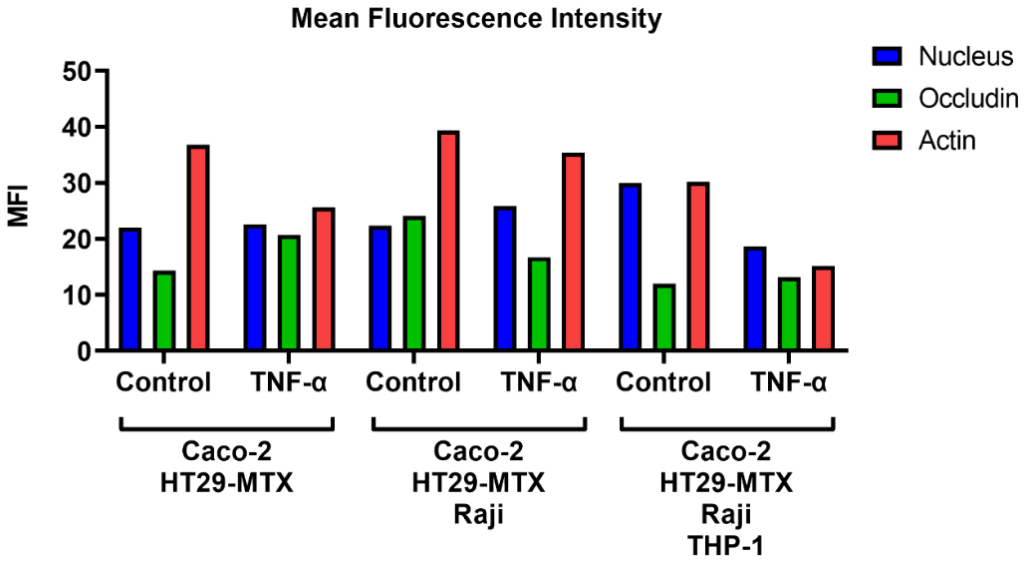

Figure 13 – Mean fluorescence intensities (MFI) obtained in ImageJ software, after z-stack merging of co-cultures' images.

Discussion

In this work we aimed at assessing the cytotoxic effects and the oxidative and antioxidant properties of three digested microalgae species (*C. vulgaris*, *N. oceanica* and *Tetraselmis* sp.) on intestinal epithelial cells. We also proposed a novel *in vitro* 2D model of intestinal epithelium, by combining an already validated triple co-culture model (6) and an *in vitro* model of intestinal inflammation (11). These models are extremely helpful in the initial phases of screening for dietary compounds namely to assess their bioaccessibility and bioactivity (19). However, it is still difficult to extrapolate results obtained *in vitro* and draw conclusions based on the observed effects. The complexity of the gut (peristalsis, mucus, microbiome, cell dynamics and immune surveillance) is not easily reproduced in static cell cultures. Thus, more and more complex models are being proposed, namely co-cultures of epithelial cells, co-cultures of epithelial and immune cells, organoids and gut-on-a-chip (10, 20, 21).

Results presented in this chapter are preliminary, as sample size was a major limiting factor restricting the analysis of the obtained results. We observed that incubation with non-differentiated, non-confluent Caco-2 cells with digested microalgae at a 10-fold dilution significantly affected cell metabolic activity. However, digested CV and T at a 20-fold dilution demonstrated to be safe for cells, contrastingly to digested NO that presented cytotoxic effects at both dilutions. Most studies evaluate the effects of non-digested microalgae or microalgae extracts. Hence, our results pinpoint the importance of including *in vitro* digested products when screening for bioactivity of dietary supplements, not only to better mimic *in vivo* processes, but also because bioactivity may be changed, either depressed or enhanced, upon digestion. There are at least two possible hypotheses for the observed cytotoxicity of particulate digested microalgae-stimulated cells: 1) the compounds formed upon digestion are toxic to cells; 2) there is a synergic effect of toxicity of digested particulate compounds and toxicity caused by enzymes and digestive fluids. The cytotoxic effect observed upon direct contact of dietary compounds with epithelial cells or immune cells *in vitro* does not mimic completely the events happening *in vivo*, where more complex processes and protective barriers exist, which will protect the epithelium from direct contact with particulate products and cell damage. However, it is known that even particulate compounds are sampled and screened in the gut by innate immune cells, in the process of immune surveillance, either through M cell transport or directly by transepithelial dendrites projected into the gut lumen by dendritic cells (2).

Digested CV, both in the particulate and soluble forms, and soluble NO, appeared to induce reactive-oxygen species production by co-cultures of Caco-2/HT29-MTX. However, when oxidative stress was induced with H_2O_2, both microalgae appeared to present ROS scavenging effects, evaluated by the less pronounced relative increase in ROS production. Microalgae are mainly studied due to their antioxidant effects (25), but it is reported that antioxidant compounds may present prooxidant activity, depending on the concentration of the antioxidant, the presence of metal ions and its redox potential (26). Digested NO should have been more diluted in order to assess its bioactivity on intestinal *in vitro* models since there was still some degree of toxicity at 20-fold dilution. However, due to the potential antioxidant activity of NO (27, 28), we still aimed at assessing its scavenger ability upon oxidative stress induced by H_2O_2. Soluble NO compounds also presented antioxidant potential after oxidative stress induction with H_2O_2. More assays must be performed to draw sound conclusions since sample size was too low. However, these preliminary results allowed us to identify CV as the most promising dietary supplement to be used in *in vivo* trials with newborn calves. Supporting this decision were the safety of the CV 20-fold dilution and CV's ROS scavenging effects.

Besides, CV is one of the most studied and used microalgae, with benefits from dietary supplementation reported in several other species (29)

Unravelling immune modulating properties of dietary compounds *in vitro* is complex, as dietary compounds are sensed by innate immune cells either through microfold cell (M-cell) sampling and transport, or directly by dendritic cells through transepithelial dendrites that reach the gut lumen (9, 24). Thus, we proposed an *in vitro* model of intestinal epithelium composed of enterocytes (Caco-2), mucous secreting goblet cells (HT29-MTX), antigen transporter cells (M-like cells), and monocyte-differentiated macrophages (THP-1) to assess the immunomodulating properties of dietary compounds. As many of these compounds are described to have anti-inflammatory properties, we also proposed the induction of inflammation in this complex model to further investigate whether this process could be prevented or reversed with the dietary compounds. Unfortunately, it was not possible to fully develop the quadruple cell model in this thesis.

Monolayers of Caco-2/HT29-MTX decreased considerably TEER values when co-cultured with Raji cells, indicating reduced barrier resistance. Although these preliminary data are in line with previously reported results, where triple co-cultures have slightly lower TEER than Caco-2/HT29 co-cultures (30), TEER was reduced to surprisingly low levels. TEER is widely used as an indicator of tight junction integrity (16), although it does not exclude cell toxicity (31). It may be impacted by temperature, media, number of cells, passage number, and the type of cells used in the Transwell™ system, and so numerous factors may contribute to TEER variability among studies (16, 32). Differentiation process of enterocytes (Caco-2) into M-like cells and the looser intracellular connections expressed by this triple co-culture (Caco-2, HT29-MTX and M-like cells), comparatively to co-cultures of Caco-2/HT29-MTX, that generally present denser intercellular junctional complexes, is expected to decrease TEER (5). In our results, although TEER was lower in Caco-2/HT29-MTX/Raji co-culture, apparent permeability of propranolol - a compound used in permeability studies that crosses monolayers through passive diffusion (33) - was identical between the three different co-cultures. Identical transcellular diffusion indicates similar cell membrane composition among the different co-cultures, as lipophilic compounds differentially cross cellular barriers according to the composition of the epithelial cell layer (5).

Mean fluorescence intensity of occluding and F-actin, evaluated by confocal microscopy, suggests there could be differences in their expression among the three different co-cultures tested. Caco-2/HT29-MTX/Raji/THP-1 model, in particular, appear to express lower levels of occludin, a tight junction protein that contributes to the integrity and stability of the intestinal epithelial barrier (34). However, further assays are needed to confirm these results since sample size was a major limiting factor in this preliminary

study.

From the analysis of TEER and quantification of cytokines accumulated in the apical and basolateral compartments after the addition of TNF-α it is suggested that, in this particular experiment, 10 ng/mL of TNF-α were not sufficient to induce inflammation. There are studies that use this cytokine to induce inflammation with the objective of testing the protective effects of dietary compounds, or their fermentation products (such as short-chain fatty acids) on the intestinal epithelial cell barrier (35, 36). TNF-α increases the monolayer permeability by activating both canonical and non-canonical NF-κB signalling, which results in increased tight junction permeability (37, 38). However, a drop in TEER of at least 20% should have been observed (11), according to previously published data, which was not the case in this preliminary study. TNF-α is also described as being able to induce the production of pro-inflammatory cytokines by Caco-2 cells, namely IL-8 (39, 40). As cytokine production was also not different between control and TNF-α-stimulated cells, the hypothesis that inflammation was not induced in this model is reinforced.

Digested microalgae, namely *C. vulgaris*, may present prooxidant and antioxidant effects, as previously addressed by other authors (26). It is plausible that, depending on the concentration, these compounds could induce oxidative stress and protect the animal from free radicals produced in the course of an infection.

It was not possible to conclude the *in vitro* model of intestinal epithelial cells and immune cells in a healthy and diseased (inflamed) state. However, it appeared that inflammation was not successfully induced in this case. Further assays are required to validate this model.

Author Contributions

ARVP performed the experiments, data acquisition and analysis, and wrote the manuscript. CG and PR participated in the experiments. ML, MIG and IA participated in the multiple co-culture assays. MX assisted in the interpretation of data. AC, MRGM, MV, CG and ARVP, conceived and designed the experiments. CG supervised the experimental work at INL and assisted in data acquisition and analysis. AC, MRGM and MV assisted in data analysis and manuscript writing.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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CHAPTER V

Immune and health status parameters of Holstein-Friesian calves fed milk replacer supplemented with *Chlorella vulgaris*

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Immune and health status parameters of Holstein-Friesian calves fed milk replacer supplemented with *Chlorella vulgaris*

Ana R. V. Pedro[1,2,3,†], Margarida R. G. Maia[3], Elisabete G. Martins[4,5,6], Carla Mendonça[7,8], Isabel C. Ramos[8], Ana R. J. Cabrita[3], António J. M. Fonseca[3], Manuel Vilanova[1,2] and Alexandra Correia[1,2*]

[1] Immunobiology Group, i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal

[2] Laboratório de Imunologia, DIMFF, ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[3] LAQV, REQUIMTE, ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[4] ADM Portugal, SA, Murtede Portugal

[5] EPIUnit, Instituto de Saúde Pública, Universidade do Porto, Porto, Portugal

[6] Escola Universitária Vasco da Gama, Coimbra, Portugal

[7] Departamento de Clínicas Veterinárias, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[8] Centro de Estudos de Ciência Animal-Instituto de Ciências, Tecnologias e Agroambiente – UP, Porto

[8] Cooperativa Agrícola de Vila do Conde, Vila do Conde, Portugal

[†] Present Address: Escola Universitária Vasco da Gama, Coimbra, Portugal

Abstract

Calf rearing is still a challenge in many farms. Infectious neonatal diarrhoea and respiratory syndromes impair calf health, growth, and performance, thus increasing costs and impacting economic management. Several approaches have been discussed and addressed, namely the modulation of calves' immune response by dietary supplementation. Microalgae, such as *Chlorella vulgaris*, may be interesting solutions since they not only provide valuable nutrients as they are valuable sources of bioactive compounds. However, information on the immune effects and health of calves upon supplementation with microalgae is still scarce. This work aimed at evaluating the performance, immune parameters and faecal fermentation profile and microbiome of calves fed *Chlorella vulgaris*. Fourteen calves aged 12.1 ± 2.92 days were randomly allocated into two experimental groups: a non-supplemented (control) and a *C. vulgaris* supplemented (supplemented). Calves from the control group received 7 L of milk replacer and calves from the supplemented group received 7 L of milk replacer plus 1% *C. vulgaris* (w/w), each day, for six weeks. Compound feed, hay and freshwater were administered *ad libitum*. Milk refusals were recorded every day, weighing and blood collection were performed in the beginning and in the end of the supplementation period, and faeces were collected in the end of the trial. After six weeks of trial, erythrocyte distribution index, platelet counts, neutrophil counts, inflammation associated ratios (neutrophil to lymphocyte ratio, platelet to lymphocyte ratio and systemic immune-inflammation index) and serum IL-8 were significantly higher in calves from the supplemented group comparatively to control group. Interestingly, global levels of IL-6 produced by monocyte-derived macrophages (MDM) from supplemented group were significantly lower, while global *IL10* mRNA expression was significantly higher, upon *in vitro* challenge with several PRR-agonists. Calves supplemented with *C. vulgaris* also had lower proportion of faecal propionic acid, and higher proportions of isobutyric, valeric, isovaleric, and isocaproic acids. No difference in faecal microbiome composition were observed between both groups. Our results indicate 1% *C. vulgaris* supplementation does not negatively impact calves' intake, growth and immune system. Differences observed in haemogram parameters and in the response of peripheral-blood macrophages stimulated with PRR agonists suggest milk replacer supplementation may impact immune function of newborn calves. However, further studies are needed to understand if those effects are beneficial upon challenge with a pathogen or in the course of an infection.

Keywords

Calf, milk replacer, *Chlorella vulgaris*, dietary supplementation, health promotion

Introduction

Calf management remains a particular sensitive process in most bovine farms, particularly in dairy herds. Although tremendous progresses have been made, regarding housing and management of calves, digestive and respiratory infections are still major causes of morbidity until weaning, being the primary cause of mortality in newborn calves (1). These neonatal diseases usually derive from an imbalance between pressure posed by pathogens in the surrounding environment and the ability of the host immune system to respond to those pathogens (2). Despite being considered a worldwide problem in dairy and beef farms, reported morbidity and mortality rates are extremely variable among countries, regions and even farms of the same region due to factors such as season, housing and management procedures (3-6). As such, proper management in the first weeks, namely through adequate colostrum intake – quantity, quality, cleanliness, and quickness of administration – and control of the microbial load in the surrounding environment – through cleaning and disinfection procedures – are decisive (7). Maintaining good management practices and enhancing calves' immunity are sustainable strategies to control morbidity rates and overuse of antibiotics, and to prevent disease, animal loss, and suboptimal performance (3, 8). Modulation of the immune system with dietary supplements is a sustainable health-promoting approach that could additionally contribute to reduce antibiotics use and antimicrobial resistance (9-11). The most studied and used nutraceuticals are prebiotics, probiotics, phytonutrients, and polyunsaturated fatty acids (12). Microalgae are commonly used as dietary supplements in human nutrition to enhance immune function and have potential to be used in livestock feeding (13-15). Their nutrient profile characterization has shown microalgae to be valuable sources of proteins, carbohydrates, lipids, vitamins, minerals, pigments, and bioactive compounds, such as β-glucans, bioactive peptides, pigments, and polyunsaturated fatty acids (16, 17). These compounds may reinforce the immune function, thus contributing to prevent disease. Yet, nutritional and functional profile are species-specific and greatly depend on cultivation characteristics (18, 19). *Chlorella vulgaris* was one of the first species to be studied and is one of the most commercialized microalgae (20, 21). Not only it has an interesting nutritive value (22), but also presents promising health benefits due to immunomodulatory, antioxidant and antimicrobial properties (23-26). Although the dietary supplementation of *C. vulgaris* had been shown

to promote poultry and swine immune function (26-29), no study has yet evaluated its potential as an immunomodulator in newborn calves, to the best of our knowledge. Hence, we aimed at exploring the overall health and immune effects of *C. vulgaris* supplementation, one of the most studied and consumed microalgae, in Holstein-Friesian calves.

Material and Methods

Animals, Housing and Management

Fourteen Holstein-Friesian male calves with mean ages of 12.1 ± 2.92 days, were acquired from four different farms and housed at Clinical and Research Veterinary Center of Vairão (CCIVV) of the School of Medicine and Biomedical Sciences from the University of Porto (ICBAS-UP). A complete veterinary examination was performed at arrival and blood was collected from the jugular vein into VACUETTE® EDTA (ethylenediaminetetraacetic acid) tubes (Greiner Bio-One, Frickenhausen, Germany) to perform complete hemogram analysis and into VACUETTE® Serum Clot Activator tubes (Greiner Bio-One, Kremsmünster, Áustria) to perform quantification of total serum proteins and serum albumin at Segalab (Laboratório de Sanidade Animal e Segurança Alimentar, Póvoa do Varzim, Portugal). An acclimation period of 17.4 ± 4.9 days was performed, to ensure all animals were fed the same amount of milk replacer at the beginning of the experimental procedure. Animals were handled under daily supervision of veterinarians and with strict compliance with good animal practices defined by national authorities and by European Union Directive 2010/63/EU and European Council Directive 2008/119/EC. Experimental procedures were approved by the Animal Ethics Committee of ICBAS-UP (P398/2021/ORBEA) and licensing was requested to the National Competent Authority, Direção Geral de Alimentação e Veterinária (DGAV).

After the acclimation period, at day 0 (T0), calves were weighted before the morning meal and those from each farm randomly allocated to one of two experimental groups: non-supplemented milk replacer (control) and *C. vulgaris* supplemented milk replacer (supplemented) groups, for six weeks (T6). Animals were fed 3.5 L of milk replacer, prepared in a calf automatic feeding system (Milk Express, Sylco Hellas S.A., Thessaloniki, Greece), reconstituted at 140 g/L, and distributed in teat-buckets. Calves were fed twice a day, approximately at 09:00 and 18:00 h, and had *ad libitum* access to fresh water, meadow hay and commercial compound feed for calves. Control calves were fed milk replacer without *C. vulgaris* supplementation and supplemented calves were fed milk replacer plus 1% *C. vulgaris* (w/w milk replacer solids basis) on-top. The *C. vulgaris* was added, in each meal, to teat-buckets with milk replacer in and thoroughly

homogenized before distributed to calves. Fresh water, meadow hay and compound feed were administered *ad libitum* throughout the experimental period. A schematic illustration is presented in Figure 1 and summarizes the experimental procedure.

Milk replacer used is commercially available and was kindly provided by ADM Portugal S.A. (Cantanhede, Portugal). *Chlorella vulgaris* was locally produced in photobiorreactors and was kindly provided by Allmicroalgae (Pataias, Portugal) as a spray-dried powder in air-tight bags protected from light. Commercial compound feed for calves was kindly provided by Cooperativa Agrícola de Vila do Conde (Vila do Conde, Portugal). The ingredient and chemical composition of feeds is presented in Supplementary Tables 1 to 4.

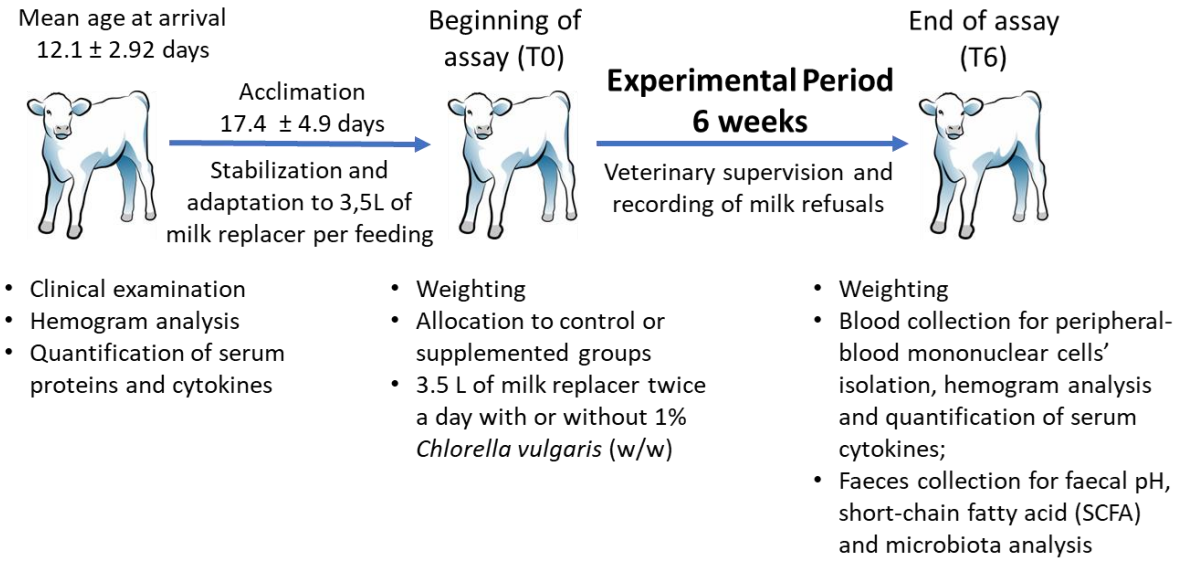

Figure 1 – Schematic overview of the experimental procedure.

Sample Collection

Veterinary supervision and animal records were granted every day. Animals were weighted at the beginning (T0) and at the end of the experimental period (T6) to assess average body weight and average daily gain. Milk replacer offered and refused, whenever existent, were recorded in each meal to determine average feed intake. At the end of the experimental period, 20 mL of peripheral blood were collected from jugular vein into BD Vacutainer® lithium heparin tubes (BD Biosciences, Franklin Lakes, NJ, US) to isolate peripheral-blood mononuclear cells, 5 mL into VACUETTE® EDTA tubes to perform complete hemogram analysis and 5 mL into VACUETTE® Serum Clot Activator tubes to perform quantification of serum cytokines. Faeces were collected after stimulation of the anus, directly into 50 mL sterile centrifuge tubes (Sarstedt, Nümbrecht,

Germany) and stored at -20 °C for faecal pH and short-chain fatty acid (SCFA) determinations. For microbiota analysis, collected faeces were snap-frozen in liquid nitrogen and stored at -80 ºC until further analysis.

Isolation of bovine peripheral blood monocytes and differentiation into monocyte-derived macrophages

Bovine monocyte-derived macrophages (MDM) were isolated from peripheral-blood as previously described (30). Whole blood collected from jugular veins was diluted 1:2 in Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich Inc., St. Louis, MO, US). It was then layered on top of Histopaque®-1077 (Sigma-Aldrich) and centrifuged at 1200 × *g* at room temperature in SepMate™ PBMC isolation tubes (Stemcell™ Technologies, Vancouver, BC, Canada). Peripheral blood mononuclear cells (PBMC) were collected and washed by centrifugation with DPBS at 400 × *g* for 10 min and 4 °C. Isolated PBMC were incubated with CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's instructions. Positive selected cells were washed at 300 × *g* for 10 min and resuspended at 2 × 10^6 cells/mL in RPMI-1640 Medium (Sigma-Aldrich) supplemented with 4 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM HEPES (all from Sigma-Aldrich) and 50 µM β-mercaptoethanol (Merck, Darmstadt, Germany). RPMI was further supplemented with 10% autologous serum. Differentiation of bovine MDM was performed in 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplates (Nunc A/S, Roskilde, Denmark) for 7 days at 37 °C and 5% CO_2. On the 3rd day of cell culture, one-third of the medium was removed and 100 µL of fresh supplemented medium was added to each well. On the 7th day, bovine MDM were observed in an inverted microscope under 200× magnification (Supplementary Figure 1) and used for stimulation and phagocytosis assays.

Cell culture and stimulation with PRR agonists

For the analysis of the mRNA expression and cytokine production upon stimulation with several Pattern Recognition Receptor (PRR) agonists, bovine MDM were cultured with 2 µg/mL *Escherichia coli* lipopolysaccharide, that functions as a TLR2/TLR4 agonist (LPS, strain O111:B4; Sigma-Aldrich), 2 µg/mL of Pam3CSK4, a TLR1/TLR2 agonist (P3C), 50 µg/mL of WGP®-Dispersible, a Dectin-1 agonist (WGP) or 50 µg/mL of Zymosan (Zym), a Dectin-1 and TLR2 agonist (all from InvivoGen, San Diego, CA, US) for 24 h at 37 °C and 5% CO_2. Supernatants were collected and the pelleted cells were treated with NZYol® reagent before being stored at -80 ºC until analysis.

Cytokine production

Cytokine production was evaluated in cell culture supernatants and in the serum of the animals, as previously described (30). Briefly, bovine Tumor Necrosis Factor Alpha (TNF-α) and bovine Interleukin (IL)-6 were assessed using Bovine TNF-alpha and Bovine IL-6 DuoSet ELISA kits (detection limit: 125 pg/mL; R&D Systems, Minneapolis, MN, US) according to manufacturer's instructions with minor modifications to improve signal-to-noise ratio: 1% molecular grade bovine serum albumin (BSA, Albumin Bovine Fraction V, NZYTech, Lisbon, Portugal), in DPBS solution, was used as reagent diluent instead of 5% Tween 20 in DPBS. IL-1β was quantified with IL-1 beta Bovine Uncoated ELISA Kit (detection limit <31.3 pg/mL; Invitrogen, Waltham, MA, US), according to manufacturer's instructions. IFN-γ was assessed with Bovine IFN-γ ELISA^{BASIC} kit (detection limit of 4 pg/mL), IL-2 with Bovine IL-2 ELISA^{BASIC} kit (detection limit of 17 pg/mL), IL-4 with Bovine IL-4 ELISA^{BASIC} kit (detection limit of 20 pg/mL), IL-8 with Bovine IL-8 (CXCL8) ELISA development kit (detection limit of 8 pg/mL) and IL-17 with Bovine IL-17A ELISA^{BASIC} kit (detection limit of 1 pg/mL), all according to manufacturer's instructions (Mabtech AB, Nacka Strand, Sweden). Bovine IL-10 was quantified using an in-house ELISA kit. Protocol for IL-10 was adapted from the standard procedure of Mabtech ELISA Bovine IL-8 kit: 1 µg/mL anti-bovine IL-10 mAb (clone CC318; Bio-Rad, Hercu, CA, US) in PBS was used to coat Nunc Maxisorp™ plates (Nunc), which were incubated overnight at 4 °C. A nine-point standard curve from 2000 to 8 pg/mL was performed with Recombinant Bovine Interleukin-10 (Bio-Rad). Supernatants and standards were incubated for 2 h at room temperature. Plates were then incubated with the detection antibody at 0.5 µg/mL (mouse anti-Bovine Interleukin-10 Biotin-conjugated; clone CC320; Bio-Rad) for 1 h, followed by 1 h incubation with Mabtech's streptavidin-HRP, according to manufacturer's instructions. Detection limit for IL-10 was 8 pg/mL. Readings were performed using Biotek™ Gen5™ Data Collection and Analysis Software at 450nm and 570nm, in a BioteK™ µQuant Microplate Reader (BioTek Instruments Inc, Winooski, VT, US).

RNA extraction, cDNA synthesis and Real-Time qPCR

RNA extraction and cDNA synthesis were performed as previously described, using NZYol protocol and NZY First-Strand cDNA Synthesis Kit (NZYtech) (30). First-strand cDNA synthesis was performed at 25 °C for 10 min, 50 °C for 30 min, and 85 °C for 5 min, in a T100^{TM} Thermal Cycler (Bio-Rad). The best combination of reference genes (geometric mean of MARVEL domain containing 1 [*MARVELD1*] and Peptidylprolyl isomerase A [*PPIA*]), determined using NormFinder software (31) (Department of

Molecular Medicine, Aarhus University Hospital, Aarhus N, Denmark), was used to perform gene expression analysis using the formula $2^{-(CT\ gene\ of\ interest-CT\ housekeeping\ gene)}$, according to the comparative threshold cycle method (32). Primers for *TNF*, *IL1B*, *IL6*, *IL10*, *IL12A*, *IL12B* and *IL23A* (Supplementary Table 5) were previously designed.. RT-PCR was performed in a CFX384 Touch Real-Time PCR Detection System, using 5 µL NZYSpeedy qPCR Green Master Mix (2×) ROX plus (NZYTech), 0.2 mM specific forward and reverse primers (all from Sigma-Aldrich), 3.6 µL H_2O and 1 µL cDNA. RT-PCR reaction ran in thin wall, skirted, clear Hard-Shell® 384-Well PCR Plates (Bio-Rad) for 5 min at 95 °C, followed by 40 cycles at 95 °C for 5 s and 62 °C for 20 s.

Phagocytosis assay

Carboxylate-modified polystyrene, 2 µm fluorescent yellow-green latex beads (L4530; Sigma-Aldrich) were used to assess non-specific phagocytosis by flow cytometry. Previously differentiated bovine MDMs were plated at 2×10^5 cells/well and incubated with 2×10^6 latex beads per well at 37 °C and 5% CO_2 for 0-, 30-, 60- or 90-min. Cells were washed twice with warm DPBS, to remove non-internalized beads and plates were kept on ice to cease phagocytosis. Cells were then detached with cold Macrophage Detachment Solution (PromoCell, Heidelberg, Germany) into flow cytometry tubes, incubated with propidium iodide (PI; Sigma Aldrich) at 1 µg/mL for 5 min, at room temperature, and analysed by flow cytometry in a BD FACSCantoII™ (BD Biosciences). Data was analysed using FlowJo software (Version 10.5.3; FlowJo LLC, Ashland, OR, US). The percentage of phagocytosis corresponded to the percentage of green-positive cells, after excluding cell debris and dead cells from the analysis.

Proliferation assay

Proliferation of PBMC response to Concanavalin A (ConA) was determined by flow cytometry. PBMC were labelled with CellTrace™ Violet Cell Proliferation Kit (Invitrogen), according to manufacturer's instructions. Labelled cells were plated in 96-Well, Nunclon Delta-Treated, U-Shaped-Bottom Microplates (Nunc) at 1×10^5 cells/well and incubated with 1 µg/mL ConA (Sigma-Aldrich) for 3 days at 37 °C and 5% CO_2. Non-stimulated labelled cells were used as negative controls of proliferation. Cells were washed with DPBS and transferred into flow cytometry tubes, incubated with PI at 1 µg/mL for 5 min, at room temperature, and analysed in a BD FACSCantoII™. Data was analysed using FlowJo software. The percentage of cell proliferation was calculated as the percentage of cells that divided ate least once, based on violet dye dilution, after excluding cell debris and dead cells from the analysis.

Faecal pH and Short-Chain Fatty Acid (SCFA) analysis

Determination of faecal pH was performed directly in thawed faeces, once at room temperature, using a potentiometer (pH and Ion-Meter GLP 22, Crison, Barcelona, Spain) (33). For SCFA analysis, procedures were performed as previously described by Pereira *et al.* (34) and Maia *et al.* (35). Briefly, 1 g of thawed faeces was diluted in 25% ortho-phosphoric acid solution with internal standard (4 mM 3-methyl valeric acid; Sigma Aldrich). The mixture was vortexed and centrifuged at 19 800 × *g* for 15 min, at 4 °C, after which the supernatant was collected and filtered through a 0.45-µm pore size polyether sulfone syringe filter (VWR International LLC, Radnor, PA, US) into chromatography vials. Analysis was performed by gas chromatography in a Shimadzu GC-2010 Plus (Shimadzu Corporation, Kyoto, Japan) equipped with a capillary column (HP-FFAP, 30 m × 0.25 mm × 0.25 µm; Agilent Technologies, Santa Clara, CA, US) and a flame ionization detector. One µL of sample was injected with a split ratio of 1:100. Injector was at 260 °C and detector at 280 °C. Oven temperature was at 80 °C for 1 min, increased at 20 °C/min to 120 °C, then increased at 6 °C/min to 175 °C and further increased at 20 °C/min to 240 °C and hold for 10 min. Short-chain fatty acids were identified by comparing retention times with a commercial standard mixture (Volatile Free Acid Mix, Sigma-Aldrich) and quantified using the internal standard (3-methyl valeric acid).

Faecal Microbiota

Pairwise comparison of the faecal microbial composition from both groups was performed by microbiome profiling at Baseclear (Leiden, The Netherlands). Analysis was conducted on genus level for 16S Ribosomal ribonucleic acid (16S rRNA) based profiling and on species level for shogun metagenome profiling. Microbiome data analysis and statistical analysis were also performed by Baseclear. All qPCR reactions were performed in 384-well PCR plates, sealed with MicroAmp Optical Adhesive Film. Reactions ran in an Applied Biosystems QuantStudio™ 5 Real-Time PCR system (all from Thermo Fisher Scientific) with QuantStudio™ Design & Analysis software v1.4.2. For the total bacteria qPCR assay, targeting the 16S rRNA gene, each reaction was carried out in a total volume of 10 µL, with 5 µL ABsolute™ Blue qPCR Mix, Low ROX (Thermo Scientific™), 0.2 µL forward primer (5'-CGGTGAATACGTTCYCGG-3'; 10 µM), 0.2 µL reverse primer (5'-GGWTACCTTGTTACGACTT-3'; 10 µM), 0.1 µL probe (FAM-CTTGTACACACCGCCCGTC-BHQ1; 10 µM), 2 µL PCR grade water and 2.5 µL undiluted template DNA. A standard curve comprising 8 serial 10-fold dilutions of a synthesized, cloned, linearized, and purified DNA of 192 bp, was generated from a work solution (0.1 ng/µL) that in turn was derived by 100 times diluting a stock solution (10

ng/µL). A positive control was performed alongside each separate amplification consisting of 2.5 µL of 0.1 ng/µL DNA (0.25 ng DNA added to a single reaction) that was derived from ZymoBIOMICS<U+2122> Microbial Community DNA Std. (D6306; Zymo Research). Negative template control (NTC) PCRs were performed alongside each separate amplification without addition of template. The PCR program started with a denaturation step at 95 ºC for 15 min, followed by 40 cycles consisting of denaturation at 95 ºC for 15 s, annealing and elongation at 52 ºC for 1 minute (with data collection). Amplification data were exported from QuantStudioTM Design & Analysis software v1.4.2 followed by determination of the target quantity per µL DNA preparations using the standard curves and calculation of the number of target per gram or mL of raw material using the following formula:

$$Quantity\ per\ unit\ material\ = \frac{Quantity\ \times D_{DNA}\ \times V_{DNA} \times \frac{V_{lysis}}{V_{lysis.extraction}}}{M_{material}\ or\ V_{material}\ or\ N_{material}}$$

Statistical Analysis

All data, except microbiome data, were analysed using the GENMOD Procedure (Generalized Linear Models) of the SAS software (SAS® OnDemand for Academics, SAS Institute Inc., Carry, NC, US). For weight, and haemogram parameters data, the model included the fixed effect of diet (control and supplemented) and farm of origin (1, 2, 3 and 4), the random residual error, and initial data (T0) as covariate. For proteinogram analysis, the model included the fixed effect of farm of origin (1, 2, 3 and 4) and the random residual error. The model used for remaining data included the fixed effect of diet (control and supplemented), stimuli (medium, LPS, Pam3CSK4, WGP®-Dispersible or zymosan for cytokine expression and production; medium or latex beads for phagocytosis assay; medium or concanavalin A for proliferation assay) and farm of origin (1, 2, 3 and 4), and the random residual error. For faecal pH and SCFA data, the model included the fixed effect of diet (control and supplemented) and farm of origin (1, 2, 3 and 4) and the random residual error. The Tukey-Kramer's post-hoc test was used for multiple comparison of means. Statistically significant results were considered when $P<0.05$ and a tendency when $0.05 \geq P<0.1$. GraphPad Prism software was used to construct the graphs (GraphPad Software Version 9.4.0, San Diego, CA, US).

For microbiome analysis, common statistical tests (alpha diversity, differential abundance test, and Linear discriminant analysis Effect Size (LEfSe) as well as higher order statistical Machine Learning methods (decision tree classification and regularised logistic regression) were performed. The outcomes of these analyses indicate Key

Biomarker and Signature Species in the microbiome dataset along with their statistical significance expressed in adjusted P-value. To perform LEfSe, a non-parametric factorial Kruskal-Wallis (KW) sum-rank test was firstly used to detect features with significant differential abundance as to the class of interest. The (unpaired) Wilcoxon rank-sum test was then used to assess biological consistency, using a set of pairwise tests among subclasses. Then, linear discriminant analysis is used to estimate the effect size of each differentially abundant feature.

Results

Serum proteinogram as a colostrum management indicator

Serum proteinogram was performed immediately upon arrival of each calf at CCIVV, to evaluate the transfer of passive immunity, since an improper management of colostrum, in the farm of origin, could result in inadequate plasma IgG levels. When measured after colostrum intake, total serum proteins (TSP), which are composed of albumin and globulins, display an altered albumin to globulin ratio that favours globulins, being most of these immunoglobulins, in particular IgG (36). Thus, TSP are highly correlated with serum IgG levels in newborn calves up to 9 days of age (37). Total serum proteins and albumin of calves to be allocated to control and supplemented groups were determined and differences among groups evaluated. No differences were observed on TSP, albumin and globulin levels, assessed by the difference between TSP and albumin, between groups (Supplementary Table 6).

Performance assessment

To understand whether microalgae supplementation could interfere with performance parameters, average feed intake, body weight at the beginning and at the end of the assay, average gain and average daily gain, in control and supplemented groups, were assessed (Table 1). Average daily milk replacer intake was identical between groups (979 g/day *vs*. 980 g/day; P=0.536). There were practically no refusals of milk intake, with the exception of one animal (Calf #11) that drunk 6 L, instead of 7 L, on two non-consecutive days. Physical examination was not suggestive of any alteration that could justify milk refusals on those days. One of the animals (Calf #5) did not eat compound feed during the experimental period, probably due to behavioural factors as no physical or clinical alterations were observed on multiple examinations. That animal was thus removed from all analyses to not bias the results.

Table 1 – Average daily milk replacer intake, body weight, average gain and average daily gain of control and *C. vulgaris* supplemented calves.

Parameter	Mean		SEM	P-value
	Control	Supplemented		
Average milk replacer intake (MR) (g/d)	979	980	0.0	0.536
Final average body weight (kg)	97.3	96.0	1.42	0.523
Average gain (kg)	42.9	41.5	1.43	0.505
Average daily gain (kg/d)	1.02	0.989	0.034	0.505

SEM, standard error of the mean.

Although final average body weight of the control group was about 1.3 kg higher (97.28 kg) than of the supplemented group (95.99 kg), this was not statistically significant (P=0.523). Average gain and average daily gain were numerically higher in the control group (42.9 kg and 1.02 kg, respectively) than in the supplemented group (41.5 kg and 0.99 kg, respectively), no significant differences being observed between groups (P>0.05).

Haemogram

The impact of the diet on haematological parameters was evaluated at the end of the assay (T6). Three distinct ratios calculated from hemogram parameters can reflect the level of inflammation. These include the ratio between neutrophils and lymphocytes counts (N/L), the ratio between platelets and lymphocytes counts (P/L), and neutrophil counts multiplied by platelets counts and divided by lymphocytes counts (Systemic Immune Inflammation, SII). Although these ratios are evaluated mostly in humans, these are currently also used in the veterinary field, including in cattle (38-40).

Haemogram parameters are shown in Table 2. There were no statistically significant differences between groups in total erythrocyte counts, haemoglobin levels, hematocrit counts, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular haemoglobin concentration, average platelet volume, leukocyte counts, basophils, monocytes, and lymphocytes (P>0.05). Erythrocyte distribution index was significantly higher (23.0% *vs*. 28.5%; P=0.001) in supplemented group. Interestingly, this parameter was significantly lower (P=0.001) in animals that were allocated to the supplemented group (20.1%) than in calves that were allocated to the control group (22.1%; P=0.001) (Supplementary Table 7). Although erythrocyte distribution indexes, at arrival, were in accordance with red cell distribution widths reported by Morita *et al.* (41), values observed in the supplemented group, at the end of the supplementation period were

above those reported by the same author. There were also statistically significant differences in platelet counts (608 × 10^3/µL *vs*. 768 × 10^3/µL; P <0.001), which were higher in animals from the supplemented group and were not significantly different between groups on the day of arrival (Supplementary Table 7). Neutrophil counts were higher in the supplemented group (1.77 × 10^3/µL *vs*. 2.55 × 10^3/µL; P <0.001). However, average neutrophil count in both groups was below levels reported previously (41). All inflammation associated ratios were significantly elevated in animals from the supplemented group in comparison to those of the control group, namely N/L (0.35 *vs.* 0.63; P = 0.005), P/L (116 *vs.* 189; P<0.001) and SII (174 *vs.* 493; P<0.001).

Table 2 – Haemogram performed at the end of the assay.

Parameter	Mean		SEM	P-value
	Control	Supplemented		
Total erythrocyte count (× 10^6/µL)	9.69	9.69	0.186	0.993
Haemoglobin (g/dL)	9.44	9.12	0.163	0.171
Haematocrit (%)	29.5	28.8	0.4800	0.364
Mean Corpuscular Vol. (fL)	30.8	29.3	0.64	0.098
Mean Corpuscular Hg (pg)	9.85	9.37	0.253	0.198
Mean Corpuscular Hg Conc. (g/dL)	31.8	31.7	0.26	0.944
Erythrocyte distribution index (%)	23.0	28.5	1.05	0.001
Platelets (x 10^3/µL)	608	768	28.2	<0.001
Average Platelet Volume (fL)	8.54	8.35	0.162	0.453
Leukocytes (× 10^3/µL)	7.67	7,60	0.170	0.768
Neutrophils (× 10^3/µL)	1.77	2.55	0.130	<0.001
Eosinophils (× 10^3/µL)	0.214	0.100	0.0229	0.002
Basophils (× 10^3/µL)	0.0852	0.0913	0.00993	0.667
Monocytes (× 10^3/µL)	0.541	0.442	0.0561	0.226
Lymphocytes (× 10^3/µL)	4.97	4.36	0.266	0.128
N/L	0.352	0.636	0.0707	0.005
P/L	116	188	10.0	<0.001
SII	174	493	47.4	<0.001

SEM, Standard error of the mean; N/L, neutrophils and lymphocytes ratio; P/L, platelets and lymphocytes ratio; SII, systemic immune inflammation.

Eosinophil counts were significantly higher (P=0.002) in control group (0.21 × 10^3/µL) than in supplemented group (0.01 x 10^3/µL), but these were also higher on the day of arrival, not being clear if it was further affected by diet. Average platelet volume and lymphocyte count were significantly different between groups, in the beginning (P=0.003) and P=0.028; Supplementary Table 7), but not at the end of the assay (Table 2).

Serum Cytokines

Several pro- and anti-inflammatory cytokines were measured in the serum of control and supplemented calves at the end of the experimental period. Calves fed *C. vulgaris* for six weeks presented a trend to higher serum IL-8 (37 568 pg/mL) than calves fed the control diet (20 561 pg/mL; P=0.050; Table 3). Serum levels of IFN-γ, IL-2, IL-10 and IL-17 were not statistically different between groups (P>0.05; Table 3). Serum TNF-α, IL-1β, IL-6 and IL-4 were below detection limits in most samples (Supplementary Table 9). Interestingly, serum IL-6 levels were significantly different between groups of calves to be allocated to control and supplemented diets (P=0.033; Supplementary Table 8), but at the end of the assay, IL-6 levels were below detection limits in 9 out of the 13 samples (Supplementary Table 9).

Table 3 – Serum cytokines levels at the end of the assay

Parameter	Mean		SEM	P-value
	Control	Supplemented		
IFN-y pg/mL	7.95	10.1	2.785	0.580
IL-2 pg/mL	130	177	58.9	0.574
IL-8 pg/mL	20 561	37 568	6075.9	0.050
IL-10 pg/mL	435	352	36.0	0.107
IL-17 pg/mL	20.5	20.8	3.75	0.956

SEM, standard error of the mean

Cytokine production and mRNA expression by bovine MDM

Exposure to several PRR agonists, including through diet, have already been proven to induce *in vivo* trained innate immunity (42-45). Trained animals undergo epigenetic and metabolic reprogramming of monocytes, macrophages, and NK cells that render these cells more efficient in exerting their protective effects against infection, but that can contribute to increased inflammation (44, 45). Interestingly, this phenomenon also affects haematopoietic stem cells (HSC), conferring long-lasting effects (46). Here, we evaluated the magnitude of the cytokine response of bovine MDM to *in vitro* stimulation with several PRR agonists such as LPS, Pam3CSK4, WGP-Dispersible and Zymosan. Global levels of IL-6 produced by cells from the control group were significantly higher (219 pg/mL) than those produced by the supplemented group (72.6 pg/mL; P<0.0001; Table 4; Figure 2). Global *IL10* mRNA expression was, in contrast, significantly higher in bovine MDM from the supplemented group (0.90) than from the control group (0.58; P=0.026; Table 4). There was, however, no effect of treatment (P>0.05) when

comparisons were made within each stimulus (Figure 2 and Figure 3). No differences (P>0.05) were observed in the global levels of TNF-α and IL-8 production, nor in global mRNA expression levels of *TNFA*, *IL1B*, *IL6*, *IL12A*, *IL12B* or *IL23A* (Table 4) between control and supplemented groups.

Table 4 – Global cytokine production and mRNA expression in bovine MDM cultured with different PRR agonists for 24 h. Global mean response to LPS, Pam3CSK4, WGP-P and zymosan, namely TNF-α, IL-6 and IL-8 production, *TNFA*, *IL1B*, *IL6*, *IL10*, *IL12A*, *IL12B* and *IL23A* expression are displayed.

Parameter	Mean		SEM	P-value
	Control	Supplemented		
TNF-α pg/mL	34.7	24.4	4.80	0.134
IL-6 pg/mL	219	72.6	19.25	<0.001
IL-8 pg/mL	37456	34639	3726.1	0.596
TNFA	0.0332	0.0590	0.01194	0.130
IL1B	1.52	1.34	0.327	0.698
IL6	0.165	0.157	0.0416	0.889
IL10	0.582	0.901	0.1004	0.026
IL12A	0.000579	0.000624	0.0000963	0.739
IL12B	0.000662	0.000740	0.0002471	0.827
IL23A	0.00626	0.00499	0.003938	0.821

SEM, standard error of the mean

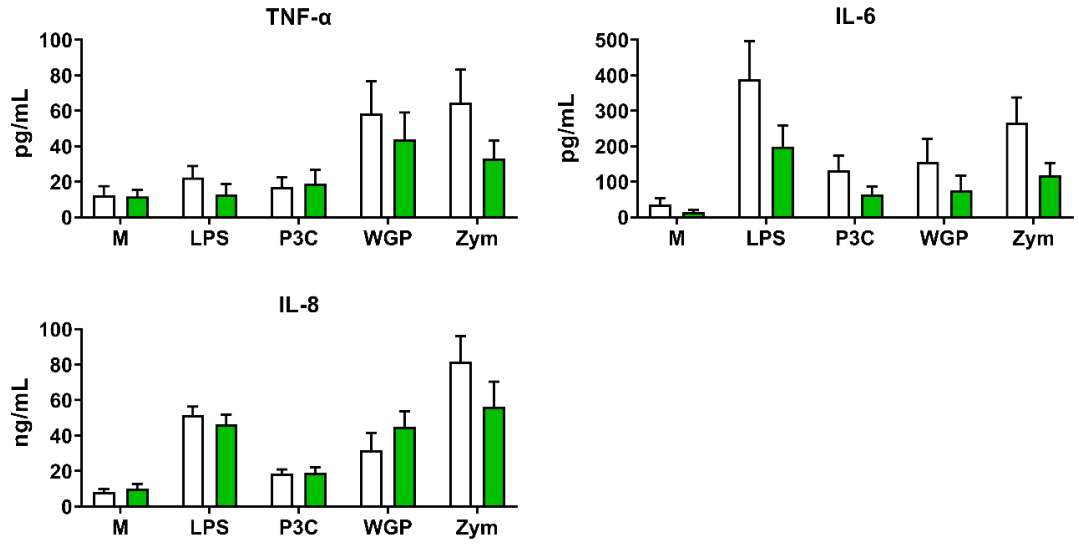

Figure 2 – Cytokine production, evaluated by ELISA in the supernatants of bovine MDM cultured for 24 h without stimulus (M) or stimulated with *E. coli* lipopolysaccharide (LPS), Pam3CSK4 (P3C), both at 2 µg/mL, WGP-P or Zymosan, both at 50 µg/mL. Bars represent mean plus SEM of cytokine production. No statistically significant differences were found between control (white columns) and supplemented (green columns) groups when comparing the same conditions (P> 0.05).

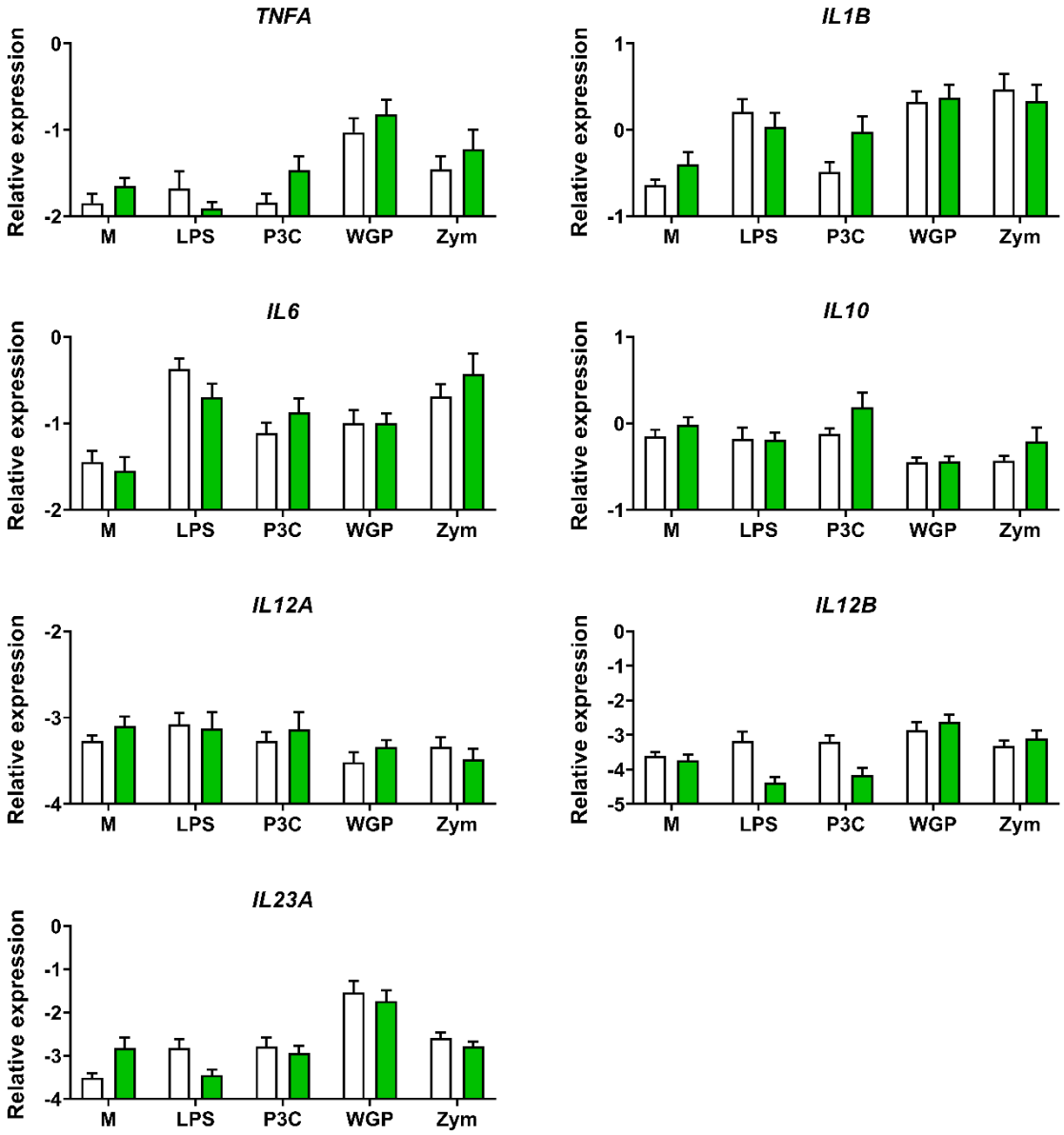

Figure 3 – Cytokine relative mRNA expression evaluated by RT-PCR in bovine MDM and normalized to the geometric mean of the mRNA expression of the reference genes *MARVELD1* and *PPIA*. Cells were cultured for 24 h without stimulus (M) or stimulated with *E. coli* lipopolysaccharide (LPS), Pam3CSK4 (P3C), both at 2 µg/mL, WGP-P or Zymosan, both at 50 µg/mL. Bars represent means plus SEM of mRNA relative expression. No differences were found between control (white columns) and supplemented (green columns) groups when comparing within each stimulating condition ($P > 0.05$). Although statistical analysis was performed using absolute values (Table 5), the scale was set to logarithmic to ease the graphical comparison between groups and conditions.

Phagocytosis by bovine MDM

Phagocytosis of latex green-fluorescent beads by macrophages differentiated from blood monocytes from both animal groups was evaluated by flow cytometry. Gating strategy used to calculate the percentage of phagocytosis, inferred from the percentage of green

positive cells (Beads) is shown in Figure 4A. No differences were observed in the percentage of bead internalization by MDM of control and supplemented groups at any of the timepoints assessed (Figure 4B).

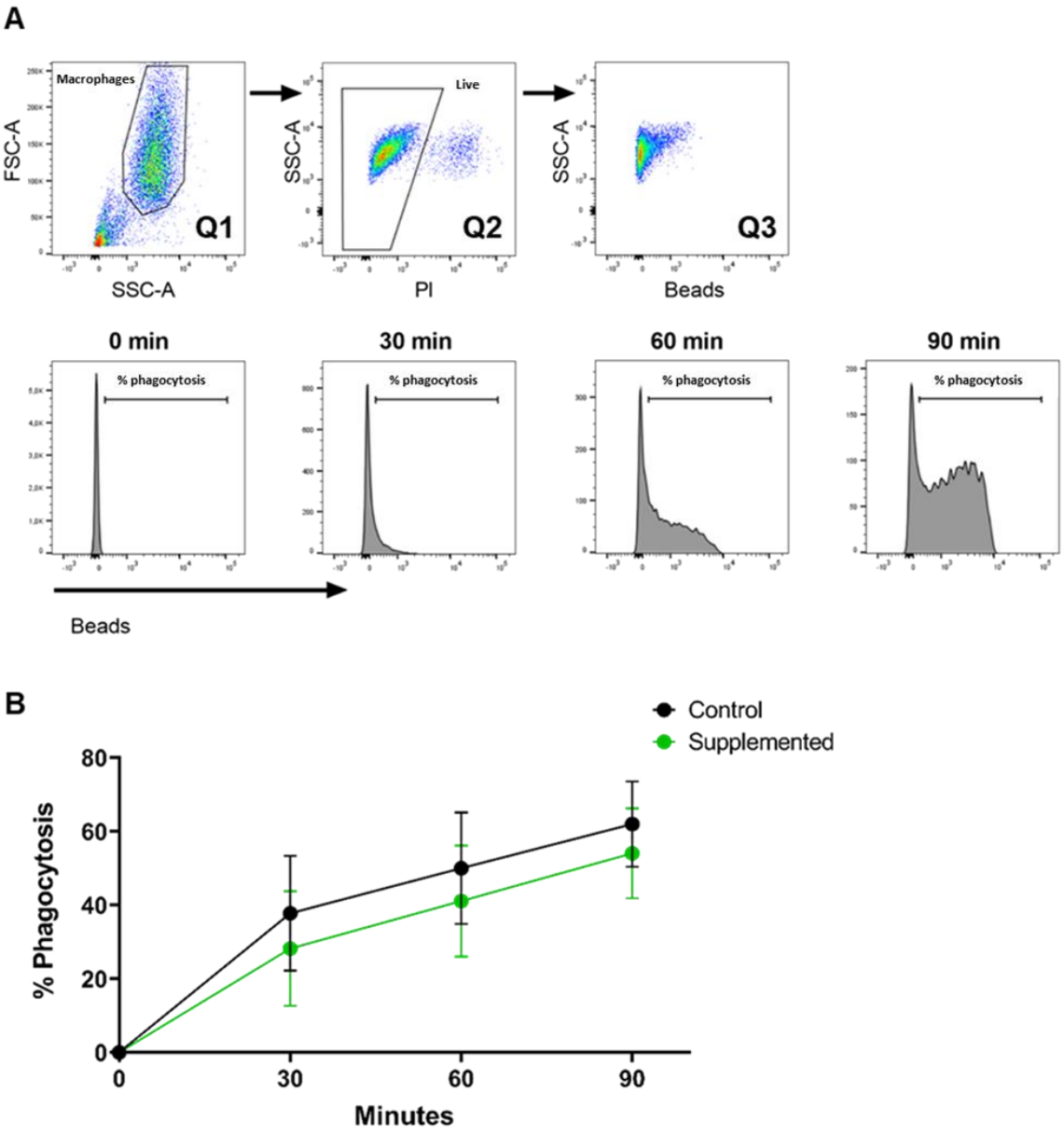

Figure 4 – Quantification of fluorescent-labelled bead phagocytosis by bovine MDM at the end of the assay, evaluated by flow cytometry. (A) Gating Strategy showing the exclusion of cell debris (A-Q1), exclusion of dead cells using propidium iodide (A-Q2), and selection of cells with internalized beads (A-Q3 and histograms). A 90-minute kinetic study was performed. Representative examples of MDM incubated with fluorescent beads for 0, 30, 60, and 90 min. (B) Results are presented as lines connecting individual data points corresponding to means ± SEM percentage of phagocytosis, at timepoints 0, 30, 60 and 90 min. No differences were found between groups regarding phagocytosis ability of bovine MDM.

Peripheral blood mononuclear cell Proliferation

To assess if the ability of lymphocytes to respond to mitogens was affected by the dietary *C. vulgaris* supplementation, PBMC proliferation in response to the T lymphocyte mitogen Concanavalin A (ConA) stimulation was analysed by flow cytometry (Figure 5A). Although PBMC from supplemented calves presented numerical higher average cell division percentage in response to ConA (Figure 5B), this was not statistically significant.

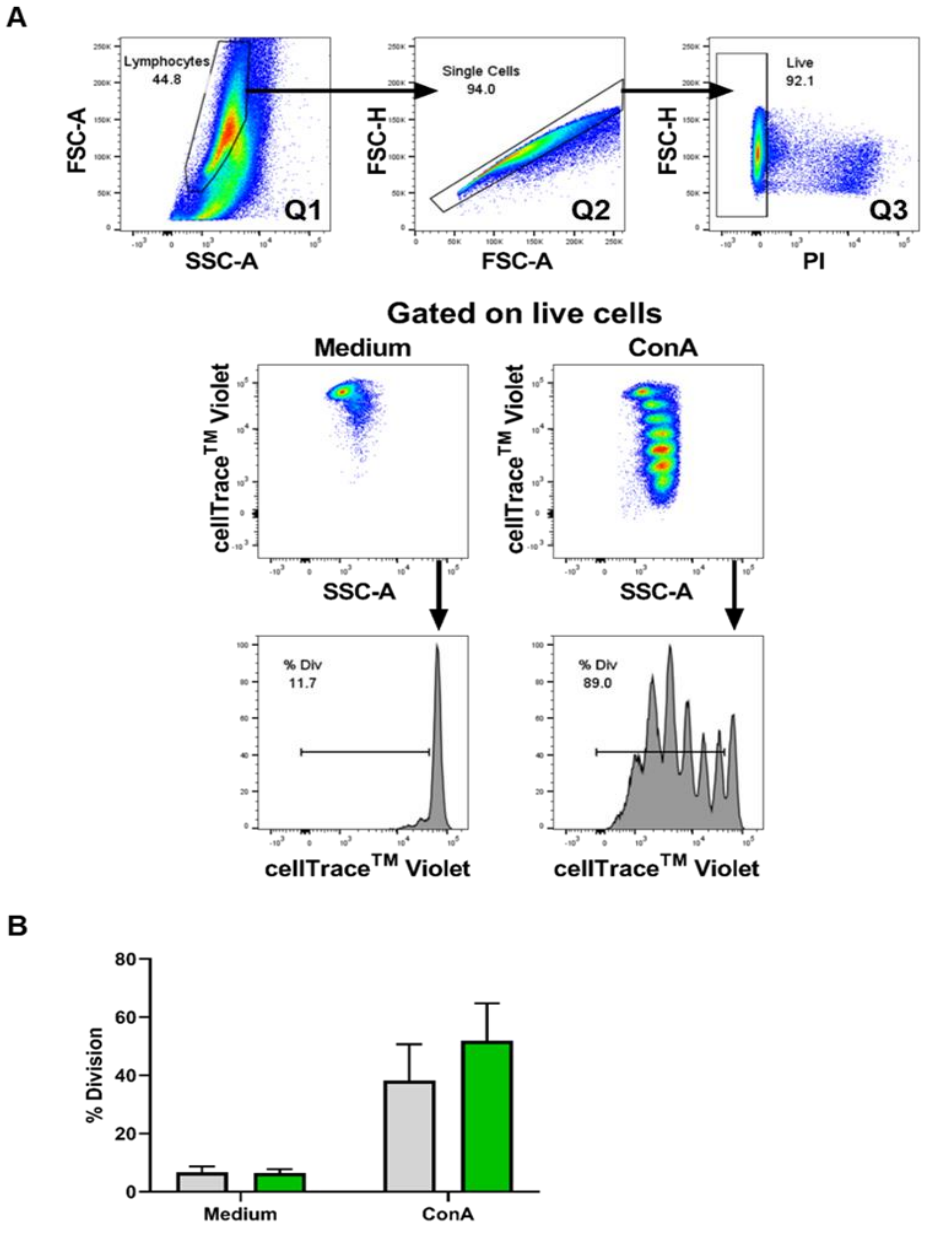

Figure 5 – Proliferation of PBMC from control (white columns) and supplemented (green columns) groups in response to ConA. (A) PBMC were labelled with CellTrace™ Violet Cell Proliferation Kit and incubated with 1 µg/mL ConA for 72 h. Non-stimulated labelled cells (Medium) were used as negative controls. Cells were incubated with PI prior to sample acquisition. Representative examples of non-stimulated PBMC or stimulated with ConA. Cell debris and dead cells were excluded from the analysis. (B) The percentage of cell division was calculated based on violet dye dilution.

Faecal pH and SCFA evaluation

To assess enteric fermentation, faecal pH and SCFA profiles were evaluated. Faecal pH and total SCFA production were similar (P>0.05) between groups (Table 5). In contrast, SCFA profile was affected by 1% dietary supplementation of *C. vulgaris*. Faeces of calves supplemented with *C. vulgaris* had lower molar proportion of propionic acid (22.62% *vs*. 20.69%; P=0.027), and higher of isobutyric (0.53% *vs*. 0.76%; P=0.002), valeric (0.51% *vs*. 1.01%; P=0.012), isovaleric (0.43% *vs*. 0.73%; P=0.001) and isocaproic acids (0.06% *vs*. 0.10%; P=0.001) (Table 5). A tendency for increased molar proportion of butyric acid (6.38% *vs.* 5.29%, P=0.085) was also observed (Table 5).

Table 5 – Faecal pH and short-chain fatty acids (SCFA) of control and supplemented groups.

Parameter	Mean		SEM	Adjusted P value
	Control	Supplemented		
pH	7.24	7.40	0.082	0.152
Total SCFA (mmol/g)	0.209	0.223	0.0137	0.490
Molar proportions (mmol/100 mmol)				
Acetic acid	69.4	71.3	0.96	0.155
Propionic acid	22.6	20.7	0.61	0.027
Butyric acid	6.38	5.29	0.443	0.085
Isobutyric acid	0.527	0.760	0.0515	0.002
Valeric acid	0.508	1.01	0.141	0.012
Isovaleric acid	0.435	0.730	0.0604	0.001
Caproic acid	0.0887	0.0845	0.01999	0.882
Isocaproic acid	0.0557	0.1017	0.00965	0.001

SEM, Standard error of the mean

Faecal Microbiome

To evaluate if *C. vulgaris* supplementation in milk replacer would impact calves' faecal microbiome, shotgun metagenomic data were analysed to identify the most significant microbial species (key microbial biomarkers) associated with faecal microbiome of each animal group. Operational taxonomic unit (OTU) abundance for top phyla and top genera are displayed in Figures 6A and 6B, respectively, for control group (group 1) and supplemented group (group 2). Most abundant phyla were Bacteroidetes, Actinobacteria, Firmicutes, followed by unclassified phyla, Euryarchaeota and Proteobacteria (Figure 6A).

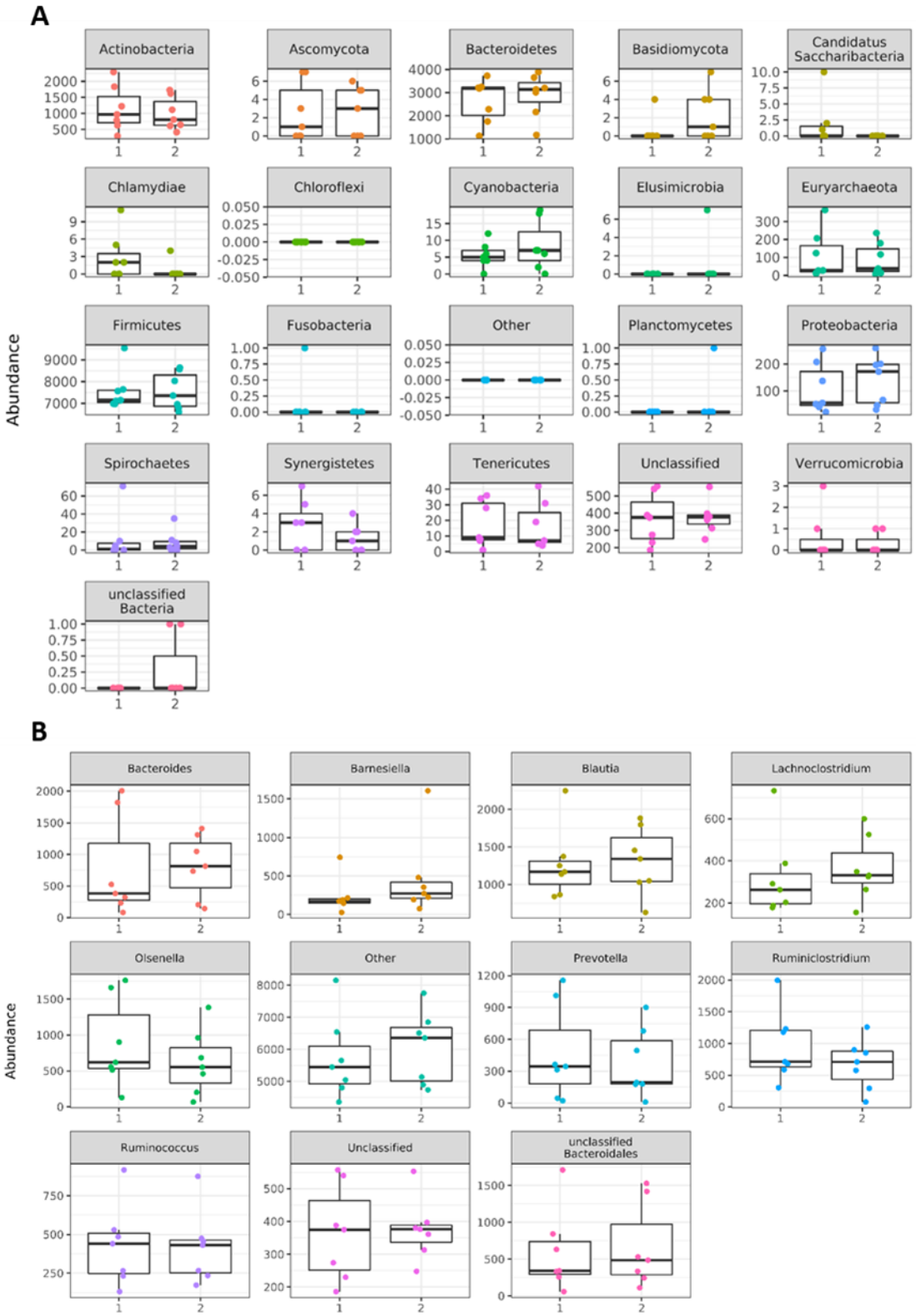

Figure 6 – (A) OTU abundance plots for top 20 phyla present in the data. Groups 1 and 2 represent control and supplemented calves, respectively. The black line on each box defines the median. Each dot corresponds to an animal. (B) OTU abundance plots for top 10 genera present in the data. Groups 1 and 2 represent control and supplemented calves, respectively. The black line on each box defines the median. Each dot corresponds to an animal.

Most abundant genera were *Blautia*, *Bacteroides*, *Ruminiclostridium Olsenella*, unclassified genera and *Prevotella*, following other genera (Figure 6B).

Microbial diversity within each sample (alpha-diversity, Figure 7A) and microbial composition (Figure 7B) between calf faecal samples were not significantly different between groups (*P*>0.05).

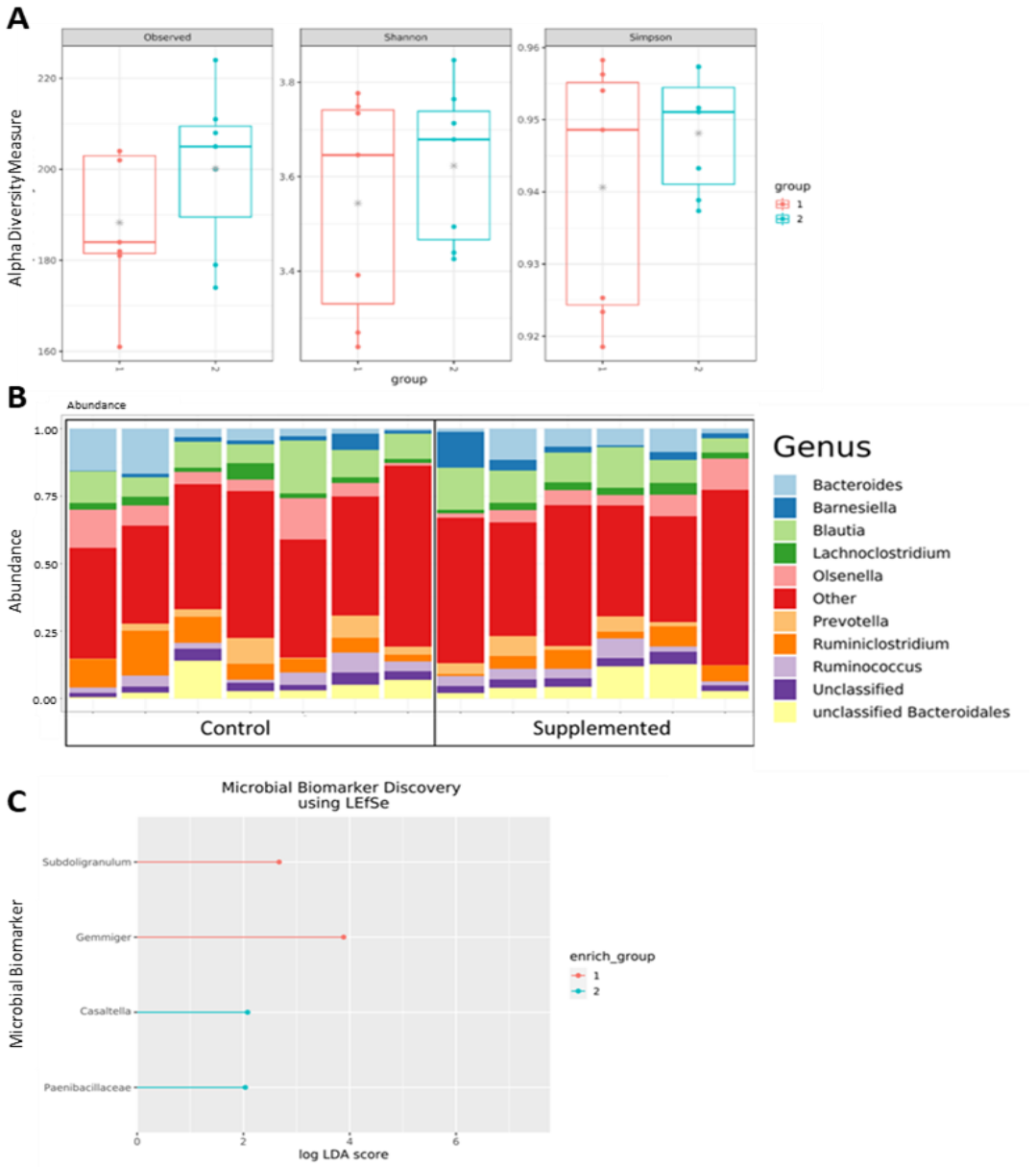

Figure 7 – (A) Alpha diversity richness between groups. Groups 1 and 2 represent control and supplemented calves, respectively. The line and asterisk on each box define the median and mean, respectively. Each dot corresponds to an animal. (B) Bar plots of relative abundance, at genus level, per sample. Seven animals from control group are aligned at left and six animals from supplemented group at right. (C) Linear discriminant analysis Effect Size (LefSe) was used to select the OTUs that best discriminate groups (potential biomarkers). LefSe analysis consists of using standard tests for statistical significance coupled with tests encoding biological consistency and effect relevance to determine the OTUs most likely to explain differences between groups.

Linear discriminant analysis Effect Size (LefSe) significantly identified the *Gemmiger* and *Subdoligranulum* genera as potential biomarkers for control group and *Casaltella* and *Paenibacillaeceae* for supplemented group (Figure 7C). LEfSe is an algorithm that determines features, namely operational taxonomic units, that explain the differences observed among conditions. It relies on the use of standard tests to determine statistical significance and tests encoding biological consistency and effect relevance. Overall, microbial composition of faecal samples was not significantly different between groups.

Discussion

Microalgae, namely *C. vulgaris*, are promising alternatives to health supplementation and have been promoted as functional food and feed, due to their diverse bioactive compounds (47). In human medicine, several studies have also been conducted to assess the effect of microalgae and microalgae-derived products supplementation on the prevention of cardiovascular disease (48, 49) and fatty liver disease (48), on the anti-inflammatory effects in LPS-stimulated cell lines (50), on the inhibitory effects on human colon cancer cells (51) and even as nutraceuticals to help fight COVID-19 (52). Interestingly, Capelli and colleagues have also reported that the antioxidant effects of synthetic astaxanthin do not equal the antioxidant power of microalgae-derived astaxanthin (53), indicating that bioactive properties of microalgae-produced molecules might not be completely mimicked by synthetic components. Studies performed in livestock indicate supplementation may also improve animal performance and overall health. In broilers, dietary supplementation with microalgae improved body weight, daily gain, feed efficiency, serum composition and antioxidant status (54, 55). Piglets supplemented microalgae *Chlorella vulgaris* shown decreased incidence of diarrhoea (56), decreased growth retardation after weaning and increased IL-1β expression in the jejunum (57) and a healthier gut microbiota (27). Calves fed a commercial product based on *Schizochytrium* spp. increased starter intake comparatively to control calves, which may contribute for a successful weaning, and lower levels of serum reactive-oxygen species (58). Microalgae can also bioconvert inorganic minerals, such as inorganic selenium, into organic minerals, with higher bioavailability and lower risk of toxicity (59). Hence, research concerning inclusion in human and animal diets has been increasing in the past few years. The present study aimed at assessing the effect of supplementing milk replacer with 1% *C. vulgaris* (w/w) in the health status, performance, and immune parameters of newborn Holstein-Friesian calves. Overall health was identical between groups. Although gains were numerically higher in control calves, there were no significant differences between groups. Lower feed intake has been reported with up to

1% *C. vulgaris* supplementation in broiler chickens (29) and dairy cows (60), which was explained by a lower palatability of the diet due to *C. vulgaris* fishy smell, taste and powdery structure (22). However, in this work there were practically no milk refusals, with the exception of one animal, from the supplemented group, that drunk slightly less (half a litre in each meal) on two sporadic non-consecutive days. Thus, suggesting that *C. vulgaris* at 1% supplementation did not negatively affect milk replacer palatability, which resulted in no differences on its intake and growth of newborn calves. In a study from McDonnell at al. 2019, calves fed a commercial supplement composed of a seaweed-derived β-glucan (laminarin from *Laminaria* spp.) and fucoidan, weighted significantly less, at the end of the experimental period, than calves from the control group (61). These authors have attributed the weight decrease in the supplemented group to reduced concentrate intake. Contrastingly, other authors observed higher body weights at the end of the assay, and a tendency for higher average daily gains, in calves fed milk replacer supplemented with a commercial β-glucan derived from the microalga *Euglena gracilis* (62). The authors hypothesise those effects could be explained by the fewer days supplemented calves had abnormal faecal consistency, comparatively to control calves, which could explain better nutrient absorption due to healthier intestinal barrier. Tomalusky and colleagues reported higher compound feed intakes, metabolic weight and total dry-matter intake, without differences in feed efficiency, in calves fed milk replacer supplemented with *Schizochytrium* spp. (58). In our study compound feed and hay intake were administered *ad libitum*, not being possible to determine differences on total feed intake between groups. However, as average gains were similar between groups, we hypothesise that no differences occurred in total feed intake. This point should be clarified in further studies.

No significant differences in total serum proteins and globulin levels were observed between groups. This indicates that calves from both groups had, in average, identical levels of maternal IgG at the beginning of the assay. Average haemogram parameters were in accordance with reference ranges for calves, except for mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration, which were slightly below the reference values. All parameters were also within 90% confidence intervals for haematology reference intervals in Holstein-Friesian calves, assessed by Panousis *et al.* (63), and described by Morita *et al.* (41), although the two above-mentioned parameters were very close to the lower limit values. There were statistically significant differences in erythrocyte distribution index, platelet and neutrophil counts and in N/L, P/L and SII inflammation-associated indicators between groups. Similarly, in a study performed by Gunun *et al.* 2022, there were significantly higher neutrophil and monocyte counts in hydrolysed yeast-supplemented growing beef cattle, without changes in growth

performance (64). In our study, eosinophil counts were also different between groups, but this difference may reflect the difference between groups at the beginning of the assay. Calves from supplemented group had higher erythrocyte distribution index which is due to higher variation of the size of red blood cells (anisocytosis). There is not much information regarding variations on the erythrocyte distribution index in cattle, but it should be noticed the expressive increase in supplemented group, from the beginning until the end of the assay. There could be a plethora of causes for anisocytosis, but one of the main reasons is iron deficiency (65). There are reports on the role of IL-6-induced hepcidin in human and mouse cells that consequently affects iron availability. Hepcidin is an iron regulatory hormone that, when in excess, leads to endocytosis and proteolysis of ferroportin (an iron exporter) lowering the transport of iron from inside the cell to the extracellular space (66, 67). Haemoglobin synthesis could be thus limited, in these cases, due to lower supply of iron, resulting in anemia (66). However, there were no significant differences between groups on the remaining haemogram parameters that could be associated with anemia in the supplemented group. Other authors reported that the structure of human erythrocytes was pronouncedly affected by IL-8, a pro-inflammatory cytokine, namely with morphological changes resembling erythrocyte programmed cell death (eryptosis) (68). We did not see any differences in serum IL-6 levels, but we hypothesize that serum IL-8, that was significantly higher in supplemented group comparatively to control group, could be affecting red blood cell size, similarly to what was previously reported (66, 67). More research is needed to understand these observations. The increased IL-8 serum levels in calves from the supplemented group are indicative of increased inflammation and are in accordance with higher neutrophil and platelet counts and with N/L, P/L and SII indexes. In fact, previous research described systemic administration of IL-8 as being able to induce proliferation of haematopoietic stem cells, with long term myelo-lymphoid repopulation potential (69). N/L, P/L and SII are novel inflammatory biomarkers that are being studied and used mostly in human medicine as sensitive prognostic tools for infectious, metabolic and tumoral disorders (70-78). N/L has also been proposed as a biomarker of stress (79), and so, several authors have been using it to evaluate physiological response to stressors in calves, such as weaning (80-82) and transport (83). In cattle, a study has also correlated N/L and P/L with risk from developing subclinical mastitis, in which cows with higher somatic cell counts exhibited higher ratios and presented lower levels of serum IL-10 and higher levels of serum IL-6 and TNF-α (40). Thus, high N/L, P/L and SII indexes are associated with poorer prognosis and with severity of disease. It has even been proposed that diets and exercise improve N/L in overweigh individuals (84) and that diets enriched with anti-inflammatory compounds such as polyphenols and ω-3 fatty

acids may impact on N/L and SII indexes as well. In fact, in a retrospective study that evaluated diet before coronary surgery, patients that consumed more eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the two most important long-chain ω-3 polyunsaturated fatty acids, had lower N/L and SII indexes, with a significantly negative correlation obtained between EPA and DHA consumption and those indexes (85). In that same line, rams supplemented with 3% *Nannochloropsis oculata,* which is a microalga characteristically rich in EPA (86, 87), showed lower N/L than control rams. However, no differences were observed between control group and rams supplemented with 1% *N. oculata* (88). Our study indicates that supplementation with *C. vulgaris* may induce a systemic inflammatory state. It would be useful, however, to evaluate if that inflammatory state would benefit the animals upon infection.

We did not observe significant differences in serum IFN-y, IL-2, IL-10 and IL-17, and serum TNF-α, IL-1β, IL-6 and IL-4 were mostly below detection limits. Very little information is available on the effects of the diet inclusion of microalgae in serum cytokine levels, particularly in the absence of infection or inflammation-inducing pathologies, which difficults data interpretation. In humans, diet supplementation of healthy subjects with *C. vulgaris* increased serum levels of IFN-y, IL-1β, and IL-12 (89). In broiler chickens, diet supplementation with *Arthrospira platensis* (spirulina) did not affect serum IL-1β levels (90), although TNF-α was reduced in the liver. In contrast, blood leukocytes from calves supplemented with DHA-rich oil extracted from *Schizochytrium* spp. in milk replacer had lower IL-1β expression than control leukocytes (91). The effects of microalgae or microalgae-derived molecules on serum cytokines are more evident in response to challenge, induced-stress or in previously existing inflammatory conditions. Studies performed in Nile tilapia showed that serum TNF-α and IL-10 were decreased in *A. platensis* supplemented groups. Yet, these groups seemed to be more protected against *Aeromonas hydrophila* infections than control group (92). In another study, piglets fed microalgae and subjected to an LPS challenge produced higher levels of TNF-α, IL1-β, IL-6 and a tendency to increased IL-10 than piglets from control group (93). In other studies, supplementation induced the reduction of the production of inflammatory cytokines in hyperinflammatory conditions. As an example, treatment of osteoporosis-induced rats with oral *Heamatococcus pluvialis* resulted in decreased IL-6 serum levels and ameliorated bone loss (94). Also, supplementation with *A. platensis* decreased serum TNF-α, IL1-β, and IL-6 in rats under fat diet-induced chronic inflammation (95). These apparently contradictory results indicate that, nevertheless, diet supplementation results in a positive outcome, depending on the underlying condition (96-101). In that sense, it would be interesting to perform a challenge, namely vaccination, infection or a stressful event (such as weaning) and compare the immune responses between

supplemented and control calves under those conditions. Accordingly, the effect of supplementation during the neonatal period in the resistance to infection and other stressors should be further investigated.

Although we observed no differences between groups when comparing macrophages' response to several TLR agonists (cytokine production and mRNA expression) within each stimulus, the overall IL-6 production was significantly lower and *IL10* mRNA expression significantly higher in supplemented calves. We therefore hypothesise that calves' macrophages may produce less IL-6 and more IL-10 upon contact with pathogens. This would be in line with the reported anti-inflammatory role of microalgae and microalgae-derived products on monocytes and macrophages (50, 102, 103). Phagocytosis was not significantly different between MDM of the two groups. In a study where heifers' diets were supplemented with a yeast-based commercial product, the authors reported increased *E. coli* internalization and ROS production by neutrophils from supplemented group comparatively to control group (104). In another study, where the same yeast-based commercial product was used in peripartum dairy heifers, authors also reported higher phagocytosis of *E. coli* in the supplemented group (105). A higher percentage of phagocytosis was observed in peritoneal macrophages from mice fed *A. platensis* in comparison to macrophages from the control group (106). The macrophages from *A. platensis* fed broiler chickens also had a higher phagocytic potential than cells from the control group (107, 108). There were also no significant differences in the proliferation percentage of PBMC from the two groups to ConA stimulation. In accordance, supplementation of veal calves with DHA from *Schizochytrium* spp. also did not affect PBMC proliferation (109). This is in contrast with the increased lymphocyte proliferative effect promoted by diet microalgae inclusion in other studies. Indeed, blood leukocyte cultures from rams supplemented with *N. oculata* proliferated more in response to the mitogen phytohemagglutinin (PHA) than leukocytes from the control group (88). These cells also produced higher amounts of IL-6, IL-12, TNF-α, and IFN-y. Despite this pro-inflammatory profile of stimulated cells, the numbers of blood neutrophils and N/L in supplemented animals were lower than those of controls, indicating that systemic inflammation was not induced. In that line, *A. platensis* supplementation increased the Con-A-mediated proliferation of mouse spleen cells (106) and the PHA-mediated lymphoproliferative response of broiler chickens (108). The high variability found in microalga-induced immune parameters among studies could be attributed to numerous factors, including differences in animal species and age, microalgae species and production conditions, composition, and percentage of inclusion. Basal diet composition may also play a role on the conflicting results obtained *in vivo*. In fact, a very recent study, performed in broiler chickens, showed that benefits of microalgae inclusion

were dependent on basal diet formulation, which explained differential performance and immune responses to the supplementation (110).

Faecal pH was not significantly different between groups, agreeing with similar total SCFA production. However, molar proportions of most individual faecal SCFA, including branched SCFA, were significantly affected by milk replacer *C. vulgaris* supplementation. Microalgae may be an important source of functional fibres and amino acids that if not digested and absorbed in the upper gut, provide substrate for colonic microbiota fermentation and production of SCFA with potential health benefits for the host (111). Major SCFA produced by intestinal microbiota are acetic, propionic, and butyric acids (111). These fermentation end-products reach systemic circulation and are known to activate genes related to inflammatory processes, thereby holding immunomodulating properties (112, 113). Butyric acid, for example, is metabolized by colonic cells and besides being used by colonocytes as an energy source, it is also the main contributor to regulatory T cells' pool in the colon, protecting from colitis and regulating systemic inflammation (111, 114, 115). Production of branched SCFAs in the gut have been less explored, but it is proposed that isobutyric acid may also play a role as an energy source for colonocytes (116) and, along with isovaleric acid, it is thought to be involved in systemic lipidic and glucose metabolisms, as studied by Heimann et al. (117) in primary rat and human adipocytes.

In our study, propionic acid was significantly decreased, and butyric acid tended to be decreased in *C. vulgaris* group, while isobutyric, valeric, isovaleric and isocaproic acids' molar proportions were increased. Propionic and butyric acids are the major SCFA produced by the gut microbiota, along with acetic acid (118, 119). Butyric acid is used as an energy source by colonocytes and they both contribute to local immune responses by downregulating the expression of proinflammatory cytokines in response to LPS-trigger, by modulating leukocyte trafficking, and also by potentiating regulatory T-cells (Treg) differentiation (114, 120). Thus, lower proportions of propionic and butyric acids may negatively impact on local immune responses in supplemented calves. Isobutyric, isovaleric and isocaproic acids are rather end-products of protein metabolism, produced in much smaller amounts, and are thereby used as faecal markers of protein fermentation (115). The increased molar concentrations of these branched SCFA may suggest a higher proteolytic microbiota and/or activity or reflect the increased protein content of the supplemented group diet due to the inclusion of *C. vulgaris*, a rich protein source. However, our results contrast with findings from a previous report where the effects of microalgae were assessed in a monogastric (canine) gut model. In that particular study, molar proportions of acetic and butyric acids were increased in an *in vitro* incubation with *C. vulgaris*, while isobutyric and isovaleric acids were decreased

(121). The authors speculated that despite the higher protein content in the experimental vessels, decreased branched SCFA may indicate decreased microbial proteolytic activities. Contrastingly, in another study performed not with microalgae but with macroalgae, four different intact seaweeds were provided to dogs and the authors did not observe any differences in faecal SCFA nor branched SCFA between groups (122). The lack of effects was attributed to the low dose used in that particular study (15 g/kg). It should be highlighted, however, that lower levels of faecal SCFA do not necessarily indicate lower production but rather better absorption through the intestinal wall (123).

The role of microbiome in disease progression and systemic immune responses is being increasingly explored (112, 124). It is possible that modulation of microbiome with a nutritional approach may benefit the animal health status. However, further research is still needed to understand the highly complex interactions between microbiome, local immune system, and systemic immune system. In our work, no differences in microbiota diversity and composition were detected between groups. A study performed in adult dogs, fed four different seaweeds, also showed no differences in microbial populations among groups. However, contrastingly to our study, these authors reported no differences in faecal SCFA (122). In other studies, microalgae supplementation promoted alterations of gut microbiome associated with gut health. Piglets fed *C. vulgaris* had increased abundance of some specific potentially-beneficial bacterial taxa (27) and *A. platensis* supplementation modulated gut microbiome in a dysbiosis scenario in rats, although no obvious differences could be observed in homeostasis (95). Broiler chickens supplemented with a *Chlorella* by-product (125) and with fresh liquid *C. vulgaris* (29) showed increased *Lactobacillus* spp. concentration in intestinal contents.

Despite not observing differences between groups, we were able to identify different potential biomarkers for both groups: *Gemmiger* and *Subdoligranulum* genera for control group, and *Casaltella* genus and Paenibacillaeceae family for supplemented group. There is not much information on these taxa in calves, or even cattle. It is known, however, that *Gemmiger* is an important genus in comparative studies of inflammatory bowel disease, being an important feature in the classification of subjects to Crohn's disease and ulcerative colitis – it is reported that *Gemmiger* genus is an important biomarker and could be decreased in inflammatory bowel disease patients. Besides, *Gemmiger* is sometimes included in probiotic supplements (126). Other authors also reported *Subdoligranulum* genus as a biomarker in Crohn's disease-patients, where levels are decreased compared to controls (127). On the contrary, *Paenibacillus* is described to be markedly increased in vancomycin-treated mice showing higher levels of gut inflammation and tumorigenesis (128). Regarding faecal microbiota, it seems reasonable to assume that dietary supplementation with *Chlorella vulgaris* did not bring

any advantage to newborn calves. Although there were only slight differences between groups, this study could be a good starting point for further assays, where an immune challenge (vaccination and/or experimentally induced infection) could bring some answers.

Author Contributions

ARVP performed the experiments, data acquisition and analysis, and wrote the manuscript. AC and CM participated in the experiments. EGM and ICR assisted in the design of the experiments. ARJC and AJMF assisted in the design of the experiments and interpretation of data. AC, MRGM, and MV supervised the experimental work, assisted in data acquisition and analysis, and manuscript writing.

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Conflict of Interest

The authors have no conflicts of interest to declare.

Supplementary Material

Supplementary Table 1 – Milk Replacer Chemical Composition

Composition	Percentage
Crude Protein	23.00 %
Crude Fat	18.00 %
Crude Fibre	0.00 %
Crude Ash	7.00 %
Calcium	1.00 %
Phosphorus	0.80 %
Sodium	0.40 %
Lysine	1.70 %
Methionine	0.60 %

Supplementary Table 2 – Milk Replacer Formula

Components	Inclusion
Skimmed milk powder[a]	-
Whey powder[a]	-
Palm oil[a]	-
Whey protein concentrate[a]	-
Copra oil[a]	-
Vitamin A	25 000 IU/kg
Vitamin D3	4 500 IU/kg
Vitamin E	100.0 mg/kg
Vitamin B1	6.5 mg/kg
Vitamin B2	6.0 mg/kg
Vitamin B6	2.5 mg/kg
Vitamin B12	0.06 mg/kg
Niacin	40.0 mg/kg
Calcium D-pantothenate	13.0 mg/kg

Supplementary Table 2 (Cont.)– Milk Replacer Formula

Components	Inclusion
Vitamin K3	2.5 mg/kg
Vitamin C	100.0 mg/kg
Chelated copper from protein hydrolysates	10.0 mg/kg
Chelated zinc from protein hydrolysates	60.0 mg/kg
Chelated manganese from protein hydrolysates	40.0 mg/kg
Chelated iron from protein hydrolysates	40.0 mg/kg
Sodium Selenite	0.10 mg/kg
Selenized Saccharomyces cerevisiae	0.20 mg/kg
Bacillus subtilis	0.64×10^9 CFU/kg
Bacillus licheniformis	0.64×10^9 CFU/kg

[a] The inclusion percentage of this ingredient is not provided due to intelectual property rights of the company responsible for the milk replacer formulation

Supplementary Table 3 – Compound Feed Composition

Composition	Percentage
Crude Protein	18.00 %
Crude Fat	2.60 %
Crude Cellulose	4.10 %
Crude Ash	6.50 %
Sodium	0.19%

Supplementary Table 4 – Compound Feed Formula

Components	Inclusion
Corn[a]	-
Barley[a]	-
Soy mea[la]	-
Wheat bran[a]	-
Calcium carbonate[a]	-
Beet molasse[a]	-
Monocalcium phosphate[a]	-
Refined sea salt[a]	-
Vitamin A	8000 IU/kg
Vitamin D3	1000 IU/kg
Vitamin E	40 IU/kg
Cobalt acetate tetrahydrate	0.40 mg/kg
Copper sulphate pentahydrate	5 PPM
Iron sulphate monohydrate	10 mg/kg
Calcium iodide anhydrous	0.20 mg/kg
Manganese oxide	25 mg/kg
Sodium selenite	0.10 mg/kg
Zinc oxide	30 mg/kg

[a] The inclusion percentage of this ingredient is not provided due to intelectual property rights of the company responsible for the compound feed formulation

Supplementary Table 5 - List of primers used for quantitative real-time PCR

Gene	Primer[a]	Sequence 5'-3'	Amplicon Size (bp)	GeneBank Accession Number or Reference
MARVELD1	F:	GGCCAGCTGTAAGATCATCACA	100	(129)
	R:	TCTGATCACAGACAGAGCACCAT		
PPIA	F:	GTGGCAAGTCCATCTATGGCG	184	Chapter III NM_178320.2
	R:	CCTCTTTCACCTTGCCAAAGTACC		
TNF	F:	CCAGAGGGAAGAGCAGTCCC	114	(130)
	R:	TCGGCTACAACGTGGGCTAC		
IL10	F:	AGAACCACGGGCCTGACAT	151	(131)
	R:	AGCTCACTGAAGACTCTCTTCACCTT		
IL6	F:	CCTGAAGCAAAAGATCGCAGA	204	(30)
	R:	ATGCCCAGGAACTACCACAA		
IL1B	F:	AAACTCCAGGACAGAGAGCAAAA	126	(132)
	R:	CTCTCCTTGCACAAAGCTCATG		
IL12A	F:	ACGCTACAGAAGGCCAGACAA	135	Chapter III NM_174355.2
	R:	ACTCTCATTCGTGGCTAATTCCA		
IL12B	F:	CCCGCATTCCTACTTCTCCC	208	Chapter III NM_174356.1
	R:	TCCTGAAGATGGGCTGTAC		
IL23A	F:	TGCACACCTACCAATGGGACA	144	Chapter III NM_001205688.1
	R:	ATTCTTTGCAAGCAGGACTGAC		

[a]Primer direction: F, Forward; R, Reverse.

Supplementary Table 6 – Proteinogram of calves to be allocated to control and supplemented groups

Parameter	Mean		SEM	Adjusted P value
	Control	Supplemented		
Total Serum Proteins (TSP) g/dL	5.74	5.28	0.219	0.147
Albumin g/dL	2.38	2.35	0.059	0.769
Globulins (TSP-Albumin) g/dL	3.36	2.93	0.201	0.134
Albumin/Globulin	0.742	0.855	0.0447	0.076

SEM, Standard error of the mean

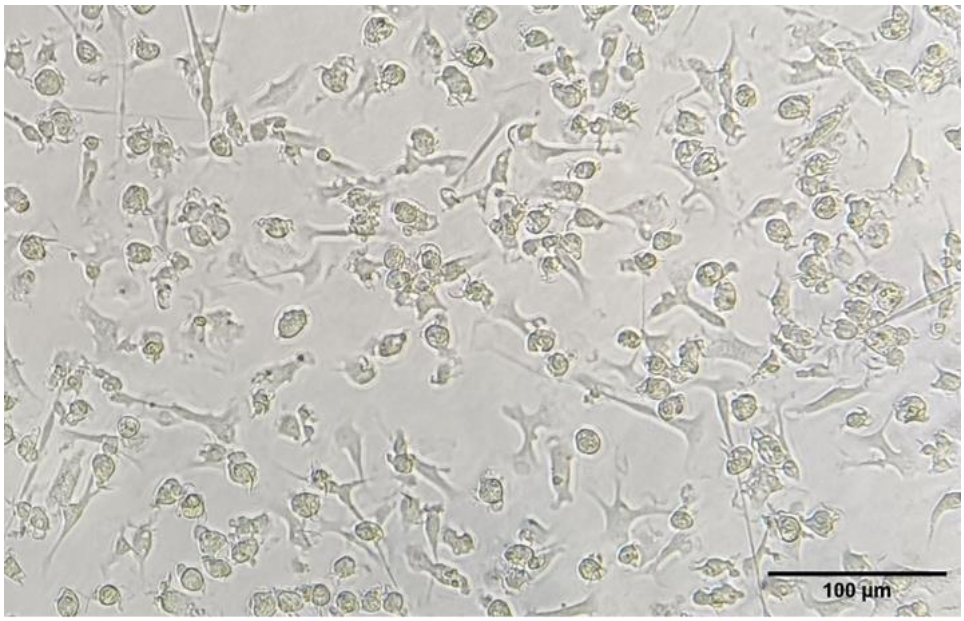
Supplementary Table 7 – Haemogram (T0)

Parameter	Mean		SEM	*P*-value
	Control	Supplemented		
Total erythrocyte count (× 10^6/µL)	7.87	7.13	0.401	0.199
Haemoglobin (g/dL)	10.05	9.67	0.609	0.660
Haematocrit (%)	32.00	30.16	1.873	0.491
Mean Corpuscular Vol. (fL)	40.93	42.06	1.136	0.484
Mean Corpuscular Hg (pg)	12.82	13.40	0.329	0.219
Mean Corpuscular Hg Conc. (g/dL)	31.37	31.91	0.470	0.418
Erythrocyte distribution index (%)	22.10	20.06	0.422	0.001
Platelets (x 10^3/µL)	667.89	791.46	99.98	0.386

Supplementary Table 7 (Cont.)– Haemogram (T0)

Parameter	Mean		SEM	*P*-value
	Control	Supplemented		
Average Platelet Volume (fL)	13.50	11.63	0.444	0.003
Leukocytes (× 10^3/μL)	11.32	11.23	1.597	0.967
Neutrophils (× 10^3/μL)	6.57	7.67	1.643	0.641
Eosinophils (× 10^3/μL)	0.27	0.18	0.021	0.005
Basophils (× 10^3/μL)	0.17	0.12	0.036	0.384
Monocytes (× 10^3/μL)	0.44	0.32	0.066	0.210
Lymphocytes (× 10^3/μL)	3.87	2.82	0.335	0.028
Neutrophil to Lymphocyte ratio	3.49	2.90	1.649	0.804

SEM, standard error of the mean.



Supplementary Figure 1 – Bovine peripheral blood monocyte-derived macrophages, at the 7th day of differentiation period with autologous serum. Magnification of 200×.

Supplementary Table 8 – Serum cytokines (IFN-y, IL-2, IL-6, IL-8, IL-10 and IL-17) evaluated by ELISA at the beginning of the assay (T0).

Parameter	Mean		SEM	*P*-value
	Control	Supplemented		
IFN-y pg/mL	7.49	4.85	1.922	0.337
IL-2 pg/mL	90.28	35.20	27.400	0.159
IL-6 pg/mL	12.77	3.95	2.905	0.033
IL-8 pg/mL	13126.0	18202.0	4408.2	0.419
IL-10 pg/mL	244.82	257.65	32.200	0.780
IL-17 pg/mL	40.96	40.96	40.955	40.96

SEM, standard error of the mean.

Supplementary Table 9 – Cytokines (TNF-α, IL-1β, IL-6 and IL-4) were below detection limits in most serum samples, evaluated by ELISA. Samples were collected at the end of the assay (T6).

Group	Calf #	Cytokine concentration (pg/mL)			
		TNF-α	IL-1β	IL-6	IL-4
Control	1	nd	0.55	nd	nd
	2	nd	0.67	nd	nd
	8	nd	nd	nd	nd
	10	nd	0.21	11.42	nd
	11	nd	nd	nd	nd
	12	nd	nd	nd	nd
	13	nd	nd	nd	nd
Supplemented	3	nd	nd	0.984	nd
	4	nd	nd	nd	nd
	6	nd	nd	55.47	nd
	7	nd	nd	nd	nd
	9	nd	2.96	nd	4.88
	14	nd	14.0	318.60	nd

nd – not detected

CHAPTER VI

General Discussion and Concluding Remarks

General Discussion

The incidence of infectious diseases is still a major concern in rearing cattle. Most of these diseases derive from an imbalance between pathogen loads and the ability of calves' immune system to respond and fight the infection. Although there is still much to unveil regarding prenatal and postnatal predisposing factors, it is well known that adequate management plays a key role in maintaining a stable, healthy herd (1, 2). In calves, management practices are particularly determinant, with adequate administration of colostrum and a suitable nutrition plan being the most important factors to enhance calves' immune function. In addition, hygiene procedures are essential to decrease the number of pathogens in the surrounding environment (3). However, even well-managed calves are at risk of acquiring infectious diseases when stressors such as adverse weather conditions and dietary changes, re-grouping, transport, weaning and veterinary procedures impact the ability of the immune system to counteract pathogen pressure (4, 5).

One of the most used strategies to enhance the immunity of young calves, thereby preventing the incidence of infectious diseases, is the use of dietary supplements in milk replacer and in compound feed. The most used products are: probiotics (live yeast, like *S. cerevisiae*, and live bacteria, such as *Lactobacillus* spp., *Enterococcus* spp., and *Bacillus* spp.) (6-8); prebiotics (fructooligosaccharides, mannanoligosaccharides, galactooligosaccharides, β-glucans and yest-cell wall) (8-11); essential oils (garlic, oregano, cinnamon, anise, rosemary and thyme oils) (12, 13); and synbiotics (combination of live microorganisms and substrates with health-promoting effects) (14, 15). Some of the above-mentioned products are thoroughly used in calves' diets (particularly yeasts and yeast-based products) and were ascribed significant immune-modulating properties (16-18). Nevertheless, there is still much to unveil regarding their mechanisms of action, from recognition by bovine immune cells to the induced response. Other dietary supplements, such as microalgae, are currently not employed in commercial farming, mostly due to their high cost (19), but are promising sources of nutrients and bioactive compounds (20, 21) and their potential as probiotics or prebiotics is being increasingly tested in livestock production (19, 22, 23).

The main goals of this thesis were: 1) to evaluate and mechanistically characterize the effects of different supplements, namely yeast-derived products and microalgae, on bovine innate immune cells and intestinal epithelial cell lines; 2) to conceptualize and design a comprehensive intestinal *in vitro* model to screen for dietary compounds that could provide more translational results than the simpler available *in vitro* models; 3) and to evaluate the immune function and health of newborn calves supplemented with the

most promising compounds, selected according to previously obtained results. Three major questions were addressed throughout this study:

1. **What are the immune effects triggered by yeast-derived products upon recognition by bovine peripheral blood monocytes?**

Yeasts – mainly *S. cerevisiae* species – and yeast cell wall components are frequently added to livestock diets to improve animal health and performance. There are several studies supporting their benefits for humans, companion animals and livestock species (24-29). Though mechanisms of action are still being investigated, it is thought that these feed additives may present probiotic, prebiotic and immunomodulating properties. They could modulate gut microbiota and enhance growth of beneficial bacteria that would directly bind pathogenic bacteria and prevent their adhesion to the intestinal mucosa, and contribute to stimulation and maturation of immune cells (28, 30, 31). Approximately 50% of *S. cerevisiae* cell wall is composed of branched β-1,3-glucans, 10% β-1,6-glucans, 40% mannoproteins and 1 to 2% chitin (32). β-1,3-glucans, in particular, have been extensively studied and are already commercially available as immunomodulators (18, 33). Their activity depends on the source, particle size, molecular structure (branching and conformation), molecular weight and solubility (34). These compounds were described to enhance phagocytosis, ROS and cytokine production, and also to induce innate immune training, a process where a first stimulus induces epigenetic reprogramming of innate immune cells, thereby altering innate cell responsiveness upon contact with subsequent non-related stimuli (35-37). Hence, it is plausible that part of the health-promoting effects observed with dietary yeast supplementation (29, 38, 39) are due to this phenomenon. The major β-glucan receptors are C-type lectin domain family 7 member A (CLEC7A) or Dectin-1, predominantly expressed in monocytes, macrophages (40) and dendritic cells (41), and Complement Receptor 3 (CR3), highly expressed in neutrophils (42). Other molecules involved in β-glucan recognition are Lactosylceramide and Scavenger Receptors (SR) (43-45). Even though the mechanisms of β-glucan recognition were extensively studied in humans and mice, specific knowledge on the response elicited by yeast-derived β-glucans on bovine innate immune cells was still lacking. In this thesis, it is shown that bovine monocytes respond to commercial pure particulate β-1,3-glucans and β-1,3-glucan containing particles (zymosan) through bovine Dectin-1 signalling. It is thus conceivable that bovine cells may also be primed by β-glucans and respond more efficiently to subsequent non-related stimuli. This is a relevant point to assess in future studies. Bovine monocytes stimulated with particulate β-glucans and zymosan increased the production of pro-inflammatory cytokines as well as the surface expression of MHC class II, CD80 and CD86 in a dose-

dependent manner, which may translate into greater stimulation T cell responses (46). Zymosan induced higher cytokine production at the same dose, comparatively to pure particulate β-glucans. This may result from the activation of different cell signalling pathways through Dectin-1 and TLR-2 receptors (47). A negative correlation between Dectin-1 expression and IL-10 expression was observed in particulate β-glucan-stimulated cells, contrastingly to zymosan, which induced the production of the anti-inflammatory cytokine IL-10. This response may be mediated through TLR-2 signalling, which is not activated by purified β-glucans and was already described to be involved in the production of IL-10 (48). It should be noted that soluble β-glucans did not induce the production and expression of any of the cytokines assessed nor the expression of MHC class II and co-stimulatory molecules, suggesting that soluble β-glucans do not activate bovine Dectin-1, similarly to what was described previously in mouse and rat macrophages (49, 50) and in porcine leukocytes (51).

2. Do microalgae display immunomodulatory properties?

The use of microalgae in animal feed has been suggested not only for their nutritional value (19, 52, 53), but also because they are sustainable sources of bioactive compounds with immunomodulatory activity (21, 54-56). Microalgae have also been proposed as promising sources of polysaccharides with prebiotic activity (22, 57). Prebiotics are thought to modulate immunity both directly, by stimulating gut-associated epithelial and immune cells through PRR binding, and indirectly, through modulation of gut microbiota (58). The immunomodulatory activity of microalgae has been mostly evaluated using whole microalgae or its extracts (59-62), and the effects of digestion on microalgae properties are not clear.

Here we evaluated the effects of three of the most produced microalgae species (*C. vulgaris*, *N. oceanica*, and *Tetraselmis* spp.) on bovine macrophages. Microalgae were digested *in vitro*, using a standardized static methodology that simulates monogastric and small intestinal digestion (63), before being used to stimulate bovine peripheral blood monocyte-derived macrophages. All microalgae species induced *TNFA*, *IL1B*, *IL6*, *IL10*, *IL12A*, *IL12B* and *IL23A* gene expression, the production of TNF-α, IL-6, IL-8, IL-1β and IL-10, and the production of ROS by bovine macrophages. Pre-incubation with TPCA-1, an IKK inhibitor, partially suppressed the production and mRNA expression of the majority of the cytokines assessed, which suggest that the previously observed effects were partially mediated by NF-κB, a transcription factor that regulates innate and adaptive immune responses and is a key mediator of inflammation (64). We have also attempted, without success, the inhibition of AP-1, another family of transcription factors involved in the inflammatory response, using the synthetic retinoid SR 11302 (65).

Interestingly, bovine macrophages previously conditioned with digested microalgae produced lower amounts of pro-inflammatory cytokines upon contact with *E. coli* LPS. Hence, despite promoting the production of cytokines, pre-incubation of bovine monocytes with these microalgae renders them less reactive to LPS, which could be beneficial in preventing hyperinflammatory responses, typical of the acute phase of infection (66). Cytokine levels and ROS production were similar between conditions and with the three microalgae species tested, despite differences in their nutritional and functional composition (67-70). These results suggest that similar immunomodulatory effects can be achieved with different microalgal compounds and species.

Digestion fluids and enzymes (blanks) added to the cells *in vitro* did not induce the production nor the mRNA expression of any of the cytokines assessed. This result thus supports the use of digested dietary supplements rather than intact products or their extracts in future studies. The use of digested dietary supplements would better mimic the contact of host gut epithelial and immune cells with dietary supplements that occurs *in vivo*.

One of the mechanisms that could explain the beneficial effects of dietary supplements is their ability to stimulate intestinal epithelial cells through activation of cell surface PRR, thereby triggering downstream cell signalling and modulation of local immune responses (58, 71). Thus, one of the objectives here was to evaluate the effect of digested *C. vulgaris*, *N. oceanica* and *Tetraselmis* sp. on intestinal epithelial cells or co-cultures of intestinal epithelial cells, namely Caco-2/HT29-MTX. Digested particulate microalgae had a negative impact on intestinal cell viability that was not observed with non-digested microalgae. It can be hypothesized that digestion products may release or generate cytotoxic compounds not present when whole microalgae are used. Whether this effect could occur *in vivo* and have detrimental effects on intestinal integrity would be worth evaluating.

The oxidant and antioxidant potential of *C. vulgaris* and *N. oceanica* were assessed in differentiated Caco-2/HT29-MTX cell co-cultures. Although sample size was too small to draw sound conclusions, preliminary results showed that soluble products of *N. oceanica* and *C. vulgaris* induced the production of ROS, but also protected cells from oxidative damage induced by H_2O_2. These results suggest an ambivalent effect of microalgae, which could on the one hand lead to the promotion of an oxidative response, but which could also function as antioxidants as previously explored for other antioxidants (72). As an example, vitamin C is an important antioxidant that has also been shown to present prooxidant activity *in vitro*. It has been suggested, however, that these effects present no relevant impact *in vivo* (73). This would be in line with the pro- and anti-inflammatory effects discussed above. However, it would be necessary to repeat these experiments

to confirm the obtained results.

3. Is it possible to improve immune parameters of newborn calves fed *Chlorella vulgaris* supplemented milk replacer?

Microalgae have been suggested to exert health-promoting effects mostly based on their bioactive compounds content and activity (74, 75). However, their use as dietary supplements in rearing cattle is still in its infancy. On one hand, few studies have assessed microalgae use as a dietary supplement in rearing cattle with the purpose of studying the impact on the host immune function. On the other hand, microalgae cost is still prohibitive for use in livestock feeding, even though the production costs are expecting to reduce, in the near future, with technological development and increased biomass production (19).

In this context, we evaluated the effect of 1% (w/w, DM basis) *C. vulgaris* supplementation to milk replacer on growth, and immune parameters of newborn calves. *C. vulgaris*, the most studied and used in feed eukaryotic microalgae species (76), was chosen since it revealed promising free radical scavenging potential *in vitro* in co-cultures of Caco-2/HT20-MTX, and low cytotoxicity. We have found that Holstein-Friesian calves supplemented with 1% (w/w) *C. vulgaris* in milk replacer presented similar growth performance comparatively to control calves. Haemogram parameters were also identical, except for erythrocyte distribution index (23.0 vs 28.5), platelet (608 vs 768) and neutrophil counts (1.77 vs 2.55), and N/L (0.352 vs 0.636), P/L (116 vs 188) and SII (174 vs 493) inflammation associated indexes, which were significantly higher in the supplemented group. These indexes are recently being used in human medicine as prognostic tools in the study of inflammatory, metabolic and tumoral diseases, and are usually associated with poorer outcomes (77-79). N/L has been studied as a marker of stress response and the average N/L in adult cattle is considered to be approximately 0.5 (80). Whenever neutrophil counts increased, due to stress or inflammation, the N/L also increased. For example, calves subjected to a road transportation had increased N/L (approximately 1.4) immediately after transport that decreased to 0.4 24 h after the stressor event had ceased (81). Also, veal calves abruptly weaned had significantly increased N/L after weaning comparatively to control calves (0.6 *vs*. 0.4, respectively) (82). Another study reported that Holstein-Friesian female calves dehorned without anaesthesia or with 2% lidocaine had increased N/L (1.37 and 1.62, respectively) comparatively to calves dehorned with 5% lidocaine or the control group (1.09 and 0.98, respectively) (83). Hence, this marker can be used to evaluate physiological response to stress in calves. To the best of our knowledge there are no studies addressing P/L and SII in ruminants. Serum IL-8, a pro-inflammatory cytokine with chemotactic activity for

neutrophils, was also increased in calves from *C. vulgaris* supplemented group, which would corroborate an inflammatory role of *C. vulgaris* supplementation. Nevertheless, all other measured serum cytokines were identical between control and supplemented groups. Moreover, no other immune parameters were affected by supplementation, including T lymphocyte function. Proliferation of peripheral-blood mononuclear cells in response to mitogens was not different between control and supplemented groups, which argues against a significant effect of microalgae supplementation in lymphocyte response.

Diet may also play a role in the epigenetic reprogramming of myeloid progenitors and thus have long term effects on innate immunity. Newborn goats supplemented with β-glucans and intraperitoneally injected with LPS had increased plasmatic IL-1β, IL-6, and TNF-α, showed increased respiratory burst activity, and elevated mRNA expression of genes encoding the macrophage surface markers CD11b and F4/80. In that same study, goat monocytes were also trained *in vitro* with yeast-derived β-glucans and subsequently challenged with LPS. Trained monocytes showed enhanced cell survival, higher phagocytic ability and increased gene expression of CD11b and F4/80 (84). In mice, in which systemic inflammation was induced by the consumption of a western diet, myeloid cells had increased and prolonged responsiveness upon challenge with LPS (85). Although potentially protective in an infectious scenario, this myeloid cell hyperactivation may have deleterious effects in inflammatory diseases (85, 86). In the present work, peripheral blood monocyte-derived macrophages from *C. vulgaris* supplemented calf group, when stimulated *ex vivo* with several TLR and Dectin-1 agonists, responded with significantly lower global levels of IL-6 and higher global mRNA expression of *IL10*, comparatively to cells from the control group. However, when comparing groups within each stimulus, no differences were observed. Moreover, phagocytosis of fluorescently-labelled beads by monocyte-derived macrophages was not significantly different between groups. These results indicate that no trained innate memory occurred and contradict our previous observations that *C. vulgaris* supplementation could enhance a pro-inflammatory response.

Overall, *C. vulgaris* supplementation had little impact on gut-associated measured parameters. Faecal pH, microbiota composition and diversity, and total short-chain fatty acids (SCFA) production were identical between groups, indicating supplementation with *C. vulgaris* had no negative impact on young calves'. However, molar proportions of individual SCFA and branched SCFA were affected by *C. vulgaris* dietary supplementation. Supplemented calves had lower faecal propionic acid and a tendency for lower faecal butyric acid, whereas valeric acid and branched SCFA isobutyric, isovaleric and isocaproic acids were increased. Differences in the proportions of

branched SCFA could be attributed to the higher inclusion of protein in the diet of *C. vulgaris* supplemented group. This could have resulted in more protein reaching the distal intestine undigested and being fermented by gut microbiota. Indeed, valeric acid and SCFA are produced by microbiota fermentation of branched-chain amino acids valine, isovaline and isoleucine (87), essential amino acids present in *C. vulgaris* (88). Similarly, calves fed higher allowances of milk replacer, and thus higher protein intake, had significantly higher SCFA content comparatively to calves fed restricted amounts (89).

From our results, low (1%) *C. vulgaris* supplementation to milk replacer presents no negative effects on calves' intake, growth and immune system. Besides, significant differences in leukocytes' counts and activity of peripheral-blood macrophages upon stimulation with several PRR agonists (evaluated by the differences in cytokine production and mRNA expression), are suggestive of an immunomodulation. However, proliferation assay, phagocytosis assay, serum cytokines, faecal pH, and faecal microbiota composition and diversity were similar between groups. As previously addressed by other authors, most of the beneficial effects upon supplementation with pro- and prebiotics are observed during disease, and it is often challenging to assess these benefits in healthy calves, where microbiome is well stablished and stable (8, 9). It is, in my opinion, difficult to ascertain the full potential of *C. vulgaris* supplementation on calf's immune system without performing further studies, namely an immune challenge.

Concluding Remarks

Although no product can replace proper management, an adequate nutrition plan and good hygiene practices in calf rearing, dietary supplementation may be a powerful ally when stressful events, often not controlled by the farmer, depress the ability of the immune system to respond to pathogen exposure, eventually leading to disease. In this framework, a work plan was designed to better elucidate the effects and mechanisms of action of dietary supplements that can be used on-farm to improve calves health and immunity.

In this thesis, we have shown that particulate β-1,3-glucans are recognized by bovine monocytes through Dectin-1, a cell surface receptor known to play an important role in the induction of innate immune memory (90). The innate immune response to pathogens could thus be enhanced in calves supplemented with yeasts or yeast-derived products. This mechanism could explain the beneficial effects of including yeasts and yeast-derived products in calf feeding. However, it remained to be elucidated whether bovine CR3 could be playing a role on recognition of yeast-derived β-glucans and if trained

immunity could be induced in bovine innate immune cells.

Digested microalgae displayed promising results by showing *in vitro* immunostimulatory properties on bovine macrophages, with potential anti-inflammatory effects when a pro-inflammatory stimulus (LPS) is added to the cells. When used in differentiated co-cultures of Caco-2 and HT29-MTX cells, microalgae also presented potential antioxidant effects when an oxidative agent (H_2O_2) is used. As such, despite increasing the production of pro-inflammatory cytokines and ROS, these compounds may be interesting sources of anti-inflammatory and antioxidant compounds. It would be interesting to deepen this work by studying the signalling pathways triggered upon stimulation with digested microalgae. This could be assessed by inhibiting upstream signalling pathways, such as those mediated by Myeloid differentiation factor 88 (MyD88) and Spleen tyrosine kinase (Syk). This would contribute to better understand which families of PRR are involved in microalgae recognition and the ensuing effects of PAMP binding (91, 92).

We did not observe clear benefits of milk replacer supplementation with *C. vulgaris*. In fact, inflammatory indexes were higher in the supplemented group, which may not be advantageous in the long term. The *in vivo* study conducted here was limited to a six-week supplementation period, which could have been insufficient to promote notorious effects on calf's health. Further studies are needed to clarify the impact of dietary supplementation with *C. vulgaris*, including a longer experimental period, the evaluation of the impact on growth and performance and the evaluation of the immune response to challenges and stressors, such as immunization and infection. It would also be important to use female calves and evaluate the impact of *C. vulgaris* supplementation in future lactation efficiency.

As the composition of microalgae varies greatly according to the species, strain and cultivation conditions, research results should not be generalized, and conclusions drawn should be cautious. Detailed characterization of the chemical composition of undigested and digested microalgae, along with their potential bioactive compounds, would partially overcome this problem and contribute to characterizing the recognition of different molecules by host cells and understanding the triggered immunological mechanisms.

Understanding the interplay between functional nutrition and the immune system is still extremely complex, as it not only depends on the functional supplement itself, but also on the physiology of the animals, on the gut microbiome and on basal diet formulation. However, once the mechanisms underlying the beneficial effects of supplementation are identified and characterized, it will be possible, in the medium to long term, to select the most appropriate feed supplement according to the particularities of the herd, disease incidence and management practices.

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