Aus dem Institut für Mikrobiologie und Tierseuchen

des Fachbereichs Veterinärmedizin

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Identification and characterization of immuno-active factors of *Enterococcus* spp. involved in immunomodulatory effects of Probiotic *Enterococcus faecium* SF68

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Abbreviations

4E-BP1	eIF4E binding protein 1
5'-P	5'- phosphate
5'-PPP	5'-triphosphate
AAR	amino acid response
AD	arginine deiminase
ADC	arginine decarboxylase
ADI	arginine deiminase
AIF	apoptosis Inducing factor
AIM2	absent in melanoma 2
Akt	AKR thymoma protein kinase B
AMPK:	adenosine monophosphate-activated protein kinase
AMP	antimicrobial peptide
AnkR	N-terminal ankyrin repeats
AP-1	Fos/Jun activator protein-1
Apaf-1	protease-activating factor-1
APC	antigen presenting cell
AS	ammonium sulfate
ASC	apoptosis-associated speck-like protein
ASCT1	alanine/serine/cysteine transporter
ATD	acidic transactivation domain
Atg4	autophagy-regulating protease 4
ATP	adenosine triphosphate
Bad	BCL2 associated agonist of cell death
BAX	Bcl-2-associated X protein
Bcl-2	B cell CLL/lymphoma-2
Bid	BH3 interacting domain death agonist
BIR	baculovirus inhibitor of apoptosis protein repeat
c/EBP	CCAAT-enhancer binding protein
c-myc	avian myelocytomatosis virus oncogene cellular homolog
C3b	complement-3b
Caco-2	cancer coli-2
CAD	caspase-activated deoxyribonuclease
cAMP	cyclic adenosine monophosphate
CARDINAL	CARD-inhibitor of NF-kappa-B-activating ligand
CARD	caspase recruitment domain
Casp2	caspase 2 coding gene
CAT1	cationic amino acid transporter
CBP	CREB binding protein
CCL2	C-C motif chemokine ligand 2
CD	cluster of differentiation
CHX	cycloheximide
cIAP	cellular inhibitor of apoptosis protein
CK	carbamate kinase
CK2	casein kinase 2

CKD6	Cyclin-dependent kinase 6
CLEC5A	C-type lectin member 5A
CLR	C-type lectin receptor
CO_2	carbon dioxide
CORM	carbon monoxide-releasing molecule
CpG	DNA containing unmethylated cytidyl guanosyl
CPPD	calcium pyrophosphate dihydrate
CRD	carbohydrate recognition domain
CREB	cAMP response element binding protein
CTLD	C-type lectin-like domains
Cxcl	chemokine (CXC) ligand
Dad1	Defender Against Cell Death 1
DAI	DNA-dependent activator of IRF
DAMP	damage-associated molecular pattern
Dap12	DNAX-Associated Protein 12
DCIR	dendritic cell immunoreceptor
DC-SIGN	dendritic cell-specific ICAM3-grabbing non-integrin
DC	dendritic cell
DD	death domain
DExD/H	D-E-X-D/ helicase box
DIABLO	direct IAP-binding protein with low PI
DNA	deoxyribonucleic acid
DR	death receptor
DRAM	damage regulated autophagy modulator
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
DSS	dextran sulfate sodium
E. coli	*Escherichia coli*
E. faecium	*Enterococcus faecium*
EDAR	ectodysplasin A receptor
EAAT3	excitatory amino acid transporter
EHEC	enterohemorrhagic *E. coli*
eNOS	endothelial NOS
EPEC	enteropathogenic *E. coli*
ERS	endoplasmic reticulum stress
ERK	extracellular signal-regulated kinase
ESP	extracellular surface protein
ETEC	enterotoxigenic *E. coli*
FADD	Fas-associated protein with death domain
FAP-1	Fas-associated phosphatase 1
Fas/TRAIL	FS-7-associated surface antigen/tumor necrosis factor-related apoptosis-inducing ligand
FasR	Fas receptor
Fc-receptor	fragment crystallizable region receptor
FcRγ	Fc receptor γ-chain
FITC	fluorescein isothiocyanate
$FLIP_{L/S}$	cellular FADD-like interleukin-1ß-converting enzyme inhibitory protein long/short

GABA	gamma-aminobutyric acid
GALT	gut associated lymphoid tissue
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSH	glutathione
HDAC-1	histone deacetylase-1
HIV-1	human immunodeficiency virus type 1
HPI	human immunodeficiency virus type 1
HSP70	heat shock protein 70
HT-29	human tumor-29
HCT-8	human colon tumor-8
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
iE-DAP	γ-d-glutamyl-meso- diaminopimelic acid
IEL	Intraepithelial lymphocytes
IFN	interferon
Ig	immunoglobulin
IκB	inhibitory proteins of kB family
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Ipaf	ICE-protease-activating factor
Irak2	interleukin-1 receptor-associated kinase 2
IPS-1	interferon- β promoter stimulator 1
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
JEV	Japanese encephalitis virus
JNK	c-Jun N-terminal kinase
LAB	Lactic acid bacteria
JAK/STAT	janus kinase/signal transducer and activator of transcription
LBP	lipopolysaccharide-binding protein
LDH	lactate dehydrogenase
LGP2	laboratory of genetics and physiology 2
LPS	lipopolysaccharide
LRR	leucine-rich repeat
M cell	microfold cell
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
MALT	mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling protein
MCP-1	monocyte chemoattractant protein-1
MD-2	myeloid differentiation factor 2
MDA5	melanoma differentiation associated gene 5
MDP	muramyl dipeptide
MEK	MAPK kinase
MH134	mouse hepatocellular carcinoma cell line
MHC	major histocompatibility complex

MIP-2α	macrophage inflammatory protein-2α
MITA	mediator of IRF3 activation
mRNA	messenger RNA
mTLR11	murine TLR11
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response 88
NACHT	NAIP, CIITA, HET-E and TP1
NAIP	neuronal apoptosis inhibitory protein
NALP	pyrin domain containing NLRs
NBD	nucleotide binding domain
NEMO	NF-kB essential modulator
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGFR	nerve growth factor receptor
NK cell	natural killer cell
NLRC	NOD-like receptor subfamily C
NLRP	NACHT leucine-rich-repeat protein
NLR	NOD-like receptor
NLS	nuclear localization sequence
NM23-H1	non-metastatic clone 23 isoform H1 metastasis suppressor genes
NO	nitric oxide
nNOS	neuronal nitric oxide synthase
NOD	nucleotide binding oligomerization domain
OTC	ornithine transcarbamylase
p38	38 kDa mitogen-activated protein kinases
p50/52	subunit protein 50/52
p100/105	precursor protein 100/105
p53	tumor protein 53
PI3K	phosphoinositide-3-kinase
PAMP	pathogen- associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCD	programmed cell death
pH	potential of hydrogen
Poly (I:C)	polyriboinosinic: polyribocytidylic acid
PPARγ	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
Ptprc	protein tyrosine phosphatase CD45
PYD	pyrin domain
Raf-1	v-raf-1 murine leukemia viral oncogene homolog-1
RAIDD	RIP-associated ICH-l/CED-3-homologous protein with a death domain
RegIIIγ	regenerating islet-derived 3 gamma
RelA/B	v-rel avian reticuloendotheliosis viral oncogene homolog A/B
RHD	Rel homology domain
RICK	RIP-like interacting caspase-like apoptosis regulatory protein kinase
RIG-I	retinoic-acid inducible gene I
RLR	retinoic acid-inducible gene-I-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species

S. suis	*Streptococcus suis*
SAGP	streptococcal acid glycoprotein
SET	endoplasmic reticulum (ER)-associated DNA repair complex
SHP1/SHP2	Src homology region 2 domain-containing phosphatase 1/2
SlpA	surface layer protein A
Smac	second mitochondria-derived activator of caspase
SMO	spermine oxidase
SODD	silencer of death domain
ssRNA	single-stranded RNA
STING	endoplasmic reticulum-resident protein stimulator of interferon genes
Syk	spleen tyrosine kinase
TAD	transcription transactivation domain
TAK1	transforming growth factor beta-activated kinase 1
TAB-1	TAK1 adaptor protein
TBK-1	TANK-binding kinase 1
TDM	trehalose 6, 6′-dimycolate
TEER	transepithelial electrical resistance
TGF-β1	transforming growth factor-β
tGPI	trypomastigote glycosylphosphatidylinositol-anchored mucin
THP-1	Acute monocytic leukemia cell line
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
TNFR	tumor necrosis factor receptor
TRAF	tumor necrosis factor receptor-associated factor
TRAM	TRIF–related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β

1 Introduction

„Nothing is constant but change. All existence is a perpetual flux of being and becoming. That is the broad lesson of the evolution of the world.“ - Ernst Haeckel (1834-1919)

There is a steady increase in the number of studies about the essential role of gut microbiota composition and function on the health status of human and animals (Marchesi et al. 2016). Additionally, alteration and perturbation in the composition and activity of gut microbiota, known as dysbiosis, may contribute to the pathogenesis of several immune-mediated and metabolic diseases (Frank et al. 2007; Qin et al. 2012; Elinav et al. 2019). Administration of several medications including a number of antibiotic agents, enteric infections like those caused by *Campylobacter* spp., pathogenic *E. coli*, *Salmonella* serovars or *Clostridium* spp., inflammatory bowel disease, e.g. Crohn's disease and ulcerative colitis, and different types of intestinal tumors present challenges, which disturb the balance of the gut (Young and Schmidt 2004; Santos 2014; Zeng et al. 2017; Lobionda et al. 2019).

Medical interventions aim to support the body's attempts to prevent or overcome imposed challenges and recuperate from their adverse consequences. The best known of these preventive and therapeutic strategies comprise administration of immunomodulatory drugs (including both anti-inflammatory medications and immunostimulatory agents), antibiotic therapy, application of biologic drugs and diet management such as fibre-carbohydrate balanced diet in human or dietary zinc and copper supplementation in animals (Isolauri et al. 2002; Starke et al. 2014; Simpson and Campbell 2015; Geva-Zatorsky et al. 2017; Telle-Hansen et al. 2018; Li et al. 2019a). It is of paramount importance to understand the delicacy of host-microbe and host-environment interplays. In this regard, the cause of a perturbation or alternation in the gut ecosystem is a crucial determinant in the selection of a preventive/therapeutic approach. For instance, any situation causing exaggerated or long-lasting inflammatory responses in the intestine, e.g. chronic inflammatory situation of Crohn's disease, can be destructive to intestinal epithelial cells and affect the microbiota (Ardizzone et al. 2010; Klein and Eliakim 2010). Therefore, such a situation is an indication for immunosuppressive and anti-inflammatory interventions in order to impede the adverse effects of exaggerated inflammatory responses (Klein and Eliakim 2010). Nevertheless, researchers and clinicians are facing challenges with these choices as by preventive and therapeutic strategies in any other physiological and clinical conditions. For instance, zinc supplementation has been shown to protect animals in poultry and pig farms against enteric pathogens and aid to control diarrhea probably by enhancing development of healthy gut microbiota (Heo et al. 2013b; Gadde et al. 2017). However, recent studies proposed that dietary zinc

supplementation increases the proportion of antibiotic resistant bacteria in the gut of piglets most likely with a co-selection mechanism (Bednorz et al. 2013). Making appropriate preventive and therapeutic choices are much more difficult when it comes to biological agents and direct administration of beneficial microbes, as their exact mechanisms of action are mostly unknown. Moreover, they can have unpredicted effects including adverse immune responses, such as allergic reactions or perturbation of microbiota leading to diarrhea (Martin-Mola and Balsa 2009; Kothari et al. 2019).

One of the beneficial bacterial strains which has been considered is *Enterococcus faecium* (NCIMB 10415). This intestinal endogenous lactic acid bacteria (LAB) strain is authorized as a probiotic for pharmaceutical applications in humans and as a feed additive in animals, and it has been administered in both human and animals over the last decades (Lewenstein et al. 1979; Franz et al. 2011). Several *in vivo* studies have shown that *E. faecium* SF68 supplementation mitigates symptoms of intestinal inflammation, diarrhea duration, and rate in human and animals. Some animal trials have demonstrated positive effects, including eliminating potential enteric pathogens from intestinal bacterial communities and a higher colonization rate of other beneficial strains (Wunderlich et al. 1989; Buydens and Debeuckelaere 1996; D'souza et al. 2002; Vahjen et al. 2002; Allen et al. 2004; Samli et al. 2007; Vahjen et al. 2007; Allen et al. 2010; Peng et al. 2019). Moreover, it has been demonstrated, that its supplementation can improve intestinal barrier integrity (Klingspor et al. 2013; Kern et al. 2017). However, in some enteric pathogen challenge studies, particularly with *Salmonella* Typhimurim serovars, higher pathogen colonization and dissemination were noted. Some of other observations made in piglets with probiotic supplementation in these studies included lower or unaffected IgA and IgG level in serum, decrease in $CD8^+$ intraepithelial lymphocytes, less potent peripheral blood mononuclear cells (PBMCs) with a late post-infection proliferative response, and dysregulation of immune-associated genes in intestinal tissues and their associated lymphoid organs (Scharek et al. 2005; Scharek et al. 2007; Szabó et al. 2009; Mafamane et al. 2011; Kreuzer et al. 2012; Siepert et al. 2014).

The overall knowledge that we have about *E. faecium* SF68, based on previous assessments, studies and observations, postulates that its described anti-inflammatory effects restrict intestinal inflammation and diarrhea. Moreover, immunosuppressive effects of this strain can facilitate colonization and dissemination of enteric pathogens (Szabó et al. 2009; Mafamane et al. 2011; Siepert et al. 2014). For a better understanding of host-microbe interactions between *E. faecium* SF68 and intestinal epithelium with the aim to adapt a considerate strategy for preventive or therapeutic application of this strain, its mechanism(s) of action should be studied more in details.

Previous infection model studies indicate that *E. faecium* SF68 properties presumably target the innate immune responses. Therefore, we decided to focus on the innate immune responses of host cells exposed to *E. faecium* SF68, to shed a light on the mechanism(s) of action of this probiotic strain and hence we selected NF-kB, one of the main inflammatory transcription factors involved in both innate and adaptive immune responses, as our main tracking target on innate immune responses (Elewaut et al. 1999). The aims of this doctoral study were identification and characterization of possible immunomodulatory factor(s) involved in above-mentioned observations, in order to have a better understanding of the risks the host can encounter by administration of such a probiotic strain. Experiments in this project were performed using *in vitro* models for a direct focus on the interactions between probiotic strain and intestinal epithelial cells.

2 Literature

2.1 Innate defense mechanisms

The immune system in vertebrates attempts to protect the body from any type of intrinsic (e.g. cancer) and extrinsic (e.g. allergy and infection) injurious factors (Chaplin 2010; Hanahan and Weinberg 2011; Platts-Mills and Woodfolk 2011). In this regard, pathogens must first break through surface barriers such as skin, mucous and defensive molecules produced by these surfaces, such as proteolytic enzymes, mucus and protective antimicrobial peptides. Once pathogens succeeded in this step and crossed surface barriers, they encounter immune system defense responses (Nicholson 2016). These defense responses are generally divided into two main types of mechanisms: “Innate defense mechanisms” and “acquired/adaptive defense mechanisms” (Chaplin 2010).

The innate or natural immune responses can recognize markers of its own normal cells to hinder initiation of defense responses against self-cells. At the same time, it can detect abnormal self-cell markers such as those markers present in tumor cells to eliminate them (Janeway et al. 2001). Major histocompatibility complexes (MHCs) play an imperative role in the recognition of self and non-self molecules. All host nucleated cells express definite MHC class I molecules on their surface, thereby they will be recognized as ‘safe’. Loss or alteration of these surface molecules destine cells to demise (Janeway et al. 2001). Moreover, the innate immune system can detect conserved structural and metabolic molecules of microorganisms reaching the body. These molecules, namely pathogen-associated molecular patterns (PAMPs), are absent in the host cells. PAMPs include peptidoglycans of bacterial cell walls, bacterial flagella, lipoproteins, lipopolysaccharides of gram-negative bacteria, teichoic acids of gram-positive bacteria and short sequences of exogenous DNA.

The foremost strategy of innate immune system for perception and detection of PAMPs is based on their identification by pattern recognition receptors (PRRs). PRRs comprise soluble receptors in blood (members of complement system), host cell membrane-bound receptors (e.g. Toll-like receptor family) and cytosolic proteins (e.g. NOD-like receptors) (Delves and Roitt 2000). Innate immune cells consist of epithelial cells and specialized immune cells. Specialized innate immune cells include dendritic cells, macrophages, granulocytes, and natural killer (NK) cells. Macrophages, dendritic cells, and epithelial cells are the main innate immune cells accounting for sensing pathogens by PRRs on their surfaces. Some specialized forms of epithelial cells such as M cells in the gut, are also able to sample presented antigens (Singh et al. 2009; Coates et al. 2018).

Occupied receptors trigger innate immune signaling pathways, followed by altered gene expression. These cascades of events lead to the production of complement components (e.g. C3b), cytokines, chemokines, acute-phase proteins (e.g. C-reactive protein), etc., in exposed cells. Altered gene expression and production of inflammatory and immunomodulatory mediators, enhance inflammation and can lead to necrosis, programmed cell death (e.g. apoptosis) or repair processes in the injured tissue (Cookson, et al., 2001; Coussens, 2002). Macrophages and dendritic cells belong to the antigen presenting cells (APCs) group of innate immune system (Sprent 1995). This means that, beside the direct action, macrophages and dendritic cells take against pathogens by recognition of them with PRRs and a subsequent attempt for their elimination via phagocytosis accompanied by the induction of inflammatory mediators in circumjacent cells, these cells also obtain a small part of antigen and present it on MHC class II molecules at their surface. With this step, the innate immune system sends a signal for specialized cells of the adaptive immune system and hence acquired antigen-specific responses will be initiated (Medzhitov and Janeway 1997). For instance, activated dendritic cells interconnect with adaptive immune responses and tender antigens directly to lymphocytes. In addition, they express B7 costimulatory molecules (CD80 and CD86) which are crucial for T-lymphocyte activation (Delves and Roitt 2000). Further signals will be sent from affected cells to recruit macrophages, dendritic cells, granulocytes, mast cells and natural killer cells to the site of infection in order to more efficiently attack invading pathogens, engulf them and clean up the mess (Janeway CA Jr, 2001). Macrophages take care of dead bodies of self-cells. However, dying cells in a necrotic tissue have a different fate than those that undergoing apoptosis. Necrotic cells trigger inflammatory responses, whereas apoptotic cells prepare themselves for phagocytosis (Aderem et al., 1999). Engulfed pathogens will be subjected then to intracellular reactive oxygen species and various degrading enzymes (Delves, et al., 2000).

Innate immune responses are rapid and rather non-specific primary defense mechanisms against threats to the body. These responses have no immunologic memory and consistently react to the invading signals with similar intensities, regardless how many times the body has been exposed to the same danger (Delves and Roitt 2000). This is the main difference between innate and adaptive immune responses. In the next subsections, regardless of the involved signaling pathways, the main consequences of activating the innate immune responses, including inflammation, apoptosis, and necrosis, will be introduced in more details.

2.1.1 Inflammation

Tissue injuries or infections induce innate immune responses such as inflammation. Previous exposure to the causative agent or injury is not a prerequisite for inflammatory

responses. Inflammation is generally a self-limiting condition but in persistent infections, neoplastic progressions or autoimmune diseases it can lead to a chronic and progressive inflammatory response due to dysregulation or dysfunction of involved effectors and molecules. Inflammatory responses will be initiated by different forms of PRRs including soluble in blood, intracellular, or cell-surface receptors. PRRs sense PAMPs or host-derived damage-associated molecular patterns (DAMPs). DAMPs contribute to sterile inflammation and include ordinary self-molecules such as ATP, IL-1α, and uric acid (Coussens and Werb 2002).

Components of the complement system comprise soluble PPRs present in blood. Confrontation of the complement system with PAMPs or DAMPs in the blood triggers a series of events leading to the activation of an initial complement key component, namely C3. Activation of C3 occurs via one of three different complement system pathways including classical, lectin, and alternative pathways. Components of complement system present microbes to the phagocytic cells to be eliminated (opsonization). Occasionally, they can also directly kill pathogens via cell lysis. Moreover, vasodilation of small blood vessels allows more blood to reach the inflamed/infected area. This leads to enhanced activation and spread of destructive properties and chemokine of complement cascades (e.g. C3a, C4a and C5a). Consequently, further phagocytic cells such as neutrophils and macrophages will migrate to the site of inflammation and besiege to eliminate the responsible cause of inflammation and damaged host cells (Delves and Roitt 2000).

Membrane-bound receptors, comprising Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), DNA sensors and C-type lectin receptors (CLRs), serve as PRRs on the cell surfaces, lumen of intracellular vesicles (e.g. endosomes or lysosomes) and in cytosol of the host cell (Table 1) (Kawai and Akira 2009; Mogensen 2009; Kumar et al. 2011; Sun and Reddy 2013; Hoving et al. 2014).

Table 1: Well-known pattern recognition receptors with their adaptor molecules and ligands (including activators and PAMPs)

	PRRs & co-receptors	***adapters***	***PAMPs/Activators***	***species***
TLR	TLR1/TLR2	MyD88, TIRAP	triacyl lipopeptides	bacteria
	TLR2/TLR6 (CD36)	MyD88 TIRAP	diacyl lipopeptides lipoteichoic acid	*Mycoplasma* spp. gram-positive bacteria
	TLR2 (Dectin-1, C-type lectin)	MyD88, TIRAP	lipoproteins peptidoglycan lipoarabinomannan porins tGPI-mucin HA protein phospholipomannan zymosan β-Glycan	different pathogens bacteria *Mycobacterium* spp. bacteria (*Neisseria* spp.) parasites (*Trypanosoma* spp.) viruses (Measles virus) *Candida* spp. fungi fungi
	TLR3	TRIF	ssRNA, dsRNA	viruses (e.g. respiratory syncytial virus, murine cytomegalovirus)
	TLR4 (MD-2, CD14, LBP)	MyD88, TIRAP, TRIF, TRAM	LPS envelope proteins glycoinositolphospholipids mannan HSP70 pneumolysin	gram-negative bacteria viruses (RSV, MMTV) protozoa *Candida* spp. host group B streptococci
	TLR5	MyD88	flagellin	flagellated bacteria
	TLR7/ hTLR8	MyD88	ssRNA bacterial RNA imidazoquinolines	RNA viruses group B streptococci purine analog compounds
	TLR9	MyD88	CpG DNA DNA malaria hemozoin	bacteria, viruses, protozoa virus DNA parasites
	mTLR11	MyD88	not determined profilin-like molecule	bacteria (uropathogenic bacteria) Parasites (*Toxoplasma gondii)*
RLR	RIG-I	IPS-1	RNA (5'-PPP ssRNA, short dsRNA with 5'-P or 5'-PPP)	viruses (e.g., influenza A virus, HCV, RSV, Ebola virus, Japanese encephalitis virus)
	MDA5	IPS-1	RNA (poly IC, long dsRNA)	viruses (picorna- and noroviruses)
	LGP2		RNA	viruses
NLR	NOD1/NLRC1	RICK, CARD9	iE-DAP	gram-negative bacteria
	NOD2/NLRC2	RICK, CARD9	MDP	bacteria
	NALP1/NLRP1	ASC	Anthrax lethal toxin, MDP	bacteria
	NALP3/NLRP3	ASC CARDINAL	MDP RNA ATP toxins uric acid, CPPD, amyloid-β	bacteria bacteria, viruses bacteria, host bacteria host
	Ipaf/NLRC4	ASC?	flagellin	bacteria
	NAIP5	?	flagellin	bacteria

CLR	Dectin-1 (lectin–ITAM)	Syk, Raf-1, CARD9, Malt1, Bcl10	β-glucan	fungi, *Mycobacterium* spp., *L. infantum*
	Dectin-2	Syk, FcRγ, CARD9, Malt1, Bcl10	α-mannans, O-linked mannobiose-rich glycoproteins	*M. tuberculosis*, *S. mansoni*, *Malassezia* ssp.
	DCIR	SHP1/SHP2	unknown	HIV-1
	DC-SIGN	Raf-1	high mannose	HIV-1
			SlpA	measles, Dengue, *Mycobacterium* spp., Influenza A, *Leishmania* spp.
	CLEC5A	Syk, Dap12	unknown	Dengue virus JEV
	Mincle	Syk, FcRγ, CARD9, Malt1, Bcl10	α-mannose mannitol linked glyceroglycolipid mannosyl fatty acids TDM	*M. tuberculosis* *C. albicans* *Malassezia* spp.
DNA sensors	AIM2	ASC	cytosolic dsDNA	bacteria, viruses
	DAI	STING, TBK-1, IRF3	cytosolic dsDNA	bacteria, viruses

These recognition receptors basically operate innate immune responses with their main functional domains including CARD, TIR, BIR, LRR, PYD, FIIND, DD, ATD, and helicase (Hansen et al. 2011). Following the recognition and binding to their ligands, these receptors interact with the relevant adapter proteins in the cell and initiate a signaling cascade, activating or deactivating other molecules via enzymatic alternations, such as ubiquitination or phosphorylation. In this process activation of several transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), Fos/Jun activator protein-1 (AP1), cAMP response element binding protein (CREB), CCAAT-enhancer binding protein (c/EBP), and interferon regulatory factors (IRF), are required to change the transcriptome of the cell and alert the immune system. Activation of these transcription factors/signaling pathways is usually synergistic, though occurring through different routes. Sequentially, transcription and expression of genes encoding different cytokines (e.g. IL-10, IL-12β, IL-1α, IL-1β, TNF-α and TGF-β1), chemokines (e.g. Cxcl1, Cxcl2), adhesion molecules and inflammatory mediators will be enhanced. These alterations and shifts in molecules generated by affected epithelial and local immune cells send a signal to recruit more scavenger cells to the site of inflammation, and furthermore, trigger activation of adaptive immune responses.

In the late nineties, a receptor protein called ‘Toll’ was demonstrated to be responsible for *Drosophila*‘s susceptibility to fungal infection (Lemaitre et al. 1996). Later, its homologue in human and other species was discovered and referred to as Toll-like receptor (Medzhitov et al. 1997). Thirteen members of this family in humans and mice have been identified. However, TLR10 has no function in mice, while TLR11, TLR12 and TLR13 are not present in human genome. TLRs are type I transmembrane proteins consisting of a large extracellular domain containing leucine-rich repeats (LRRs), a transmembrane domain and an intracellular domain known as Toll/IL-1R (TIR) domain (Figure 1). The extracellular domain mediates the recognition of PAMPs and the intracellular one takes care of downstream initiation of

inflammation. TLRs can induce inflammatory responses via three main pathways, including MyD88-dependent TAK1 and IKK activation by TLRs with an exception of TLR3, MyD88-dependent type I Interferon (IFN) induction by TLR7 and TLR9 and TRIF-dependent signaling by TLR3 and TLR4 (Abreu et al. 2002; Abreu et al. 2003).

The RLRs family are cytosolic proteins sensing the viral nucleic acids derived from replicating virus in the cytoplasm. This recognition process is independent of perception of nucleic acids by endosomal TLRs. Attachment of proper ligand to RLRs induces the activation of pro-inflammatory cytokines and type I IFN. Three known members of this receptor family are retinoic-acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). Structurally, these proteins contain a central DExD/H-box helicase domain with an ATP-binding motif and a C-terminal regulatory domain with the RNA-binding function. RIG-I and MDA5 also have caspase activation and recruitment domains (CARDs) at their N-terminal, which is necessary for protein-protein interactions and activation of downstream signaling pathways. LGP2 is more likely a regulator of RIG-I- and MDA5-dependent responses induced by viral nucleic acids (Yoneyama and Fujita 2008; Satoh et al. 2010). The activation of RLR proteins is followed by their engagement to the mitochondrial CARD-containing protein MAVS (IPS-1) which coordinate anti-viral responses and activate MAPK, NF-kB, IRF3 and IRF7 sequentially (Meylan et al. 2005). The transmembrane protein STING (MITA) located in endoplasmic reticulum is also required for the IFN induction by detection of viral nucleic acids via RLRs and MAVS signaling (Ishikawa and Barber 2008).

Nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) are cytosolic bacterial PAMPs-detectors. These receptor molecules are particularly important in immune responses against intracellular pathogens. They structurally consist of a N-terminal caspase-recruit-domain (CARD) or pyrin domain (PYD) or baculovirus inhibitor repeat (BIR) domain, a central nucleotide-binding domain (NBD) including NACHT and NAD domain and a C-terminal LRR. LRRs are involved in the ligand-binding and recognition processes the same as by LRRs in TLRs (Tschopp et al. 2003). N-terminal domain oversees signaling pathway initiation. Over 20 different types of NLR proteins have been described, some of which have an essential functional role in inflammation and pro-inflammatory activation of caspases. Generally, NLR proteins are divided into three main subfamilies based on their N-terminal domain. Two main CARD-containing NLR proteins, namely NOD1 and NOD2 are intracellular sensors of bacterial peptidoglycan subunits. However, it is unclear whether these two NOD proteins directly recognize PAMPs or indirectly via the modifications to host molecules. Regardless of the mechanism of action they activate IkB kinase complex via receptor-interacting protein (RIP) 2. Activation of IkB kinase complex leads to NF-κB and MAPK activation and subsequent

induction of inflammatory cytokine and inflammatory mediator genes. Other NLR proteins, e.g. NLRP1 (Nalp1), NLRP3 (cryopyrin) and NLRC4 (Ipaf), activate caspases (Kanneganti et al. 2007) and trigger production of IL-1β from its precursor molecule in the cytosol. These receptor molecules are involved in the TLRs-independent activation of inflammasomes. Following the detection of PAMPs by cytosolic NLRs, these receptors bind to a CARD-containing adaptor molecule, namely apoptosis-associated speck-like protein (ASC). ASC molecules recruit caspase-1 and assemble the inflammasome complex (Figure 2) which can result in inflammation and apoptosis (pyroptosis). It is important to know that TLR4-dependent induction is a prerequisite for inflammasome activation. This induction is required for both production of pro-IL-1β and expression of molecules directly involved in inflammasome function (Bergsbaken et al. 2009).

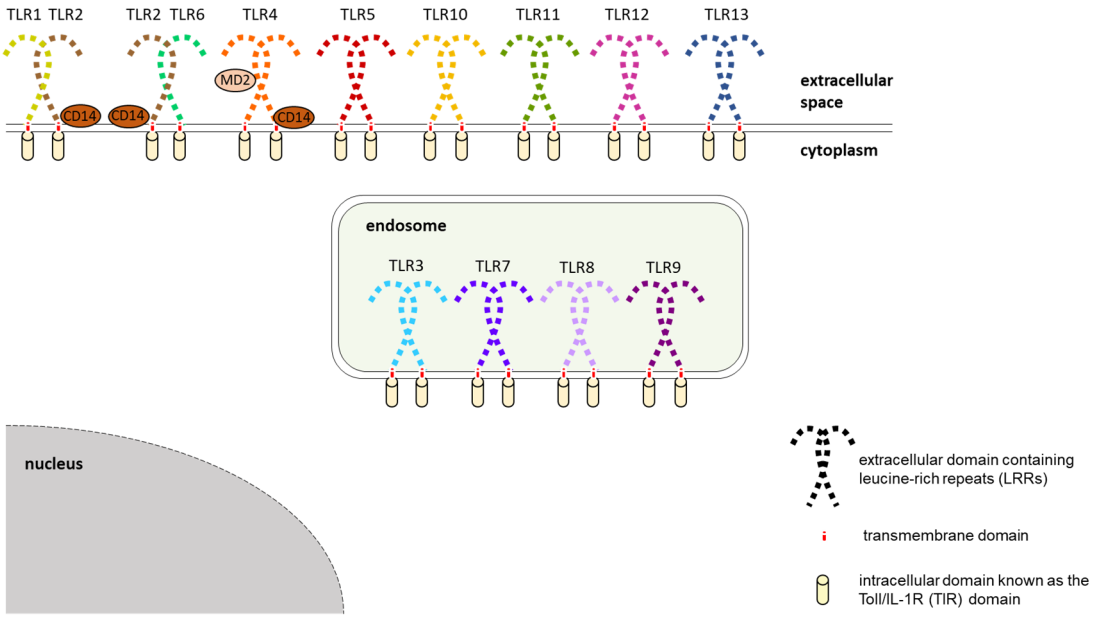

Figure 1: Localization and structure of different Toll-like receptors.

TLRs as membrane-bound receptors are located on the cellular surface (TLR1, 2, 4, 5, 6 and 10) or endosomal membranes (TLR3, 7, 8 and 9). Transmembrane TLR11, 12 and 13 are not present in human genome. Following the recognition of their ligands, TLRs recruit a TIR domain-containing adapter protein complex and activate TLR signaling cascades. CD14 and MD2 co-receptor molecules assist TLR2 and TLR2/TLR4 in the recognition of PAMPs, respectively.

C-type lectin receptors (CLRs), defined by their C-type lectin domain, are a large family of PRRs which can be found as membrane-bound (e.g. MBP and Dectin-1) or secreted protein forms (e.g. SP-A and SP-D) (Hardison and Brown 2012). These receptors include 17 different groups based on their structure and phylogenetic profile (Zelensky and Gready 2005). They can recognize carbohydrate-recognition domains (CRDs) or C-type lectin-like domains (CTLDs) in both a calcium dependent and independent manner (Figdor et al. 2002). CLRs play

an important role in the recognition of fungal pathogens (Hardison and Brown 2012). Nevertheless, they can participate in recognition of bacterial, viral, and protozoal PAMPs as well. CLRs can functionally be divided into activating and inhibitory molecules based on possessing immunoreceptor tyrosine-based activation motif (ITAM) domain or immunoreceptor tyrosine-based inhibition motif (ITIM) domain (Zelensky and Gready 2005). CLRs can coordinate and facilitate the crosstalk between other PRRs and signaling pathways involved in inflammatory responses and gene expression alterations. Moreover, they can also be involved in endocytic, phagocytic and even anti-inflammatory responses (Hardison and Brown 2012) and orchestrate the induction and development of adaptive immune system responses.

DNA sensors are less studied and defined PRR molecules in the cytosol of immune and none-immune cells, which can detect bacterial and viral dsDNA. DNA-dependent activator of IRF (DAI) has DNA-binding and TBK-1 activation abilities (Takaoka et al. 2007). AIM2 (absent in melanoma 2) is another recently identified DNA sensor molecule. It can trigger pro-inflammatory signaling pathways and maturation of IL-1β pro-forms (Hornung et al. 2009).

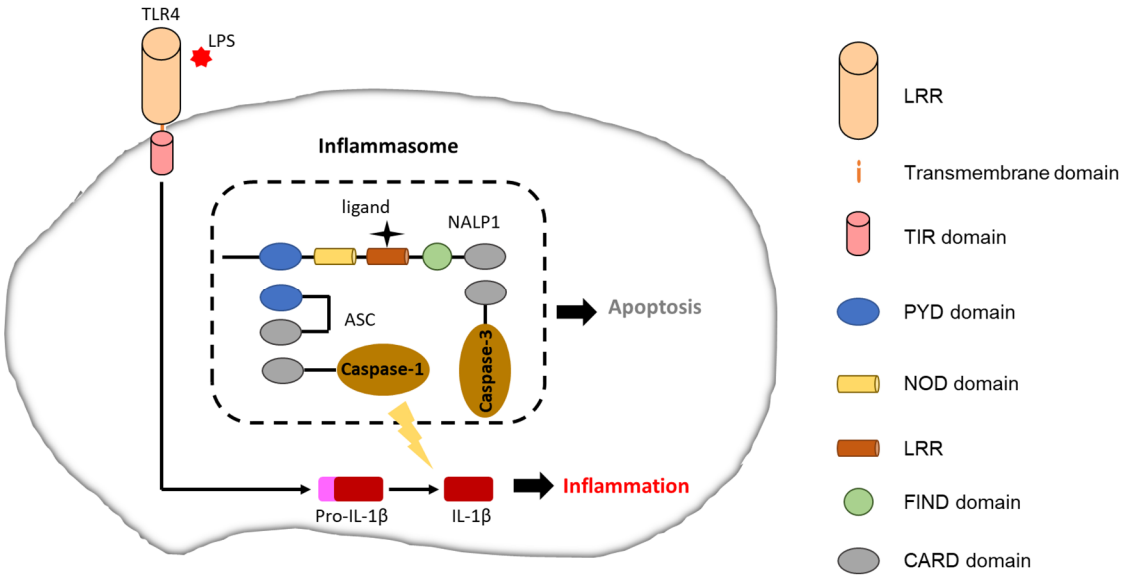

Figure 2: NLR protein and inflammasome formation (NLRP1).

NLRP1-receptor senses bacterial PAMPs with its LRR domain in the cytosol. Sequentially, N-terminal PYD-domain of NLRP1 recruits ASC adaptor molecule. The CARD domain of ASC molecule activates caspase-1. Activated caspase-1 assists in the processing of accumulated inflammatory IL-1β and IL-18 precursors. LPS interaction with TLR4 receptor enhance the production of IL-1β and IL-18 precursors. Moreover, CARD domain of NALP1 activates effector caspase-3 and trigger apoptosis in the cell.

2.1.2 Cell death

Necrosis is a passive, uncontrolled and non-programmed event occurring as a result of accidental damage to the cells, while programmed cell death (PCD) such as apoptosis is an energy-dependent and programmed process. PCD includes apoptosis, pyroptosis (an inflammatory form of apoptosis), necroptosis, and autophagy. PCD can be either a homeostatic or a defense mechanism leading to active cell suicide (Bergsbaken et al. 2009; Ouyang et al. 2012). However, in case of autophagy, as a recycling mechanism, it can be both a pro-survival and pro-death process. Apoptosis and necrosis are two independent death processes which can occur one after the other or simultaneously (Ouyang et al. 2012).

Apoptosis can be activated by two main distinct mechanisms, including extrinsic (death receptor pathway) and intrinsic (mitochondrial pathway) apoptotic pathways. A third perforin-granzyme-dependent pathway induced by cytotoxic T cells is also described (Trapani and Smyth 2002). All three of these pathways (Figure 3) meet at the same execution pathway and activate caspases which are cysteine-proteases. However, there is a form of granzyme pathway that is caspase-independent (Martinvalet et al. 2005). Fourteen types of caspases are identified and are divided into three subgroups. Apoptosis activators (initiator caspases) include caspase-2, -8, -9 and -10. Apoptosis executioners consist of caspase-3, -6 and -7 that will be activated by active form of initiator caspases and induce apoptosis. Caspase-1, -4, -5 and 11-13 are inflammatory mediators which can be activated via NLR proteins including NALPs, NAIP and Ipaf in the inflammasome signaling activation (Shi et al. 2003).

The extrinsic pathway of apoptosis will be initiated by stimulation of transmembrane receptors referred to as death receptors (DRs) (Locksley et al. 2001; Chen and Goeddel 2002). DR family consist of eight known receptors, including TNFR1 (DR1), FasR/CD95 (DR2), TRAMP (DR3), TRAILR1 (DR4), TRAILR2 (DR5), DR6, EDAR and NGFR. Following the engagement of DRs and their related ligands, the internal death domain (DD) of these receptors recruits several associated adaptor proteins. These signaling adaptor proteins differ between death receptors. TNFR1, TRAILR1 and TRAILR2 recruit the death-inducing signaling complex (DISC), which includes FADD, pro-caspase-8 and -10 and cellular FLICE-inhibitory protein ($FLIP_{L/S}$). Other molecules such as RIP and FAP-1 can also interact with DISC complex. DISC formation will be followed by activation of pro-molecules of caspase-8, and -10 (Hsu et al. 1995; Wajant 2002). Activation of initiator caspases triggers the cleavage and activation of executioner caspase-3, which is the common final terminal for the activation of all three apoptotic pathways. Moreover, the active form of caspase-8 cleaves Bid, a Bcl-2 family protein. Truncated Bid molecule facilitates release of pro-apoptotic molecules such as cytochrome c and formation of the apoptosome. Recruitment of RAIDD, another CARD-containing adaptor

molecule to TNFR1 also leads to activation of caspase-2 enzyme and cleavage of Bid molecule as well. Other death receptors comprise TNFR1, TRAMP and DR6 recruit RIP, TRADD, cIAP and TRAF1/2 adaptor molecules. Recruitment of TRAF2 and RIP leads to activation of NF-kB, JNK and p38 pathways via recruitment of TAK-1/TAB-1 complex. Furthermore, cIAP promotes the activation of the canonical NF-κB signaling pathway. Part of this adaptor complex is translocated into the cytosol to form traddosome with FADD, procaspase-8 and -10 and $FLIP_{L/S}$ proteins. This cytosolic complex leads to activation of effector caspases and apoptosis. Simultaneous activation of inflammatory and apoptotic pathways keeps the homeostatic balance in the cell population (Kim et al. 2001).

Intrinsic apoptotic pathway begins with intracellular signals originating from mitochondria. Mitochondrial permeability transition pores and mitochondrial transmembrane potential collapse caused by some molecules produced during course of infection, cancer, oxidative stress, hypoxia etc., or released molecules through extrinsic apoptotic pathway (e.g. Bid) open the mitochondrial intermembrane space. This incident releases pro-apoptotic proteins into the cytosol. Some well-known instances of such proteins are cytochrome c and Smac/DIABLO. These proteins trigger formation of apoptosome with cytochrome c and Apaf-1. Apoptosome activates caspase-9 which finally induces caspase-3 activation. After initiation of apoptosis, another surge release of new pro-apoptotic molecules, including AIF, endonuclease G and caspase-activated deoxyribonuclease (CAD), into the cytosol will happen. Integration of signals to the mitochondria, changes in its permeability and further release of pro-apoptotic proteins from mitochondria are controlled by Bcl-2 family proteins. Bax, Bak and Bid are pro-apoptotic, while Bcl-2 and $Bcl\text{-}X_L$ are anti-apoptotic proteins, all belonging to the Bcl-2 family proteins. p53, a tumor suppressor protein, plays an essential regulatory role in the pro- or anti-apoptotic functions of Bcl-2 family proteins (Miyashita et al. 1994).

Activation of the perforin-granzyme pathway induces apoptosis in virus-infected cells with both caspase-independent (granzyme A) and caspase-dependent (granzyme B) mechanisms, executed by cytotoxic T lymphocytes and NK cells (Brunner et al. 2003). In addition to the activation of the extrinsic apoptotic pathway via FasL/FasR, transmembrane pore-forming molecules, namely performin, assist in the delivery of toxic granules (e.g. granzymes) into the cytosol of the infected cells (Trapani and Smyth 2002). Granzyme A cleaves SET complex, which inhibits DNAse activity of NM23-H1 molecule and plays a role in DNA repair. Consequently, apoptosis occurs via DNA degradation and loss of chromatin integrity (Fan et al. 2003). Granzyme B activates caspase-3 either directly or via caspase-10 activation and leads to apoptosis induction (Sakahira et al. 1998). Granzyme B molecules are also able to cleave Bid protein and subsequently activate the mitochondrial apoptotic pathway, via leakage of pro-apoptotic mediators into the cytosol (Alimonti et al. 2001).

Pyroptosis is a caspase-1 dependent programmed cell destruction. It employs a different mechanism than apoptosis and has distinct consequences from it. Cells infected with intracellular microorganisms causing a pronounced pro-inflammatory response, such as *Salmonella* spp., produce noticeable amount of caspase-1 via NLRs recognition (e.g. NLRP1b, NLRP3 and NLRC4) and inflammasome formation. Normally, production of caspase-1 induces the active form of IL-1β and IL-18 from their precursors. Released pro-inflammatory mediators further activate inflammatory pathways. However, activation of caspase-1 can also lead to the formation of plasma-membrane pores, which eventually causes cell swelling and lysis (Fink et al. 2008). Moreover, caspase-1 leads to DNA cleavage via endonucleases.

Necroptosis is another programmed cell death which has the morphological features of necrosis including swelling of organelles, karyolysis, vacuole formation, and nuclear condensation except that it is not a passive mode of cell death as necrosis is (Vanden Berghe et al. 2010). The molecules involved in the necroptosis pathway are to a great extent similar to those inducing apoptosis (Vandenabeele et al. 2010). Normally, signals recognized by TNFR trigger either pro-survival/inflammatory signaling pathways or caspase-dependent apoptosis pathways. Under specific conditions where caspase-8 is blocked (e.g. via cFLIP), RIP1 will be phosphorylated by RIP1 kinase. Phosphorylated RIP1 interacts with RIP3, which initiates necroptosis via activation of pro-necrotic kinases (Gunther et al. 2013). As cells undergo the necrosis process, inflammatory responses will be activated.

Autophagy or self-cannibalization of cells is also a form of PCD, important in developmental processes, turnover of aged proteins and organelles, hypoxia, lack of nutrients, and starvation stress (Huett et al. 2010). Typical autophagic feature is cytoplasmic macromolecules and organelles enclosed in double membrane vesicles. Content of these vesicles will be recycled under specific conditions, e.g. nutrients depletion (Li et al. 2011). p53 under conditions of nutrient depletion activates the AMPK signaling pathway, followed by inhibition of the mTOR pathway which leads to an attenuation in mRNA translation rate. These modifications and alternations induce autophagy (Finch and Ruvkun 2001). Cytoplasmic p53 is known to play a role in transcriptional activation of pro-apoptotic molecules such as Bax and Bak but p53 aside from mTOR inhibition can induce autophagy via activation of damage regulated autophagy modulator (DRAM) (Crighton et al. 2006). Additionally, ROS production during hypoxia, starvation, and oxidative stress causes DNA damage and activation of different molecules, such as Beclin-1, Bax, Bcl-2, Atg4 and Atg5, which can conduct cells toward autophagy or apoptosis in a context-dependent process (Galonek and Hardwick 2006; Yousefi et al. 2006).

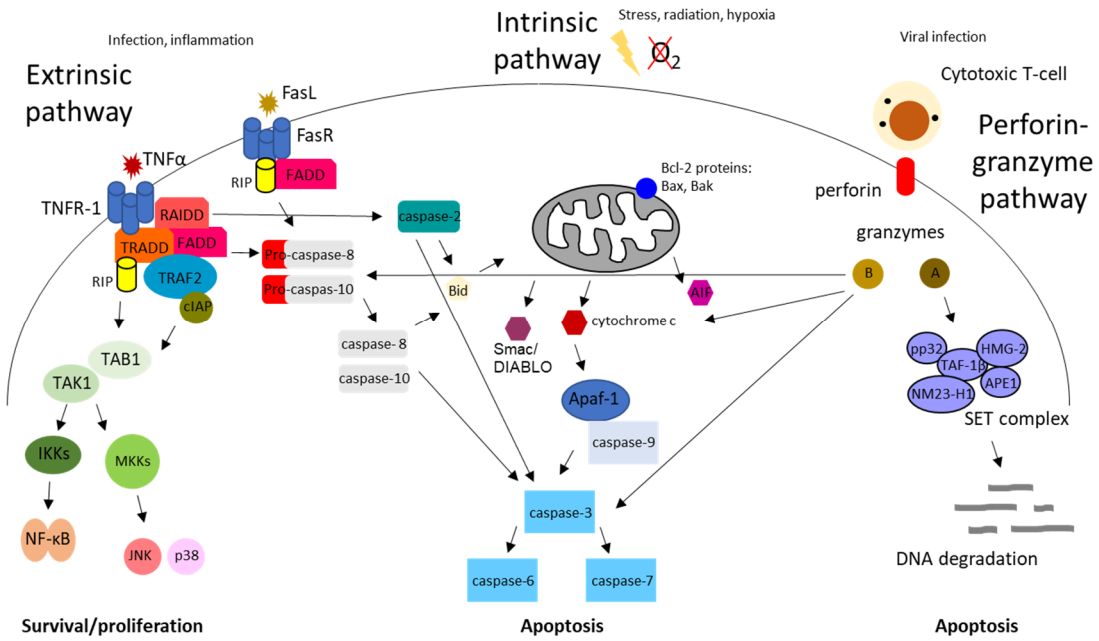

Figure 3: Schematic illustration of three main apoptotic pathways.

Apoptosis can be triggered via extrinsic, intrinsic, and perforin-granzyme pathways. Extrinsic pathway: Stimulation of death receptors (e.g. TNFR and FasR) recruit a complex of adaptor proteins including RAIDD, TRADD, FADD, RIP, TRAF2 and cIAP. On one hand these adaptor molecules activate initiator caspase-2, -8 and -10 molecules. Processed caspase-8 and -10 molecules activate effector caspase-3, -6 and -7 and cells undergo apoptosis process. Moreover, Bid a pro-apoptotic molecule is a substrate for activated initiator caspases. Bid causes loss of mitochondrial membrane potential, leakage of pro-apoptotic molecules and formation of apoptosome. On the other hand, recruited adaptor proteins activate inflammatory signaling pathways including NF-kB, JNK and p38. RIP, TRAF2 and cIAP molecules trigger inflammatory signaling pathways by activation of TAB1 and TAK1 molecules. Subsequently, kinase complexes involved in these inflammatory pathways (IKKs and MKKs) will be phosphorylated. Intrinsic pathway: This pathway will be stimulated by factors that can cause loss of mitochondrial transmembrane potential such as oxidative stress, radiation and hypoxia or Bid protein released during extrinsic apoptotic pathway. Releasing of different pro-apoptotic proteins including cytochrome c and Apaf-1 result in formation of apoptosome and activation of caspase-9. Active caspase-9 molecule transforms executioner caspases (3, 6 and 7) to their active forms. Other pro-apoptotic proteins freed during intrinsic pathway are AIF and Smac/DIABLO. Bcl-2 family proteins control mitochondrial permeability transition pores and release of pro-apoptotic proteins. Bax and Bak are two pro-apoptotic proteins from Bcl-2 family. Perforin-granzyme pathway: Cytotoxic T cells inject their toxic granules (containing granzyme A and B) to the virally infected cells via pore-forming perforin molecules. Granzyme A molecules cleaves DNA-protective complex, namely SET. Consequent DNA-degradation causes apoptotic cell death. Granzyme B molecules trigger apoptosis either by direct activation of caspases or assistance in release of pro-apoptotic proteins.

Necrosis is an irreversible form of cell death which almost invariably occurs as a result of a pathological process. Extreme environmental conditions (e.g. high temperature, extreme pHs), trauma, exposure to reactive oxygen species, ischemia, pathogens, and tumors can cause cell necrosis. Pathogen-induced cell necrosis confer disease pathogenesis, particularly in infections with intracellular pathogens. Demise of immune cells help pathogens to survive host immune defenses. Moreover, intracellular pathogens need to lyse cells to be able to spread in the body (Brennan and Cookson 2000; Fink and Cookson 2005). Thus, pathogen-induced necrosis is well studied. Despite the definition of necrosis as non-programmed cell death, a series of controlled events happens in necrosis process. These events include disruption in mitochondrial functions, ATP depletion, increased production of reactive oxygen metabolites and proteolytic activity by calpains and cathepsins (Abdelal 1979). Normally, necrosis elicits

uncontrolled inflammatory responses, because the plasma membrane integrity of necrotic cells will be lost before phagocytic cells can recognize and eliminate them and hence contents of necrotic cells will be exposed to the intercellular spaces. One of the important enzymes released after plasma membrane rupture in necrotic cells is cytosolic lactate dehydrogenase, which accounts for a certain indicator of cell death (Scaffidi et al. 2002; Shi et al. 2003).

Cells modulate immune system responses differently during infection by choosing one of these fates and activating distinct signaling pathways. However, there is crosstalk between different cell death processes. They have some overlaps in their pathways and can switch from one process to another under some circumstances (Fink and Cookson 2005). Type of cells, the nature of the damaging factor, degree of the damage, and duration of exposure to the factor determine whether cells undergo an apoptosis or necrosis process. Weak pro-apoptotic signals are reversible (Chen et al. 2018).

2.2 NF-κB signaling pathway

Nuclear factor binding near the κ light-chain gene enhancer in activated B cells (NF-kB) is a family of rapid inducible transcription factors. Researchers in cellular molecular biology have been intrigued by the broad regulatory role of NF-κB in expression and transcription of an enormous number of target genes. This transcription factors family is involved in many physiological and pathological processes, such as both innate and adaptive immune responses, inflammation, proliferation, survival, and apoptosis (Zhang et al. 2017). NF-κB molecules can be found as heterodimers or homodimers. There are five monomeric constituents of NF-κB including p50, p52, RelA (p65), RelB, and c-Rel. Fifteen potential combinations of these monomers can form NF-κB dimers (Smale 2012). All NF-κB monomers have a 300 amino acids Rel homology domain (RHD) at the N-terminal segment. This domain is a site for DNA recognition and binding, dimerization and binding of inhibitory proteins. RHD segment is directly connected to a nuclear localization sequence (NLS). Following the NLS sequence, in p50 and p52 molecules there is a region of ankyrin repeats. Three other monomers, RelA, RelB and c-Rel, have a transcription transactivation domain (TAD) directly after NLS in their C-terminals. Heterodimers containing TAD-domain, such as p50-p65 or p52-RelB molecules, are involved in transcription's activation, while homodimers of p50 and p52 act as repressors (Hayden and Ghosh 2008; Smale 2012). In latent state, NF-κB molecules are located in cell cytoplasm in an inactive form, bound to proteins of the κB inhibitor family (IκB). In fact, there is always an active flow of NF-κB paired to IκB between nucleus and cytoplasm, but the nuclear export is more proficient. Therefore, the outcome is in favor of cytoplasmic localization of NF-kB. Activation of NF-κB is a complicated process and involves several molecules and their modifications (Hayden and Ghosh 2008; Hayden and Ghosh

2012). These modifications basically comprise phosphorylation of different amino acid residues, polyubiquitination and dimerization of proteins. Activation of NF-κB happens via canonical (classical), non-canonical (alternative) or atypical pathways (Karin and Ben-Neriah 2000).

The canonical pathway begins with phosphorylation of IκB proteins by IκB kinases (IKKs). Consequently, active transport of NF-κB will be stopped and the accumulation of NF-κB molecules in nucleus induce changes in gene expression. IKK enzymes participating in canonical NF-κB activation pathway are IKKα, IKKβ and IKKγ (NEMO) (Hayden and Ghosh 2008). A stimulatory ligand like TNFα sends a signal via TNF-receptor which releases SODD molecule and recruits a number of adaptor molecules into a huge complex. These molecules comprise RIP-1, TRADD, TRAF2, cIAP, TAB and TAK1. Depending on the nature and intensity of the received signal, various modifications occur in the protein members of this complex and fate of the cell will be determined. For instance, recruitment of RIP kinases can induce cell death, but IKKs recruitment and their interaction with the complex mainly leads to ubiquitination of NEMO and phosphorylation of IKKs (IKKα and IKKβ) (Hayden and Ghosh 2008; Dondelinger et al. 2016). These series of events finally cause phosphorylation, ubiquitination, and degradation of IκB via the proteasome pathway and release of NF-kB. Freed NF-κB will be phosphorylated at different amino acids of TAD region depending on the characteristic of stimulant and the intended plan for a response to it. Comprehensively, for a better coordination of involved cells and a balanced response to the stimulant, different and sometimes opposite pathways (e.g. inflammation, apoptosis, and necrotic cell death) will be in parallel incorporated (Hsu et al. 1995; Karin and Ben-Neriah 2000).

The nonconical or alternative pathway occurs via other sensors and adaptor molecules. CD40 is an important receptor which conveys stimulatory signals such as CD154, lymphotoxin β and BAFF, through TRAF molecules into the cell. TRAF molecules recruit NIK and prompt its phosphorylation. Modified NIK phosphorylates only IKKα. Phosphorylated IKKα further phosphorylates p100-RelB dimer molecules. After ubiquitination and proteolysis of ANKR domain of p100-RelB, modified p52-RelB molecules will be translocated into the cell nucleus (Claudio et al. 2002; Sun 2011).

The atypical pathway is completely IKKs independent. Instead of IKK molecules, CK2 targets IκB proteins. Subsequently, released NF-κB heterodimers like p50-p65 will be translocated into the nucleus to alter the expression of target genes. Stimulation of the atypical NF-κB pathway occurs following DNA-damages caused by radiation or oxidative stress. DNA-damage signal recruits p38 molecules which recognize and activate CK2 enzymes (Kato et al. 2003).

Direction of responses after NF-κB activation relies on the cell type, nature and intensity of stimulant and phosphorylation sites of IκB. However, phosphorylation of NF-κB monomers at different residues is mainly determinant for the outcome of NF-κB activation. For instance, p65 is a key functional NF-κB monomer. Apart from phosphorylation and degradation of IκB molecules which is the main trigger of NF-κB activation pathway, p65 phosphorylation at different amino acids shape and optimize various active sites of this protein for DNA-binding and -recognition. Moreover, p65 phosphorylation improves its potentials to recruit histone acetyltransferases, e.g. CBP and p300, and remove the p50-HDAC-1 complex from DNA (Zhong et al. 1998). Phosphorylation at serine residues 276 and 536 are two best known important modifications required for transcriptional transactivation abilities of p65. Interestingly, a protein such as p65 can have a dual role as suppressor or activator of expression in the same gene. Transcription factors such as p65 switch their roles by their interaction with different kinases at distinct phosphorylation sites (Okazaki et al. 2003; Christian et al. 2016).

Pro-inflammatory cytokines stimulate NF-κB activation. Therefore, immense NF-κB activity will be detectable in cancers, infections, inflammations, and chronic inflammatory diseases. Innate immune responses are tightly tied with NF-κB activation, particularly during infection and inflammation. In the innate immune system, NF-κB activation will usually be triggered by a stimulatory signal carried by pro-inflammatory cytokines or PAMPs to epithelial or immune cells. Received signals will be conducted via PRRs into the cell and activate NF-κB via one or more of its three known signaling pathways. NF-κB plays an important role in adaptive immunity as well. On one hand it provides ways for equipped and forceful acquired immune responses by its assistance in specialization, maturation, and survival of immune cells in the healthy latent state. On the other hand, after receiving signals from the innate immune system NF-κB will be instantaneously activated in T and B lymphocytes. This immediate activation of NF-κB temporarily leads to a greater proliferation rate of immune cells, higher antibody and cytokine production, and acceleration in anti-pathogen strategies applied by immune cells, especially T cells. Mentioned rapid induction of NF-κB in lymphocytes occurs via recruitment and modification of CARD molecules (specially CARD11), Bcl10 and Malt1. The induction signals will be further processed via canonical pathway. Additionally, the combination of adaptor proteins including CARDs, Bcl10 and Malt1 can activate other signaling pathways, such as mTOR and JNK (Hayden and Ghosh 2008; Blonska and Lin 2011; Hayden and Ghosh 2012).

2.3 Intestinal immune system

The intestinal tract is unique in many different aspects and is a remarkable part of the immune system in vertebrates. First, the bowels have a huge functional surface. Moreover, the

intestinal tract is colonized with a large community of microbial population and hence is in close contact with a great number of antigens. Furthermore, the intestinal mucosa is continuously exposed to non-self signals including stimulatory antigens, allochthonous microorganisms, invading pathogens, nutrients, chemicals, and drugs (Nochi and Kiyono 2006; Zoetendal et al. 2008; Singh et al. 2009). Therefore, the gut requires a fine and well-regulated recognition system for simultaneous tolerance to beneficial commensal and gut microbiota and a proper immune response to pathogens (Macpherson and Harris 2004). For this purpose, the immune cells are properly integrated among gut epithelial cells (Figure 4). Immune cells together with epithelial cells form a complex immunomodulatory system. Intestinal epithelial cells (IECs) are involved in gut innate immune responses to infectious or non-infectious stimulants via their diverse defensive strategies and mechanisms. Secretion of proteins and antimicrobial peptides (AMPs) into the gut lumen is an important gut defense mechanism on the front line of combat with pathogenic microorganisms. Mucus produced by goblet cells and α-defensins synthesized by Paneth cells are examples of defensive secretory proteins produced by intestinal epithelial cells. Moreover, proteins generated by immune cells, such as IgA produced by plasma cells, are secreted into the gut lumen via transcytosis and are also involved in defense mechanisms (Bevins and Salzman 2011; Tezuka and Ohteki 2019). IECs possess different PAMPs-sensing receptors on their cellular membrane which can convey a danger signal into the cell cytosol and activate inflammatory pathways (Peterson and Artis 2014). However, studies have shown that the expression level of the aforementioned receptors, e.g. TLR2 and TLR4, is not high in epithelial cells in regular conditions, but is enhanced over the course of infection by immune-mediated signals. This normal down-regulation of transmembrane receptors such as TLR4 in epithelial cells is reasonable due to the constant exposure of these cells to PAMPs particularly LPS (Abreu et al. 2001; Melmed et al. 2003; Suzuki et al. 2003). Despite the scarcity of cell surface PRRs, intact cytosolic PRRs (e.g. cytosolic TLRs or NOD1) play an essential role in the gut recognition of intracellular pathogens or flagellin of bacteria like enteropathogenic *E. coli* and *S.* Typhimurium (Kim et al. 2004). However, intact pathogenic organisms (e.g. *Escherichia coli*, *Vibrio cholerae*, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Shigella flexneri*) can manage to get translocated through epithelium via M cells and later local macrophages (Jensen et al. 1998; Roberts et al. 2010). Intestinal intraepithelial lymphocytes

(IELs) are single lymphocytes which are scattered among epithelial cells. They fight directly and robustly against invasive pathogens via inflammatory responses (Cheroutre et al. 2011).

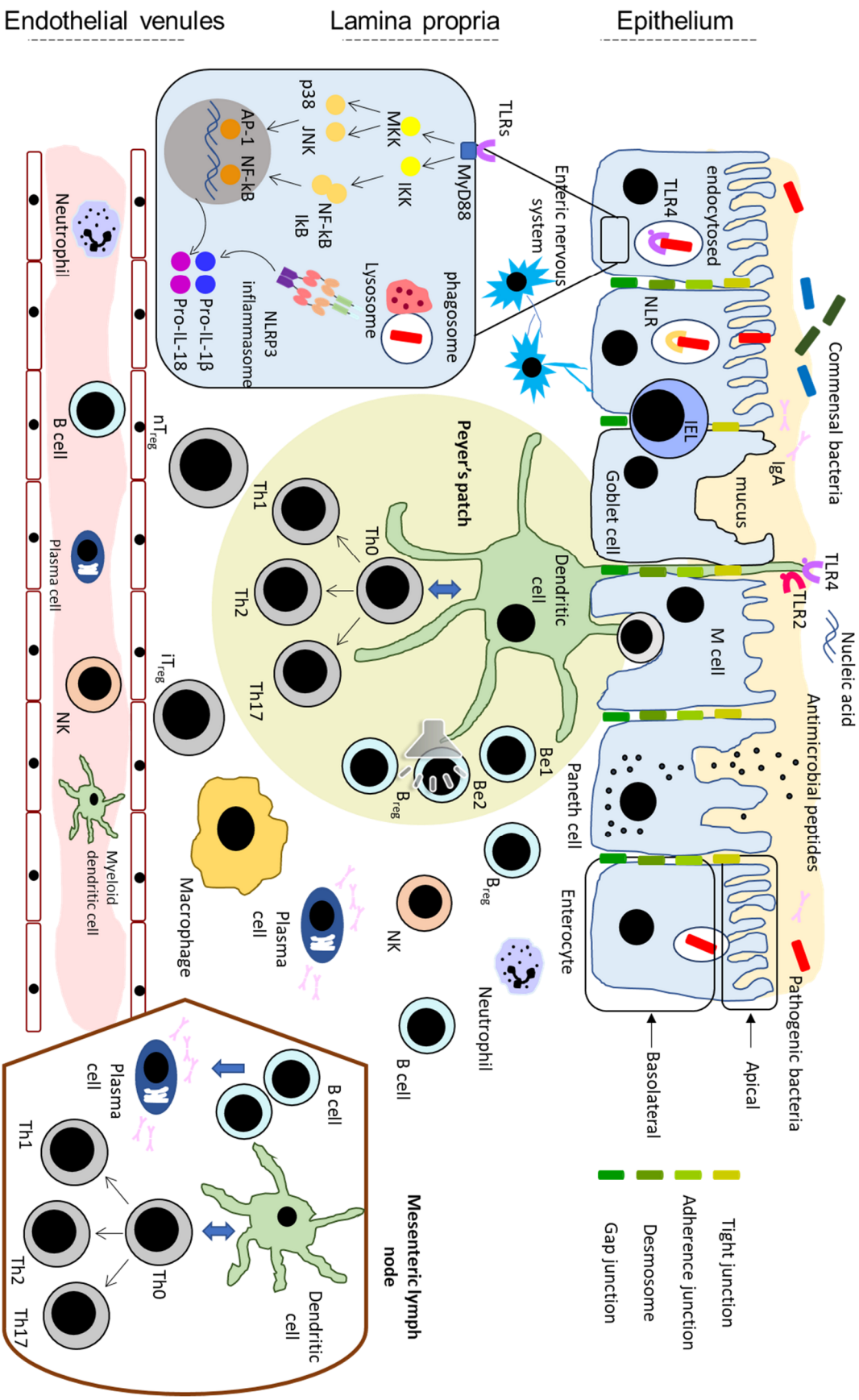

Figure 4: An overview of gut immune system.

Gut lumen is inhabited by a large number of harmless commensal bacteria, fungi, archaea and viruses. Moreover, secreted antimicrobial peptides, IgA molecules, bacterial structural compounds and metabolites and nucleic acids float in the lumen full of digestive contents. Intestinal epithelial cells, covered in a thick layer of mucus, consist of normal enterocytes and specialized epithelial cells including goblet cells (mucin production), Paneth cells (secretion of antimicrobial peptides) and M cells (antigen sampling). These cells are connected to each other through basolateral cell junctions including tight junctions, adherence junctions, desmosomes and gap junctions. Intraepithelial lymphocytes (IELs) and membrane extensions of dendritic cells located in Peyer's patches can be found between epithelial cells. Membrane-bound and cytosolic PRRs of enterocytes, IELs and membrane extensions of dendritic cells detect PAMPs and foreign antigens in the gut content. Engagement of ligands to the receptors recruits adaptor proteins in the cytosol of the affected cells and activate inflammatory signaling pathways including NF-kB, JNK and p38. Inflamed cells produce various inflammatory cytokines (e.g. IL-1β and IL-18) to directly attack the invader pathogen and send a danger signal to immune cells in the underlying layers of the gut, particularly to lamina propria and Peyer's patches. Moreover, cytokines and chemokines recruit further immune cells from blood. Various types of innate immune cells are resident of lamina propria including granulocytes, macrophages, dendritic cells and natural killer cells. These cells orchestrate responses to the received signals from epithelium and communication to the cells of adaptive immune system. Adaptive immune cells such as naive lymphocytes, regulatory B and T cells and plasma cells are located in Peyer's patches and are also dispersedly present in lamina propria. Primed lymphocytes and activated dendritic cells get to the mesenteric lymph nodes via high endothelial venules and lymphatic vessels and activate more of B cells and T cells. In mesenteric lymph nodes B cells will be transformed to plasma cells (for antibody production) and memory B cells. Naive T cells (Th0) become activated into different types of functional T-helper cells (Th1, Th2, Th17). Moreover, enteric nervous system, which plays an important role in maintenance and function of intestinal epithelia, is also involved in pathophysiology of intestinal inflammation. Inflammation, particularly in a chronic form results in degeneration and destruction of enteric neural cells. Inflammatory neuropathies disrupt gut digestive, secretory and absorptive functions leading to disorganized contractile behavior and abdominal pain. Modified from figure 1 of Crohn's disease seminar published in The Lancet (Baumgart and Sandborn 2012).

Comprehensively, transcriptional alterations resulting from activation of inflammatory signaling pathways lead to inflammatory cytokines release (TNF-α, IL-1β, IL-6, IL-12, IL-23, and type I interferons) and subsequent recruitment of more phagocytic and inflammatory cells to the site. They take a sample of detected antigens and present it to lymphoid tissues, namely gut-associated lymphoid tissue (GALT). GALT is essential for further assessments of antigens, activation of adaptive immune responses and antibody production (Nochi and Kiyono 2006; Round and Mazmanian 2009; Singh et al. 2009). The production and secretion of antimicrobial proteins such as RegIIIɣ, S100 proteins, and β-defensins is enhanced as well (Sonnenberg et al. 2011). Among the activated immunomodulatory molecules NF-κB is one of the main transcription factors which trigger inflammatory responses of the gut. NF-κB also prevents apoptotic cell death and promotes proliferation and repair mechanisms in cells (Elewaut et al. 1999; Pasparakis 2009). Perturbation of the delicate balance between activation and inhibition of inflammatory responses in the gut can be detrimental. For instance, two types of inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, represent results of exaggerated inflammatory responses triggered by overreaction of innate immune responses of epithelium to antigens and commensal bacteria (Baumgart & Sandborn, 2012). Balanced composition of commensal gut bacteria and administration of beneficial probiotics promote gut health and constructively manipulate epithelial innate immunity in either stimulatory (pro-inflammatory) or inhibitory (anti-inflammatory) direction (Pagnini et al. 2010).

2.4 *In vitro* study models

In vivo intestinal research models are advantageous tools to maintain and mimic the natural intestinal environment. Following the exposure to the intended pathogen or drug, alterations can be concurrently determined in different cell types and hence their crosstalk can be studied in *in vivo* models. Moreover, the influence of the microbiota on gut both in health and diseased conditions can be incorporated in these study models (Lennernas 1998; Cronin et al. 2009; Possemiers et al. 2009; Zhao et al. 2018). However, ethical limitations, complicated processes of animals maintaining and feeding, length of experiments and excessive expenses oblige researchers to avoid *in vivo* experiments so far possible. One main alternative research tool is the application of *in vitro* study models. Target cells for these kinds of studies are mostly derived from naturally occurring cancers or normal cells which were transformed/immortalized to proliferate permanently or for a longer time (Berwald and Sachs 1965; Rhim 1993; Counter et al. 1998). Selection of a cell line for research purpose will be based on the target site of the study within the body, species of interest and the purpose of the experiment (Schierack et al. 2006; Schwerk et al. 2013; Li et al. 2019b).

There are several well-studied human and animal intestinal epithelial cell lines (Chopra et al. 2010). Some of these known cell lines are HuTu-80 (duodenal adenocarcinoma), HT-29 (colon adenocarcinoma), Caco-2 (colon carcinoma) and T84 (lung metastasis of colon adenocarcinoma) with human origin, IPEC-1 (jejunum/ileum), IPEC-J2 (jejunum), IPI-2I (ileum) derived from porcine tissues and MODE-K (duodenum-jejunum), IEC-1 (small intestine), IEC-6 (small intestine) and IEC-18 (ileum) with murine origin (Fogh and Trempe 1975; Fogh et al. 1977; Forgue-Lafitte et al. 1989; Ma et al. 1992; Ametani et al. 1993; Kaeffer et al. 1993; Vidal et al. 1993; Schierack et al. 2006; Schwerk et al. 2013; Devriese et al. 2017). Normally, a single cell line at time will be employed in an experiment. However, occasionally an exposure will be assessed in a mix of different cell types. In co-culture models, immune cells are often added to epithelial cells for simulating the natural microenvironment of epithelial cells or their differentiation (Barrila et al. 2017; Nielsen et al. 2017). One example for this kind of *in vitro* study model is the co-culture of Caco-2 cells and B lymphocytes in order to transform epithelial cell to M cell (Beloqui et al. 2017).

In vitro models are applicable for long-term experiments or for the assessment of toxic and harmful reagents and performed experiments are easy to monitor and repeat. Gene expression patterns, proteomics, absorption and uptake mechanisms and activation/inhibition of different signaling pathways have been determined in different intestinal epithelial cell lines in health and disease conditions (Schierack et al. 2006; Sun et al. 2008; Gagnon et al. 2013; Nossol et al. 2015; Pajarillo et al. 2017; Ponce De Leon-Rodriguez et al. 2019).

Epithelial cells are connected through a network of proteins forming adherence junctions (e.g. desmosomes, β-catenin and E-cadherin), tight junctions (e.g. claudin and occluding) and gap junctions (connexins) (Van Roy and Berx 2008; Goodenough and Paul 2009; Zihni et al. 2016). These transmembrane proteins and their related internal adaptor proteins regulate the communication between epithelial cells, exchange of ions and other molecules, in addition to the epithelial barrier function (Hatayama et al. 2018). Tight junctions divide cell surfaces into apical (luminal) and basolateral (abluminal) sides. This polarization plays an important role in transport of molecules and pathogenesis of different microorganisms. In fact, pathogen binding epithelial surface determines pathogen invasion strategy and its encounter tactics to face the local immune cells (Hatayama et al. 2018; Co et al. 2019) and *in vitro* models provide an opportunity for a closer examination of these aspects of host-microbe interplays.

Invasion of pathogens and non-infectious intestinal inflammations such as Crohn's disease and gastrointestinal cancers alter junctional proteins and the permeability of epithelial barrier. As a result, epithelial barrier loses its integrity and becomes leaky (Groschwitz and Hogan 2009; Bhat et al. 2019). Transepithelial electrical resistance (TEER) is an indicator for the integrity of tight junctions and cellular barriers in cell culture models (Srinivasan et al. 2015). Additionally, using permeability assays like P-glycoprotein transport and FITC-dextran permeability assays, the permeability and transport of labeled molecules from apical to basolateral direction can be evaluated (Kauffman et al. 2013).

Reporter assays, which are invaluable tools for tracking gene expression and transcription can perfectly be adapted into *in vitro* models. Reporter genes (e.g. firefly luciferase) or detectable fluorescent substances (e.g. GFP) will be coupled to the promoter or response element of the target gene or transcription factor, respectively. Cells will be transfected with a plasmid harboring the reporter apparatus. Therefore, these cells could be easily monitored for alterations in the gene or transcription factor of interest after exposure or treatment with different stimuli (Brasier et al. 1989).

2.4.1 IPECJ-2

Swine has an important place in human health and disease studies, regarding both its similarities and differences; accordingly, determination of alterations caused by human pathogens and immune responses in porcine models is considerable (Litten-Brown et al. 2010; Deglaire and Moughan 2012). Moreover, pig farming constitutes a large part of animal husbandry and livestock production. Thus, porcine-specific pathogens, immune responses and host-microbe interplays should be well studied. Therefore, for explicit research in this species an *in vivo* or an *in vitro* model of the same species should be considered.

IPEC-1, IPEC-J2 and IPI-2I are few available non-tumor intestinal epithelial cell lines derived from porcine small intestine for *in vitro* experiments (Kaeffer et al. 1993; Nossol et al. 2015). IPEC-J2 is a non-transformed jejunal cell line in which cells were initially isolated from an unsuckled piglet (Berschneider 1989). These porcine epithelial cells build a tight epithelium with TEER of 2000 Ω cm^2 on average. They produce membrane-bound mucin and form a polarized monolayer of cells with microvilli on their apical surface. Their junction complexes consist of tight junctions, adherens junctions and desmosomes (Schierack et al. 2006). Several studies demonstrated that IPEC-J2 cells preserved structural and immune-related gene expression patterns of their obtained tissue of origin. mRNA expression of cytokines, which can potentially be found in small intestinal epithelial cells, has been reported in IPEC-J2 cells with the two exceptions of TGF-β and MCP-1 (Schierack et al. 2006; Mariani et al. 2009; Arce et al. 2010). It has been shown in numerous studies that IPEC-J2 is a permissive *in vitro* model to study of host-pathogen interactions, as well as the relationship between epithelial cells and commensal bacteria (Skjolaas et al. 2006; Liu et al. 2010b; Marcinakova et al. 2010; Brosnahan and Brown 2012). The IPEC-J2 cell line, unlike many other intestinal epithelial cell lines, is not of a cancerous origin, therefore it is an appropriate model for studying the normal intestinal epithelium in physiological and pathological conditions. Additionally, since these cells form a polarized monolayer in the culture, microorganisms can choose their natural communication path with epithelial cells, as in the gut lumen. Taking all these traits into consideration, IPEC-J2 cells constitute an opportunity to have a deeper look into the primary role of intestinal epithelial cells in innate immune responses, communication of epithelial cells with pathogenic and non-pathogenic microorganisms and direct adhesion/invasion mechanisms of microorganisms to epithelial cells in the absence of immune cells interference.

The most studied bacterial enteric pathogens in IPEC-J2 cells are human and porcine infecting *S*. Typhimurium, porcine infecting *S*. Choleraesuis, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC) (Schierack et al. 2006; Koh et al. 2008; Johnson et al. 2009; Veldhuizen et al. 2009; Liu et al. 2010a). Bacterial properties involved in pathogenesis and the subsequent induced immune responses and signaling pathways in host cells after exposure were the focus of these studies. For instance, high pathogenicity island (HPI) positive pathogenic *E. coli* strains promote inflammation via upregulation of IκB-α expression and subsequent release of TNF-α and IL-1 through ubiquitin-proteasome pathway (Liu et al. 2018). Depending on the infecting strain adhesion of bacteria, accumulation of actins, cytoskeletal dearrangement, development of lesions, formation of microcolonies and, eventually, invasion and internalization of bacteria, can be reflected in *in vitro E. coli* infection model in IPEC-J2 cells (Kaper et al. 2004; Schierack et al. 2006; Johnson et al. 2009). Invasion and replication of *Salmonella* serovars in IPEC-J2 cells are comparable with their invasion and proliferation ability in *in vivo* infection models. IL-8 and TNF-α

expression levels (indicators of inflammatory responses) will be upregulated in IPEC-J2 cells challenged with genuine pathogenic organisms such as different serovars of *Salmonella* (Skjolaas et al. 2006; Burkey et al. 2009). However, it has been shown that relative TLR4 gene transcript level and IL-8 secretion level in response to low dose LPS-stimulation remains unaffected. This incident is probably due to the relative insensitivity of intestinal epithelial cells to LPS in a lumen full of commensal bacteria and their floating products, including LPS (Abreu et al. 2003). But the direct contact between intact pathogens and epithelial cells, similar to the *in vivo* situation, can upregulate and increase expression level of various PRR genes (e.g. TLRs) and enhance production of their downstream cytokines and chemokines via diverse extrinsic and intrinsic pathways (Arce et al. 2010; Brosnahan and Brown 2012).

IPEC-J2 cells are an invaluable tool to determine immunomodulatory effects (e.g. anti- or pro-inflammatory effects) of probiotics and determine their adhesiveness to the epithelium in physiological and pathological conditions (Larsen et al. 2007; Bevilacqua et al. 2010; Schierack et al. 2011; Valeriano et al. 2016; Heo et al. 2018; Xiao et al. 2018). In this regard, several studies have been focused on the direct interaction of a probiotic strain and epithelial cells. As an instance, using proteomics data obtained from IPEC-J2 cells in the presence of *Lactobacillus mucosae* it has been demonstrated that intestinal epithelial cells and beneficial microorganisms can reciprocally have an influence on proteins production level. In this case, *L. mucosae*, a beneficial microbe and resident strain of porcine and human mucosa, manipulates the cell structure, proliferation, signaling pathways and tight junctions of this intestinal cell line at protein level (Pajarillo et al. 2017). Another example is given by *Lactobacillus gasseri*, a dominant member of commensal bacteria in weaned piglets, which can promote cell proliferation and enhance metabolism in cells via oxidative phosphorylation pathway (Hu et al. 2018). Furthermore, in some experiments, effects of probiotics properties were determined on transepithelial ion transport, metabolic activity and immune responses of IPEC-J2 cells (Grzeskowiak et al. 2020; Ho et al. 2020; Lan et al. 2020; Yang et al. 2020). For instance, it has been shown that porcine colostrum or fermented milk with live *Lactobacillus helveticus* can protect IPEC-J2 cells against *Escherichia coli* infection and *Clostridioides difficile* toxin, respectively, since treatment with this strain enhances inflammatory responses in the cells (Ho et al. 2020).

2.4.2 Caco-2

*Ca*ncer *co*li-2 (Caco-2) is an important human derived intestinal epithelial cell line (Fogh et al. 1977) isolated from a human colorectal adenocarcinoma. They form naturally differentiated polarized monolayers in cell culture (Lea 2015). Caco-2 cells basically have morphological and biochemical characteristics of enterocytes from small intestine (Pinto et al. 1983). However, it

has been shown that they express a blend phenotype of both colonocytes and enterocytes (Engle et al. 1998), and are heterogenous. Therefore, culture conditions and the number of passages influence characteristics of the cells and for data consistency experiments should be performed under the same conditions (Ishikawa and Barber 2008).

Studies using Caco-2 cell line are mainly focused on absorption of drugs or food metabolites, permeability of bioactive molecules, cell uptake and transport (Yee 1997; Hubatsch et al. 2007; Shimizu 2010). Moreover, the adhesiveness and immunomodulatory effects of different probiotics on the expression of immune-associated genes, such as different TLRs, NODs and cytokines, in Caco-2 cells were determined (Tuomola and Salminen 1998; Kingma et al. 2011; Bourgine et al. 2012; Maccaferri et al. 2012) and few studies were focused on the host-pathogen interaction. Finaly and Falkow demonstrated that Caco-2 cells are a proper *in vitro* study model for *S*. Choleraesuis or *S*. Typhimurium, comparable with their *in vivo* infection models, considering their ability to invade Caco-2 epithelial cells (Finlay and Falkow 1990).

Transformed human intestinal epithelial cells such as Caco-2, HT-29 and T84 have different transcriptional profiles from each other (Bourgine et al. 2012). Comparing to IPEC-J2, Caco-2 cells make a more prominent mucus layer (Navabi et al. 2013) but their tight junctions are looser than in IPEC-J2 cells (Pisal et al. 2008). Regardless of divergences between various epithelial cell lines and their differences with normal epithelial cells, they can be a valuable tool for epithelium and mucosa studies. Nevertheless, conclusions should be drawn cautiously and preferably parallel to *in vivo* experiments.

2.4.3 MODE-K

MODE-K is a murine small intestinal epithelial cell line derived from duodenum/jejunum of a 2-weeks-old mouse. Cells are not of tumor origin but were immortalized using the large T gene of SV40 virus. These cells have the characteristics and properties of normal murine enterocytes. They constitutively express MHC class I molecules, but the MHC class II molecules have a regulated expression and can be induced upon appropriate stimulation. Hence MODE-k is a desirable study model for the antigen transport, process and presentation and the communication of epithelial cells with lymphoid cells for a local response to invasive enteric pathogens (Vidal et al. 1993; Vidal et al. 1995).

MODE-K is a less employed *in vitro* model comparing to the other well-known human and porcine intestinal epithelial cell lines. Apoptotic pathways induced by bacterial products or mycotoxins are the best studied models in MODE-K cells. These experiments determined the response of epithelial cells to the inflammatory cytokines such as TNF-α and IFN-ɣ. These

responses included induction of oxidative stress responses, production of reactive oxygen species (ROS) and subsequent stress-associated induction of apoptotic pathways (Song et al. 1999; Jia et al. 2012; Babu et al. 2017; Long et al. 2018). These studies have been shown that the exposure to TNF-α and cycloheximide (CHX) can induce apoptosis, increase caspase-3 and -7 activities, and decrease reduced glutathione level (GSH). Furthermore, in these experiments anti-apoptotic effects of carbon monoxide-releasing molecules (CORMs) like CORM-A1, CORM-2 and CORM-401, nitrite, resveratrol (a polyphenolic compound) and proanthocyanidins were determined. These substances can impair the activation of caspase 3- and -7, oxidative stress responses, reactive oxygen formation and endoplasmic reticulum stress (ERS)-induced apoptosis.

2.5 Probiotics

Supplementation with nutrients and administration of probiotics and prebiotics have been shown to stimulate gut health in human and animals (Allen et al. 2004; Wald and Rakel 2008; Allen et al. 2010; Sanders et al. 2019; Hujoel 2020). For instance, zinc, an essential trace element, has been suggested as a therapeutic option in patients with irritable bowel syndrome (Wald and Rakel 2008). Moreover, zinc and copper have been used as alternatives to antibiotics growth promoter in animal husbandry (Heo et al. 2013a; Gadde et al. 2017) and several studies reported positive effects of zinc supplementation on prevention/control of diarrhea and enteric infections specially with pathogenic *E. coli* (Melin and Wallgren 2002; Crane et al. 2014). The initial idea of probiotics was proposed over a hundred years ago by Elie Metchnikoff who indicated an association between the consumption of fermented dairy products containing live *lactobacilli* cells and prolongation of life (Mackowiak 2013). Presently, probiotics are viable non-pathogenic beneficial microorganisms, which in adequate amount, can confer health benefits to their host. Probiotics are mainly administered for both preventive and therapeutic purposes in gastrointestinal and limited non-gastrointestinal conditions (Hill et al. 2014). Probiotics can be applied in different forms, from dietary supplementations to pharmaceutical formulations (Hill et al. 2014). The most common medical conditions which probiotics can be used for are acute diarrhea and intestinal inflammatory diseases (Hart et al. 2003; Schultz et al. 2004; Allen et al. 2010).

Induction of proliferation and differentiation in epithelial cells, maintaining the mucosa permeability, improvement of barrier function via upregulation of tight junction proteins and enhanced TEER are among determined probiotics tactics to regulate the intestinal development and function and to protect host cells against physiologic stresses, inflammatory diseases and infections (Ljungh and Wadström 2006; Sonnenburg et al. 2006; Preidis et al. 2012). Furthermore, probiotics exhibit immunomodulatory effects via proximity of the probiotic

strain or its metabolites and products (e.g. surface and secreted proteins) to the host cells (mostly intestinal epithelial cells). These effects can be in both pro- and anti-inflammatory directions. NF-κB, MAPKs, Akt/PI3K and PPARγ signaling pathways involved in homeostasis and inflammation along with their downstream mediators and cytokines are best-known targets of probiotics (Wu et al. 1999; Kelly et al. 2004; Ruiz et al. 2005; Frick et al. 2007; Thomas and Versalovic 2010). These modulatory effects subsequently lead to alterations in the differentiation of T cells and epithelial cells and production of β-defensins and IgA by host cells (Delcenserie et al. 2008; Ivanov et al. 2008; Mazmanian et al. 2008). It should be noted that different probiotic strains have divergent and strain-specific approaches to modulate epithelium responses (Van Baarlen et al. 2011).

Beside immunomodulatory effects of probiotics on host cells, they can ultimately interact with invasive pathogens. Colonization and adhesion of pathogens can be directly hampered by physical blocking of beneficial microbes. It is evidenced that some probiotic strains belonging to *Enterococcus* and *Lactobacillus* genera produce glycosaminoglycans degrading enzymes which confine attachment of pathogens to epithelium (Kawai et al. 2018). Moreover, probiotics can protect host cells from pathogens or at least mitigate the burden of infection by enhancing the production of mucin, antimicrobial peptides (e.g. β-defensin and reuterin), heat shock proteins, pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α and increase in acidity (Macpherson and Uhr 2004; Tsai et al. 2005; Kim et al. 2008; Fukuda et al. 2011).

Moreover, microbiota composition and alterations are interconnected with several health conditions (De Vos and De Vos 2012). Probiotic bacteria are believed to manipulate and restore intestinal endogenous microbial populations in their composition or function by altering genes expression, transcriptional networks or metabolic pathways (O'toole and Cooney 2008; Thomas and Versalovic 2010; Thomas et al. 2012).

Different *Bifidobacterium* and *Lactobacillus* strains are among the best characterized probiotic bacteria. Other bacterial genera that mediate a balance in the microbiota inhabitants, promote immune system responses in health and diseased conditions, enhance digestion and improve host health comprise *Bacillus* spp., *Butyricicoccus* spp., *Enterococcus* spp. as well as *E. coli* Nissle 1917 strain and few *Streptococcus* species, such as *S. thermophilus* and *S. salivarius* (Bolotin et al. 2004; Kruis et al. 2004; Burton et al. 2006; Franz et al. 2011; Eeckhaut et al. 2013).

Aside from all above-mentioned effects proposed and determined in probiotics, probiotic related experiments have some limitations. Most of the study models for crosstalk and dynamics between probiotics and host cells or probiotics and pathogens are in cell culture systems and hence cannot fully reflect the *in vivo* conditions. Furthermore, a limited number of

performed *in vivo* studies cannot be easily translated from one species to another. Moreover, human clinical trials are mostly based on stool sample analysis. Therefore, the interpretation of the outcomes from these studies can be easily biased and these assays cannot give a general impression of the effect that administered probiotic strain has on the microbiome, intestinal immune and metabolic homeostasis (Suez et al. 2019). Finally, modifications have person-to-person variations and the experiment results are often not reproducible in other clinical conditions or study designs. In other words, complexity of the microbiome itself, its variation between the individuals and disease/infection related alterations have a great impact on the shifts in the microbiome composition and immune responses which can outshine beneficial effects of probiotics (Mcfarland 2014; Kristensen et al. 2016).

2.5.1 *Enterococcus* spp.

Genus *Enterococcus* includes gram-positive and catalase negative lactic acid bacteria (LAB) that can ferment a wide range of carbohydrates to lactic acid. They are facultative anaerobic and non-spore forming by their nature (Teixeira et al. 2015). With the isolation of *Enterococcus xiangfangensis* in 2014 nearly 50 species of *Enterococcus* are described (Li et al. 2014). The best-known species of this genus consist of *E. faecalis*, *E. faecium*, *E. durans*, *E. hirae*, *E. avium*, *E. cecorum*, *E. mundtii*, *E. raffinosus*, *E. gallinarum* and *E. casseliflavus*. *E. gallinarum* and *E. casseliflavus* are the only two motile enterococci species (Facklam and Collins 1989; Lebreton et al. 2014). *Enterococcus* spp. are ubiquitous microorganisms, spread in the environment. Genus *Enterococcus* forms one of the main constituents of microbiome and enterococcal strains are commensal residents of gastrointestinal tract of humans and animals. Even the designation of genus *Enterococcus* stems from initial description of its members as cocci of intestinal origin. In human *E. faecium* and *E. faecalis* strains are the main enterococcal inhabitants of the gastrointestinal tract. In gut of farm animals, along with two mentioned species, strains from *E. hirae*, *E. durans* and *E. cecorum* species can be found (Devriese et al. 1991; Devriese et al. 1994; Franz et al. 2011; Lebreton et al. 2014). These LAB bacteria have a wide host range and can be detected in mammals, birds, reptiles, and insects. Broad host range indicates a great adaption and survival ability of *Enterococcus* spp., despite the diverse environment and innate defense mechanisms in the gut of different hosts (Lebreton et al. 2014).

Several strains of *Enterococcus* spp. have been employed in food industry and food fermentation, since they can survive a wide range of pH and salt concentrations (up to 6.5% NaCl and 40% bile salts) and can grow in a wide temperature range (Teixeira et al. 2015). They also possess natural antimicrobial substances such as enterocins, which can be advantageous in food preservation processes or as replacement for antibiotic therapy,

particularly against multi-drug resistant pathogens (Arques et al. 2015). Furthermore, some strains have been applied as probiotics or feed additives to improve animal growth rate, prevent or treat diarrhea in humans and animals and exploit their immunostimulatory and anti-inflammatory effects. Besides aforementioned functions, positive effects of enterococcal strains on digestion and maintenance of microbiome have been documented (Franz et al. 2011; Carasi et al. 2017; Fiore et al. 2019). Some examples of these beneficial microbes and their observed effects are as follows: administration of *E. faecium* NCIMB 10415 (SF68) has been shown to reduce the duration of diarrhea in general and to prevent antibiotic-associated diarrhea (Wunderlich et al. 1989; Buydens and Debeuckelaere 1996). *E. faecalis* Symbioflor 1 can help to treat and prevent recurrent infections in patients with chronic bronchitis and sinusitis (Habermann et al. 2001; Habermann et al. 2002). *E. faecium* LCW 44 and *E. durans* 6HL demonstrated antibacterial and inhibitory activities against various pathogenic bacteria in *in vitro* models (Nami et al. 2014; Vimont et al. 2017). *E. gallinarum* L-1, a fish commensal strain, has great adhesion and colonization abilities in fish intestinal epithelium and produces antibacterial molecules which act against fish pathogens, like *Vibrio anguillarum* (Román et al. 2015). The two *E. durans* strains M4-5 and EP1 have anti-inflammatory potentials against inflammatory stimuli (Avram-Hananel et al. 2010; Carasi et al. 2017).

Despite of all observed profits recorded in beneficial enterococcal strains, their incautious usage has been declined and decisions on deliberate usage of advantageous strains or their development at commercial level will be made more carefully (Van Tyne and Gilmore 2014). The reason for this caution is that the distinction of commensal enterococci from pathogenic ones is poorly defined. Several commensal enterococcal species can be opportunistic pathogens. Urinary tract infections, bacteremia, endocarditis, and infection of implanted medical devices are among the most reported infections caused by *Enterococcus* strains. In fact, enterococcal species are included in the major players in health care-associated infections in human (Fiore et al. 2019).

Enterococcus species are intrinsically resistant to diverse antimicrobial compounds with cephalosporins, aminoglycosides, trimethoprim-sulfamethoxazole, lincosamides and streptogramins among these agents (Garcia-Solache and Rice 2019). Moreover, a great pathogenicity of enterococcal isolates due to the acquirement of virulence factors such as aggregation substance, extracellular surface protein (ESP), gelatinase, pili, hemolysin and cytolysin has been shown in several studies, as well as the acquisition of antibiotic resistance, particularly vancomycin resistance (Sahm et al. 1989; Mundy et al. 2000; Fiore et al. 2019). Additionally, strains of *Enterococcus* genus have an excellent genome plasticity which allows virulence factors and antibiotic resistance determinants to circulate in the bacterial population via mobile genetic elements. Besides, some virulence and antimicrobial resistance traits can

be vertically transferred from one generation of bacteria to the other. Moreover, gut is a great medium for genetic exchanges and interplays between divergent autochthonous and probiotic/digested bacterial strains (Giraffa 2002; Franz et al. 2011; Van Tyne and Gilmore 2014).

Altogether, there are immense concerns about administration of *Enterococcus* strains in food industry or its use as probiotic to gain health benefits in humans and animals. Safe strains from this genus should be selected through an intricate and strict process. Therefore, among diverse enterococcal species with shown probiotic properties and health benefits only limited strains of *E. faecium* and *E. faecalis* are allowed to be used as authorized probiotics (Franz et al. 2011). Two well-established probiotic strains from *Enterococcus* genus are *E. faecium* SF68 (NCIMB 10415) and *E. faecalis* Symbioflor 1, which are manufactured as pharmaceutical formulations (Lewenstein et al. 1979; Domann et al. 2007).

2.5.1.1 *Enterococcus faecium* SF68 (NCIMB 10415)

E. faecium SF68 (NCIMB 10415) is a commensal *Enterococcus faecium* strain which was isolated for the first time from stool of a healthy infant (Holzapfel et al. 2018). *E. faecium* SF68 has no adherence ability to epithelial cells, maybe therefore, it cannot constantly colonize intestinal epithelium and gradually will be eliminated from the gut microbiome community after ceased intake (Lund et al. 2002; Holzapfel et al. 2018). The same as other enterococcal strains, this LAB strain is intrinsically resistant to some antibiotics such as kanamycin and erythromycin. Nevertheless, it is sensitive to glycopeptides and vancomycin. More important, *E. faecium* SF68 is a poor donor for transmission of antibiotic resistance determinants to other enterococcal strains and has a scare ability for exchanging mobile genetic materials such as virulence factors. This strain, so far, does not contain functional virulence factor genes (Holzapfel et al. 2018). Using multiple typing methods, it has been shown that *E. faecium* SF68 stands genotypically far from clinical strains and in the same group as other *E. faecium* strains of food origin (Vancanneyt et al. 2002; Werner et al. 2011).

Following manifold *in vitro* and *in vivo* assessments on safety and efficacy, *E. faecium* SF68, has been accepted as a probiotic strain for therapeutic purposes in human and authorized for pharmaceutical applications (Lewenstein et al. 1979; Holzapfel et al. 2018). Indeed, over last decades, this strain has been used in human as an effective treatment of diarrhea with different causes, particularly in cases of antibiotic-associated diarrhea (Wunderlich et al. 1989; Buydens and Debeuckelaere 1996; D'souza et al. 2002). This positive effect on reduction of diarrhea duration and rate has also been demonstrated in piglets (Taras et al. 2006; Zeyner and Boldt 2006). Furthermore, several studies carried out in different hosts demonstrated inhibitory

activity of *E. faecium* SF68 against pathogenic bacteria, such as members from family of *Enterobacteriaceae* (Canganella et al. 1996; Beirão et al. 2018; Peng et al. 2019).

E. faecium SF68 is also permitted as feed additive in pets and livestock animals (e.g. calves, piglets and chicken broilers). Therefore, it is administered as performance/health and growth promoter in different animals (Franz et al. 2011; Busing and Zeyner 2015; Xie et al. 2018). For example, *E. faecium* SF68 has been observed to promote growth in turkey and broiler chickens via production of lactic acid in the gut and its indirect effect on colonization of other LAB strains (Vahjen et al. 2002; Samli et al. 2007). Probiotic *E. faecium* SF68 has attracted a considerable attention particularly in porcine research, because it can survive in the pig intestinal tract with an efficient high number without colonizing it after ceasing intake (Vahjen et al. 2007). Moreover, this probiotic strain can be naturally transferred from sows to their litters via the fecal-oral route (Taras et al. 2006; Zeyner and Boldt 2006). Weaning transition is a stressful process and can lead to perturbation of microbiota which make animals more prone to diarrhea and intestinal infection. Therefore, preventive measurements should be provided during this time (Nabuurs 1998; Wieler et al. 2001; Melin et al. 2004; Taras et al. 2006) and different *in vivo* trials determined beneficial potentials of *E. faecium* SF68 during weaning process. Pretreatment of sows and their piglets with *E. faecium* SF68 has been shown to reduce pre- and post-weaning diarrhea cases, *E. coli* induced diarrhea, and also it accelerates the recovery of these diseased animals (Underdahl et al. 1982; Taras et al. 2006). Administration of *E. faecium* SF68 in Chlamydia-positive sows and their litters showed a lower carryover rate in the piglets comparing with the control group (Pollmann et al. 2005). In another study, a decline in the number of *E. coli* pathoserovars in intestinal content of piglets supplemented with this probiotic strain was observed (Scharek et al. 2005).

Despite various positive effects which have been reported on the use of *E. faecium* SF68 in human and animal species, there are some controversial observations on this probiotic strain in the literature. Some of these studies and their main results are listed as follow: in a feeding trial, effects of oral supplementations with *E. faecium* SF68 and *Saccharomyces cerevisiae* on mediators of acute phase response in feedlot steers were determined. The result of this study showed that administration of this live cultures combination causes an inflammatory response against the yeast strain (Emmanuel et al. 2007). In another experiment, young healthy dogs were supplemented with this LAB probiotic strain. The presence and frequency of the three enteric pathogens *Salmonella* spp., *Campylobacter* spp. and *Clostridium* spp. were compared before and after administering the probiotic strain. *Salmonella* and *Campylobacter* species were detected more frequently after application of probiotic, albeit the decrease in the *Clostridium* spp. counts (Vahjen and Männer 2003). Different trials on healthy sows and their piglets or on enteric infection models of *Salmonella* Typhimurium showed that applications of

E. faecium SF68 can cause dysregulations in immune responses of its recipients, which can be a game changer in favor of pathogen in case of infections. A reduction in peripheral blood mononuclear cells (PBMCs) and intraepithelial lymphocytes (IELs) populations, in particular CD8+ T-cells, in animals with probiotic supplementation has been demonstrated (Szabó et al. 2009; Mafamane et al. 2011). These cells were shown to be less efficient and to delay their response to *Salmonella* infection in an *in vitro* model (Mafamane et al. 2011). Moreover, reduction of inflammatory cytokines expression in immune cells of ileal Peyer's patches and simultaneous unvaried expression of inhibitory molecule for T cells activation (CTLA-4) were observed. These observations proposed a hindrance in activation of T cells (Siepert et al. 2014). Anti-inflammatory effect of *E. faecium* supplementation in healthy piglets resulted in lower serum total IgG and fecal IgA as well. Supplemented animals showed compromised innate immune responses of epithelium (Scharek et al. 2007). In animals challenged with *Salmonella* Typhimurium, higher number of *Salmonella* in tonsils and colons, greater fecal excretion and shedding of *Salmonella* and higher IgA and IgM levels in the blood of animals supplemented with *E. faecium* SF68 indicates the enhanced course of infection (Szabó et al. 2009; Kreuzer et al. 2012; Siepert et al. 2014).

The exact mechanisms of action of *E. faecium* SF68 are still not known and there are several studies proposed anti-inflammatory effects of this strain (Siepert et al. 2014; Kern et al. 2017). Enhanced intestinal barrier function (Klingspor et al. 2013; Kern et al. 2017), production of enterocins and its related antibacterial effects (Foulquie Moreno et al. 2003), production of lactic acid and metabolites such as alkaline phosphates (Ljungh and Wadström 2006; Chen et al. 2010; Martin et al. 2012) are other proposed mechanisms of action for *E. faecium* SF68 in the literature. Nevertheless, dose of probiotic applied in different studies, form of application, total length of experiment, length of exposure to the probiotic strain, host species, studied pathogens in infection models, age of recipient hosts and their immunity status are determinant in the outcomes of these sort of experiments.

2.6 Arginine deiminase

Arginine deiminase (ADI) pathway (also known as arginine dihydrolase pathway) is one of the main arginine catabolism routes of prokaryotes, in particular eubacteria, as well as of some archaea and unicellular eukaryotes (e.g. *Giardia intestinalis* and *Trichomonas vaginalis*). This pathway leads to generation of energy in form of ATP at the cost of arginine (Abdelal 1979; Novák et al. 2016) and was first identified in *E. faecalis* (Slade and Slamp 1952). This pathway will typically be induced under anaerobic conditions (Abdelal 1979; Gamper et al. 1991). However, in species such as *E. faecalis*, where ADI is the single recognized arginine catabolism pathway, arginine can trigger the initiation of catabolic pathway under aerobic

conditions as well (Simon et al. 1982). Moreover, in some bacteria the presence of carbon as energy source suppresses arginine catabolism by a carbon catabolite repression mechanism. In these strains, the presence of arginine in the environment can increase the AD-activity, while the presence of glucose will decrease it (Abdelal 1979; Dong et al. 2004). ADI pathway constitutes by three main enzymes: arginine deiminase (AD), ornithine transcarbamylase (OTC) and carbamate kinase (CK), encoded respectively by *arcA*, *arcB* and *arcC* genes. Additionally, the arginine-ornithine antiporter ArcD is involved in arginine uptake and arginine-ornithine countertransport (Fulde et al. 2014). In most of the microorganisms with arginine deiminase activity, the genes encoding mentioned enzymes (*arcA*, *arcB*, *arcC*) and their related regulatory molecules, such as *arcR* are clustered into one operon. However, the number, arrangement and regulation of genes present in the operon vary among different species (Barcelona-Andres et al. 2002). For instance, *arcA*, *arcB* and *arcC* genes of *arc*-operon are located on the sense strand in the majority of bacterial strains possessing ADI genes but in *E. faecium* GR7 and *E. faecium* Aus004 they are placed on the anti-sense strand (Lam et al. 2012; Kaur and Kaur 2015). The order of genes in *Bacillus licheniformis* and *Clostridium perfringens* is *arcABDCR*, whereas in *Lactobacillus sakei* and *E. faecalis* is *arcABCRD* (Barcelona-Andres et al. 2002). Nevertheless, in biochemical terms, enzymatic reactions are the same in different bacteria (Figure 4). In the first step, arginine deiminase turns arginine into citrulline, and this reaction is irreversible. In the next step, ornithine transcarbamylase phosphorylases citrulline to ornithine and carbamyl phosphate. Finally, carbamate kinase catalyzes formation of ATP from ADP and production of carbamyl, ammonia and carbon dioxide from carbamyl phosphate (Abdelal 1979).

Arginine deiminase, the first enzyme in the ADI pathway, has been shown to have other functions rather than only its direct metabolic role in providing energy and helping bacteria to survive nutritional and oxygen deficit. In some species like *Streptococcus pyogenes* and *Streptococcus suis*, arginine deiminase is proposed as a virulence factor. It can protect bacteria against low pH through the production of ammonia and it can also play a role in adhesion and invasion of bacterial cells to epithelium (Marquis et al. 1987; Champomier Verges et al. 1999; Degnan et al. 2000; Gruening et al. 2006). It has been shown in a study on *Streptococcus gordonii* that this inhabitant of oral cavity can regulate the presence of other bacterial species and their biofilm formation abilities by a cross-feeding mechanism and an elevation in ornithine concentration, produced by ADI pathway (Sakanaka et al. 2015). Arginine deiminase can inhibit LPS-induced nitric oxide synthesis (Park et al. 2003) and its inhibitory effect on proliferative response of human T cells facing antigens has been reported (Degnan et al. 1998).

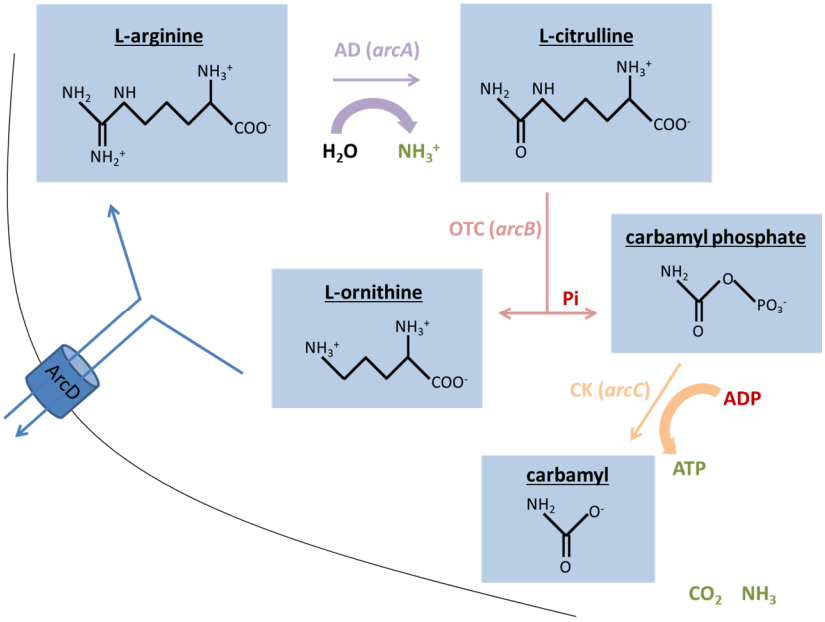

Figure 5: Arginine deiminase pathway.

Arginine deiminase expressed by arcA gene, ornithine transcarbamylase expressed by arcB gene and carbamate kinase expressed by arcC gene are three main enzymes of ADI pathway. Transmembrane ArcD oversees countertransport of arginine and ornithine into and out of the cell, respectively. Input sources of phosphate are shown in red and by-products of catabolic pathway are in green.

It has been demonstrated that AD can prevent proliferation of different tumor cells, especially arginine auxotrophic cancerous cells (Patil et al. 2016). Thus, modified bacterial AD (mainly driven from *Mycoplasma arginini*) with less antigenicity and higher efficiency has been recognized as an anti-cancer drug and is being used in treatment of a number of tumors, e.g. hepatocellular carcinomas and melanomas (Ni et al. 2008). Several studies proposed different mechanisms of action to explain the inhibition of proliferation and growth in cells, especially cancer cells, by arginine deiminase. Arginine depletion, inhibition of de novo protein synthesis, suppression of nitric oxide synthesis, inhibition of NF-κB and MAPK signaling pathways, apoptosis induction and G1-phase cell cycle arrest are postulated as arginine deiminase strategies to interfere with cell growth and metabolism (Shen et al. 2006; Madhuchhanda et al. 2009).

3 Publications

3.1 Publication I

A virulence factor as a therapeutic: the probiotic *Enterococcus faecium* SF68 arginine deiminase inhibits innate immune signaling pathways

Fereshteh Ghazisaeedi (FG), Jochen Meens (JM), Bianca Hansche (BH), Sven Maurischat (SV), Peter Schwerk (PS), Ralph Goethe (RG), Lothar H Wieler (LHW), Marcus Fulde (MF), Karsten Tedin (KT)

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Authors' contributions:

LHW, MF and KT provided funding and supervision of the work in this study. KT conceived the study. FG, JM, BH, SM, PS and KT designed the experimental work and generated data. FG, MF and KT were involved in analysis and interpretation of the data. FG and KT drafted the manuscript. All authors read and approved the final manuscript.

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A virulence factor as a therapeutic: the probiotic *Enterococcus faecium* SF68 arginine deiminase inhibits innate immune signaling pathways

Fereshteh Ghazisaeedi[a], Jochen Meens[b], Bianca Hansche[a,c], Sven Maurischat[a,d], Peter Schwerk[a], Ralph Goethe[b], Lothar H. Wieler[a,e], Marcus Fulde[a], and Karsten Tedin[a]

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ABSTRACT

The probiotic bacterial strain *Enterococcus faecium* SF68 has been shown to alleviate symptoms of intestinal inflammation in human clinical trials and animal feed supplementation studies. To identify factors involved in immunomodulatory effects on host cells, *E. faecium* SF68 and other commensal and clinical *Enterococcus* isolates were screened using intestinal epithelial cell lines harboring reporter fusions for NF-κB and JNK(AP-1) activation to determine the responses of host cell innate immune signaling pathways when challenged with bacterial protein and cell components. Cell-free, whole-cell lysates of *E. faecium* SF68 showed a reversible, inhibitory effect on both NF-κB and JNK(AP-1) signaling pathway activation in intestinal epithelial cells and abrogated the response to bacterial and other Toll-like receptor (TLR) ligands. The inhibitory effect was species-specific, and was not observed for *E. avium, E. gallinarum*, or *E. casseliflavus*. Screening of protein fractions of *E. faecium* SF68 lysates yielded an active fraction containing a prominent protein identified as arginine deiminase (ADI). The *E. faecium* SF68 *arcA* gene encoding arginine deiminase was cloned and introduced into *E. avium* where it conferred the same NF-$_{\kappa}$B inhibitory effects on intestinal epithelial cells as seen for *E. faecium* SF68. Our results indicate that the arginine deiminase of *E. faecium* SF68 is responsible for inhibition of host cell NF-κB and JNK(AP-1) pathway activation, and is likely to be responsible for the anti-inflammatory and immunomodulatory effects observed in prior clinical human and animal trials. The implications for the use of this probiotic strain for preventive and therapeutic purposes are discussed.

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Introduction

The alarming increase in bacterial antibiotic resistance worldwide has lead to a fundamental reassessment of the use of antibiotics in both therapeutic and prophylactic applications.[1] Increased restrictions in the therapeutic application of antibiotics in human and veterinary medicine, and the ban of antibiotics as growth promoters in livestock, have heightened the need for alternatives to antibiotics in both human and animal health and the food industry.[2,3] As a result of these developments, increased interest and research has focused on the use of beneficial microbes as both prophylactics and therapeutics in human and animal health.[4,5] Probiotic microorganisms have been found to confer health benefits to the host through modification and modulation of microbiota, alleviation of dysbiosis, niche shifts in favor of colonization by beneficial microorganisms, exclusion of potential pathogens, stimulation or inhibition of the immune system, and dampening of pro-inflammatory properties of altered, damaged, or infected cells.[6,7] Likewise, both natural and engineered probiotic microbes have been reported to enhance absorption and digestion in the gut, increase antibacterial activities and elimination of pathogens, and have been used for the prevention and treatment of diarrhea and reduction of inflammatory responses in chronic inflammatory diseases, *e.g.* inflammatory bowel disease, Crohn's disease, cancers, and enteric infections.[8,9]

Enterococcus faecium SF68 (NCIMB 10415), is an endogenous, intestinal commensal isolate, and a well-characterized member of the lactic acid bacteria (LAB), which has been authorized for use as a probiotic in pharmaceutical preparations and food

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supplements in humans and animals.[10–13] As with many other LAB, the strain metabolizes carbon sources through fermentation and substrate level phosphorylation reactions rather than oxidative phosphorylation for generation of ATP.[8,9,14] Glycolysis and fermentation of sugars generates acetate and lactic acid, providing a growth advantage for LAB which are generally resistant to low pH.[8,15] Furthermore, production of ATP through catabolism of arginine by the arginine deiminase pathway (ADI) generates ammonia as an end-product contributing to survival in acidified environments, although *E. faecium* is generally not thought to use arginine as a source of ATP.[8]

E. faecium strain SF68 has been shown to mitigate symptoms of human and animal intestinal inflammation. In humans, it is used as a treatment for diarrhea, particularly in cases of antibiotic-associated diarrhea. Positive effects of *E. faecium* SF68 on the reduction and duration of diarrhea have also been demonstrated in animals.[12,13] Despite the generally positive effects reported for *E. faecium* SF68, there are conflicting reports concerning the beneficial effects of this probiotic strain. Results of some *in vivo* trials in different host species supplemented with *E. faecium* SF68 found no significant beneficial effects or even adverse effects, such as a higher bacterial loads and shedding of enteric pathogens including *Salmonella* serovar Typhimurium in animal infection studies.[16–20]

In a number of *in vivo* studies in post-weaning piglets treated with *E. faecium* SF68, we and others observed an apparent immune dysregulation in intestinal tissues, including lower serum total IgG and fecal IgA, and an attenuation of $CD8^+$ intraepithelial lymphocyte populations.[16,17] Post-weaning piglets supplemented with *E. faecium* SF68 followed by a challenge with *Salmonella* Typhimurium aggravated the course of infection, characterized by higher rates of dissemination and colonization of the pathogen in host organs, elevated fecal shedding of *Salmonella*, and reduced or delayed proliferative responses to *Salmonella* antigens by peripheral blood mononuclear cells.[19–21] Furthermore, in an animal feeding trial, we observed significantly reduced expression of immune-associated genes of intestinal and associated lymphoid tissues in post-weaning piglets supplemented with this strain.[21]

As the factors and mechanism(s) involved in the probiotic effects of *E. faecium* SF68 are not very well known, the aim of this study was the identification and characterization of possible immunomodulatory factors involved in the observed effects on immune-associated gene expression in previous animal trials in post-weaning piglets, with the larger goal of explaining prior clinical studies reporting beneficial, probiotic effects on recovery from intestinal inflammation and diarrhea in humans and animals.

Results

E. faecium SF68 inhibits NF-κB activity of intestinal epithelial cells

As our previous *in vivo* studies indicated an inhibitory effect on both pro- and anti-inflammatory immune-associated gene expression by *E. faecium* SF68,[21] we focused on the activation status of nuclear factor-κB (NF-κB), a central host cell transcription factor involved in regulation of genes involved in growth and metabolism, as well as innate immune responses of many cell types, including intestinal epithelial cells.[22,23] The NF-κB signaling pathway consists of the NF-κB proteins NF-κB1(p50), RelA(p65), and c-Rel, among others. In resting cells, NF-κB is retained in an inactive form in the cytosol bound by the inhibitor of κB (I_kB), which must first be phosphorylated followed by ubiquitylation and degradation by the proteosome, resulting in release (activation) of NF-κB which then translocates to the nucleus to activate NF-κB-dependent gene expression.[23,24]

To determine the possible effects of *E. faecium* SF68 on NF-κB activation, we initially performed co-incubation experiments with various concentrations of *E. faecium* SF68 and a porcine, intestinal epithelial cell line (IPEC-J2/K6) harboring an NF-κB transcriptional reporter responsive to NF-κB activation. In preliminary studies, it was not possible to treat cells with viable *E. faecium* SF68 for times longer than 4 h, due to the rapid acidification of the cell culture media as a result of bacterial metabolism. To avoid this complication, we first treated *E. faecium* SF68 with gentamicin at concentrations resulting in bacterial killing, but which left the bacteria intact. As shown in Figure 1(a),

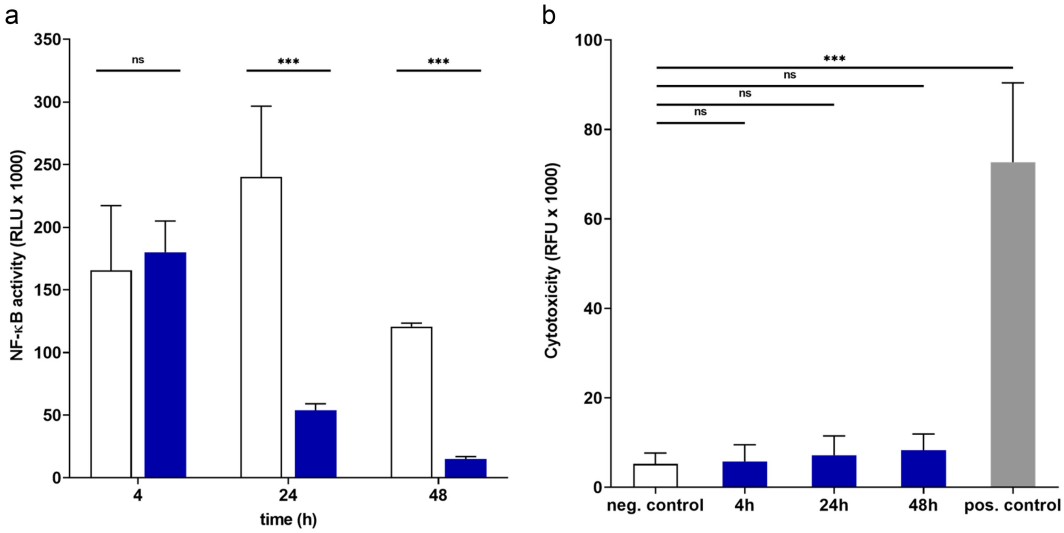

Figure 1. The probiotic *E. faecium* SF68 inhibits NF-κB activity in intestinal epithelial cells. (a) The porcine, intestinal epithelial cell line IPEC-J2 harboring an NF-κB responsive luciferase reporter fusion (IPEC-J2/K6) was incubated for the times indicated in the presence of gentamicin-killed, intact *E. faecium* SF68 at a multiplicity of infection (MOI) of 100:1 bacteria: host cells. The basal levels of NF-κB activity are indicated by open bars, and the treated cells with filled bars. (b) Cytotoxicity assays performed in parallel to the NF-kκ B assays shown in panel A. The results shown are representative of at least three, independent assays.

treatment of host cells with killed but intact bacteria resulted in an initial activation of NF-κB, as expected, but longer incubations showed an inhibition of NF-κB activity to levels below the basal levels of control, untreated cells. Notably, the multiplicity of infection (MOI) of bacterial cells:host cells in these assays (100:1) are at the lower range observed for *E. faecalis* microcolonies adherent to the intestinal wall *in vivo* studies in mice.[25] Furthermore, we observed no acidification of the cell culture medium using killed *E. faecium* SF68, indicating that the NF-κB inhibition was not the result of accumulation of acidic metabolites derived from glycolysis such as acetate or lactate. Likewise, incubation of host cells with the killed but intact bacteria did not show indications of host cell cytoxicity (Figure 1(b)).

The results shown in Figure 1 indicated that some factor(s) present in killed but otherwise intact *E. faecium* SF68 was capable of inhibition of NF-κB activity. Due to the long incubation times in the presence of gentamicin required in order to achieve sufficient bacterial killing (≥4 h), it was possible that protein turnover/degradation during the treatment resulted in low levels of the putative inhibitory factor of NF-κB activity. In order to avoid the complication of bacterial metabolism of the cell culture medium and possible loss of putative protein factors due to degradation, we therefore chose to treat the host cells with cell-free, bacterial lysates of *E. faecium* SF68. In order to standardize the assays, the total protein concentrations of the bacterial lysates were determined, and corrected for the efficiency of lysis to yield CFU equivalents/μg lysate (see Materials and Methods). In all assays, 5 μg of these cell-free, bacterial lysates were used in the cell culture co-incubation assays, corresponding to an approximate MOI of 300:1 bacterial CFU equivalents:host cells, a ratio within the same range of *E. faecalis* microcolonies to enterocytes observed *in vivo*.[25]

As shown in Figure 2(a), treatment of host cells with bacterial lysates of *E. faecium* SF68 showed the same time-dependent reduction in NF-κB activation, to levels below that of untreated, cells within 24 h of incubation. To determine whether this effect was specific for *E. faecium* SF68 or a general feature of enterococcal strains, we also performed the same treatments using cell-free, bacterial lysates of a well-characterized pathogenic strain of *E. faecium*, TX0016. As seen in Figure 2(b), we observed the same time-dependent inhibition of NF-κB activity. In contrast, treatment of host cells with bacterial lysates of other genera including *Staphylococcus aureus* ATCC 29213 or *Escherichia coli* K-12 MG1655 showed an initial increase in NF-κB

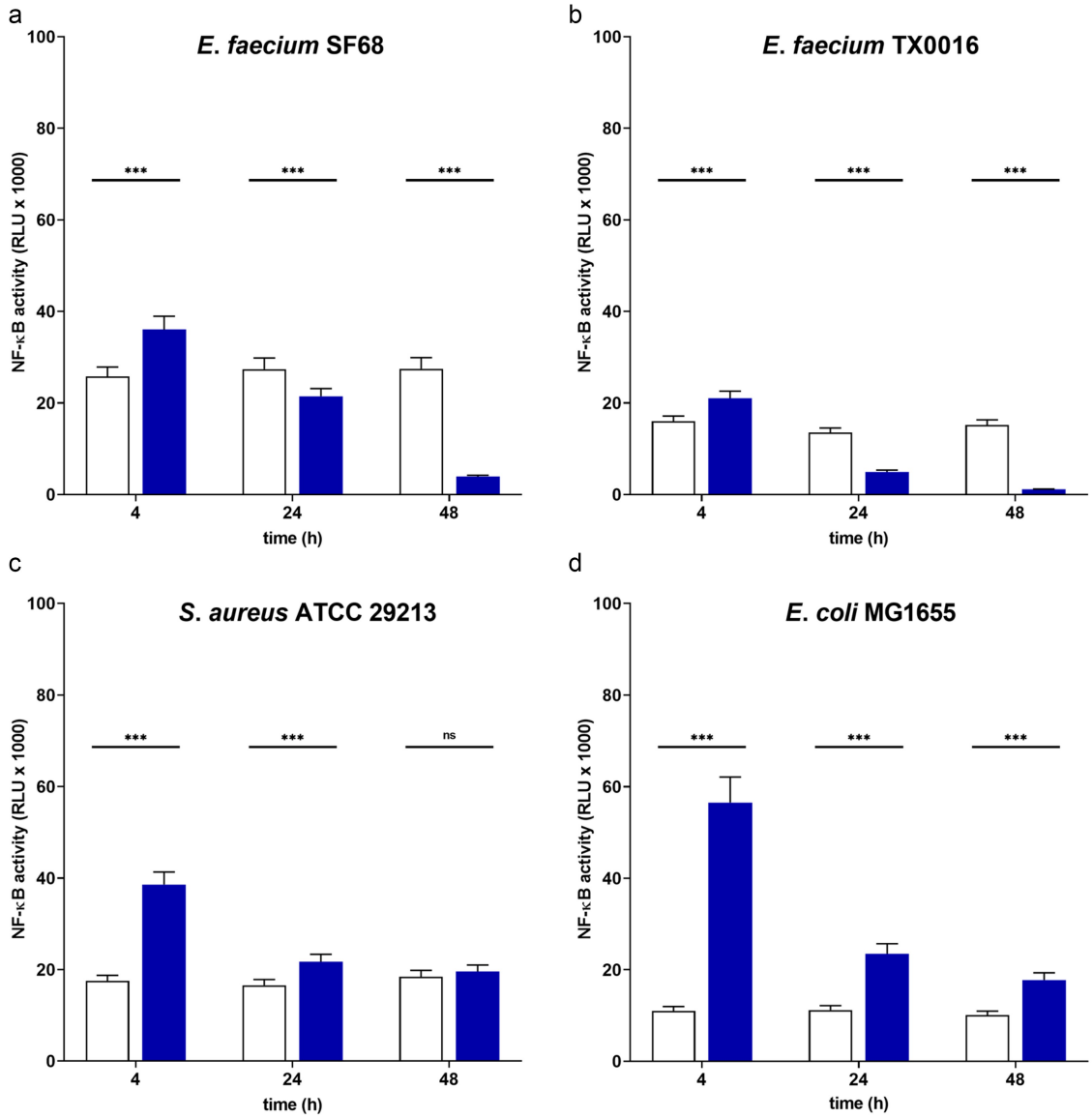

Figure 2. The probiotic *E. faecium* SF68 and pathogenic *E. faecium* TX0016 strains show severe inhibition of NF-κB activity in intestinal epithelial cells. The IPEC-J2/K6 cell line was incubated for the times indicated in the presence of cell-free, whole cell bacterial lysates of (a) *E. faecium* SF68, (b) *E. faecium* TX0016, (c) *S. aureus* ATCC 29213, or (d) *E. coli* K-12 strain MG1655. In all panels, the basal levels of NF-κB activity are indicated by open bars, and the treated cells with filled bars. The results shown are representative of at least three, independent assays.

activation 4 h post-treatment, but which declined to the basal levels of NF-κB activity at later time points (Figure 2 (c) and (d), resp.).

E. faecium SF68 treatment inhibits host cell proliferation but is not cytotoxic

Despite no obvious cytotoxic effects on the host cells such as rounding-up or detachment of monolayers during treatment with the bacterial lysates, a possible explanation for the loss of NF-κB activity could have been cytotoxic effects not immediately visible during the course of the treatments. We therefore examined the treated cell cultures by microscopy and after staining cells with antibodies targeting the cell proliferation marker Ki67.[26,27] In addition to its role in regulation of immune responses, NF-κB also regulates genes involved in epithelial cell homeostasis and proliferation, examples of which can be found at http://www.bu.edu/

nf-kb/gene-resources/target-genes.[28–30] Cultures of untreated cells showed intact monolayers with isolated cells in various stages of proliferation, as judged by the degree of staining with Ki67. In contrast, cells challenged with *E. faecium* SF68 cell lysates clearly showed intact monolayers, but only rare cells showed staining with anti-Ki67 antibodies, indicating the majority of cells had ceased to proliferate (see supplementary Figure S1).

While visual and microscopic inspection of the cell monolayers showed intact monolayers with no obvious morphological changes suggestive of cell death, it remained possible the *E. faecium* SF68 cell lysates induced cytotoxic effects not immediately obvious by visual inspection. We therefore performed a number of cell cytotoxicity and viability assays with cell cultures treated with the cell-free bacterial lysates. Cell viability assays comparing cells treated with the *E. faecium* SF68 lysates and untreated cells, indicated no major effects on cell viability during the first 24 h of incubation (supplementary Figure S2A). An approximately 30% reduction in viability of host cells was observed after 48 h of co-incubation with the *E. faecium* SF68 lysates, but which was attributable to the severely reduced NF-κB basal activity to only around 10% that of untreated, resting cells as seen in Figures 1 and 2. In addition, cells treated with *E. faecium* SF68 lysates showed no increase in lactate dehydrogenase (LDH) release into the cell culture media, indicating no loss of cell membrane integrity, an indicator of cell cytotoxicity (supplementary Figure S2B). Furthermore, co-treatment of cells with the caspase inhibitor Z-VAD-FMK did not improve the NF-κB activation levels (supplementary Figure S2C), indicating that the *E. faecium* SF68 lysates were not involved in activation of apoptosis, as previously reported for other *E. faecium* and *E. faecalis* isolates.[31–34] Finally, it has previously been reported that *E. faecium* and *Streptococcus* spp. isolates produce cytotoxic levels of hydrogen peroxide leading to an oxidative stress-associated death of host cells.[35–37] We therefore also determined the NF-κB activation levels of cells treated with *E. faecium* SF68 lysates in the presence of catalase, which would detoxify any hydrogen peroxide present in the medium. The presence of catalase did not affect the NF-κB inhibition by the *E. faecium* lysates (supplementary Figure S2D), indicating that the inhibitory effects on NF-κB activation was not due to production of hydrogen peroxide by the bacterial lysates.

Enterococcal inhibition of NF-κB activity is species-specific

The inhibitory effects of bacterial lysates on NF-κB activation shown in Figure 2 appeared to be bacterial genera-specific, characteristic only for the *Enterococcus faecium* isolates tested, and not a general response of the cell line to treatments with bacterial cell lysates. To explore this

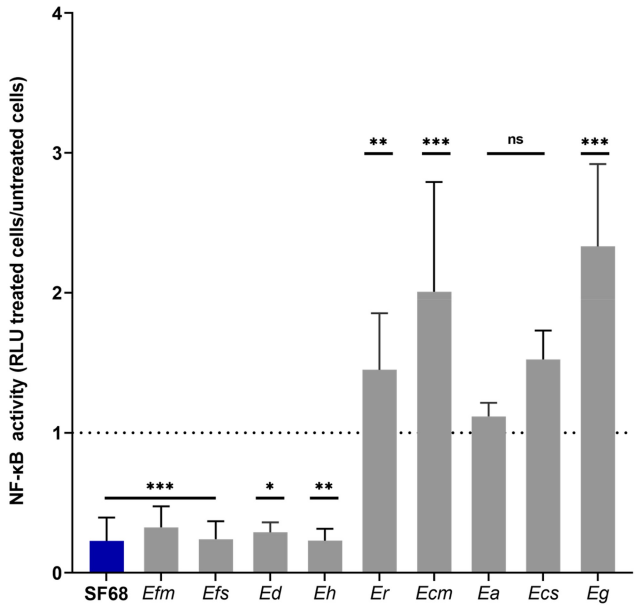

Figure 3. Inhibition of NF-κB activity is *Enterococcus* species-specific. Confluent monolayers of the IPEC-J2/K6 NF-κB reporter cell line was incubated in the presence of 5 µg of cell-free, bacterial lysate of either *E. faecium* SF68 (blue bar) or other representative *Enterococcus* species (gray bars) as indicated below the graph for 24 h followed by determination of NF-κB (luciferase) activity. The dotted line indicates the basal NF-κB activity of untreated cells determined in parallel. SF68, *E. faecium* SF68; Efm, *E. faecium* TX1310; Efs, *E. faecalis* ATCC 29212; Ed, *E. durans* IMT38978; Eh, *E. hirae* ATCC 9790; Er, *E. raffinosus* IMT23827; Ecm, *E. cecorum* IMT19051; Ea, *E. avium* IMT39925; Ecs, *E. casseliflavus* IMT39928; Eg, *E. gallinarum* IMT12257. The results shown are the averages of at least three, independent assays for each *Enterococcus* strain, and are reported as the relative NF-κB activity of treated cells compared to untreated, control cells from the same experiment. See supplementary Figure. S3 for additional isolates of all species and Table S1 for additional strain information.

observation further, we screened additional clinical and commensal isolates of *E. faecium* as well as isolates of other *Enterococcus* species to determine whether the inhibitory effects were a general characteristic of the genus *Enterococcus*. We tested a total of 48 isolates of *E. faecium, E. avium, E. casseliflavus, E. cecorum, E. durans, E. faecalis, E. gallinarum, E. hirae*, and *E. raffinosus*. As shown in Figure 3, cell-free lysates of representative isolates of *E. faecium, E. faecalis, E. durans* and *E. hirae* isolates showed the same inhibitory effects on NF-κB activation of intestinal epithelial cells, whereas isolates of *E. avium, E. casseliflavus, E. cecorum, E. gallinarum*, and *E. raffinosus* showed either unchanged or elevated NF-κB activity. Similar results were found for additional isolates of these *Enterococcus* species (supplementary Figure S3 and Table S2). These results indicated that the inhibition of NF-κB activation was a characteristic which the probiotic *E. faecium* SF68 strain shared with other *Enterococcus* species, including both commensal and pathogenic strains, but which was not a general feature of all *Enterococcus* species.

The inhibitory effect of E. faecium SF68 on NF-κB activation levels is independent of the host cell background

To exclude the possibility that the apparent inhibitory effects of the cell-free, bacterial lysates on NF-κB activation was a characteristic of the IPEC-J2 porcine intestinal epithelial cell line, we also constructed cell lines harboring the same NF-κB luciferase reporter in intestinal epithelial cell lines of both human (Caco-2) and murine (MODE-K) origin, and performed the same assays using cell-free lysates of both *E. faecium* SF68 and *E. avium*. As shown in Figure 4(a), although the degree of inhibition for the *E. faecium* SF68 treatments were slightly different in the different host cell backgrounds, in all three host cell lines there was a clear inhibition of NF-κB activity relative to untreated cells. In contrast, treatments with lysates of *E. avium* showed either unchanged (IPEC-J2), or higher levels of NF-κB activity (Caco-2, MODE-K). Notably, the relative degree of NF-κB activity in all three host cell backgrounds comparing the effects of *E. avium* to *E. faecium* SF68 lysates were within

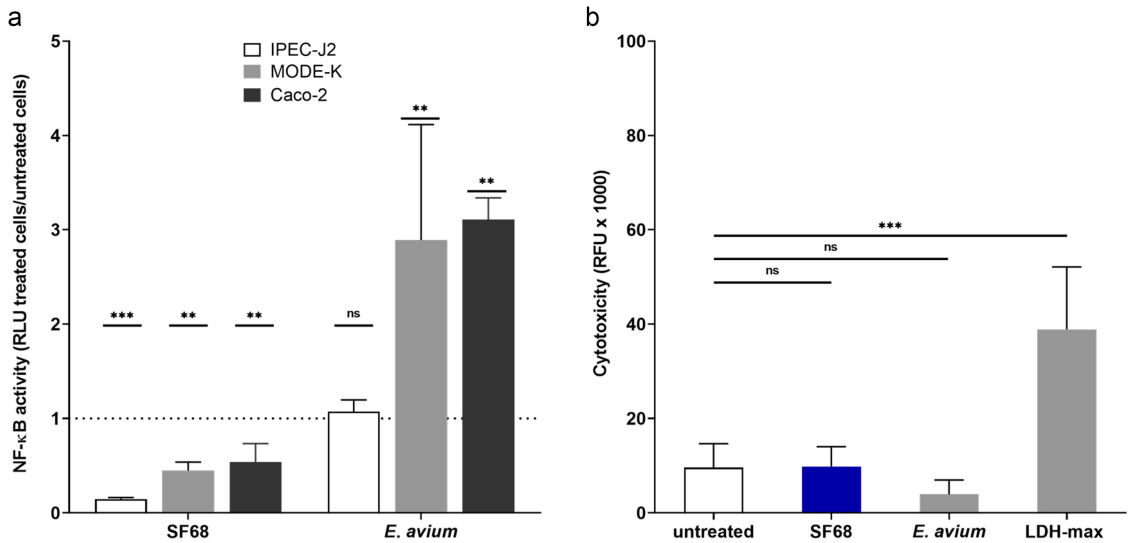

Figure 4. *E. faecium* SF68 inhibition of NF-κB activity is independent of the host species origin of intestinal epithelial cells. (a) Confluent monolayers of NF-κB-luciferase reporter cell lines of porcine IPEC-J2 (open bars), human Caco-2 (gray bars) or murine MODE-K (black bars) intestinal epithelial cells were treated with 5 µg of total protein of cell-free, bacterial lysates of either *E. faecium* SF68 or *E. avium* IMT39925, for 24 h prior to determination of NF-κB activity. The results shown are reported as the averages of the ratios of NF-κB activity of treated/untreated cells from at least three, independent assays. The dotted line indicates the normalized, average basal NF-κB activity determined for untreated cells. (b) Cytotoxicity assays performed in the IPEC-J2 cell line treated with 5 µg of total protein of cell-free, bacterial lysates of either *E. faecium* SF68 (blue bar) or *E. avium* IMT39925 (red bar) for 24 h prior to the assays. Controls included untreated cells (open bar) or cells treated with lysis buffer to determine the maximum LDH release (LDH-max). The results shown are the averages of at least three, independent assays.

the same range, six- to eightfold, indicating the elevated levels of NF-κB activity in the Caco-2 and MODE-K cells lines were likely a characteristic of the cancerous or transformed nature of the two cell lines, but which were similarly inhibited by the *E. faecium* SF68 bacterial lysates. These results supported the conclusion that the inhibitory effects on NF-κB activation were neither host cell background nor NF-κB reporter-dependent. Likewise, consistent with the results shown in Figure 4(a), neither the *E. faecium* SF68 nor the *E. avium* lysate treatments showed signs of increased host cell cytotoxicity (Figure 4(b)).

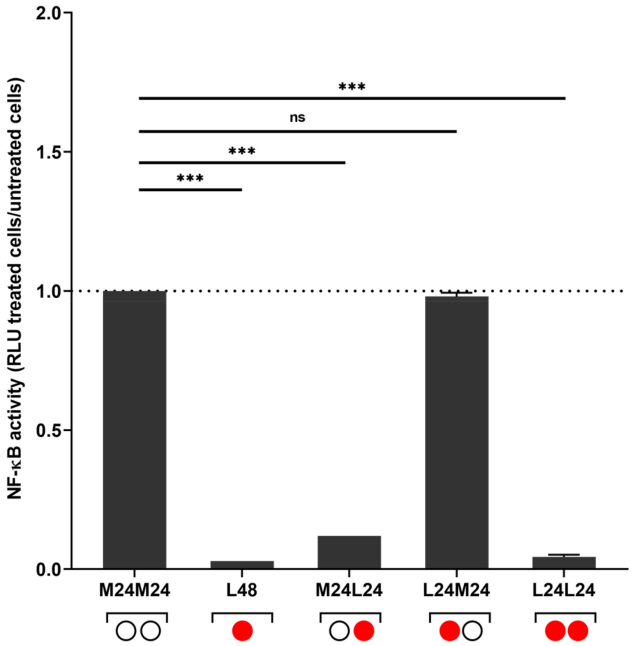

Figure 5. The inhibitory effect of *E. faecium* SF68 on NF-κB activation is reversible. Wells containing confluent monolayers of the IPEC-J2/K6 reporter cell line were treated with 5 µg of total protein of whole-cell, bacterial lysates of *E. faecium* SF68 (red circles) 48 h prior to determination of NF-κB activity (L24 and L48, red circles), or left untreated (M24, open circles). 24 h post-challenge, the cell culture medium was removed and replaced with either cell culture medium alone (L24/M24), or medium containing 5 µg of protein of *E. faecium* SF68 lysate (L24/L24), and incubated an additional 24 h. Controls included wells with no change of medium (L48), untreated cells (M24/M24), or cells left untreated for the first 24 h, followed by addition of 5 µg of lysate protein for the remaining 24 h (M24/L24). At 48 h, the NF-κB activity for all combinations were determined. The results shown are the relative NF-κB activities (ratio RLU treated/untreated cells) compared to untreated cells (M24/M24; dotted line). The results shown are representative of at least two, independent assays.

The inhibitory effect of E. faecium SF68 on NF-κB activation levels is reversible

To determine whether the inhibitory effects of *E. faecium* SF68 lysates on NF-κB activity was reversible, cells were pre-treated with the lysates for 24 h followed by replacement of the cell culture medium with either fresh medium alone or containing bacterial lysates again for an additional 24 h. As shown in (Figure 5), replacement of the cell culture medium without lysate resulted in full recovery of NF-κB activity, whereas replacement and incubation with fresh medium containing *E. faecium* SF68 bacterial lysates continued to suppress NF-κB activity below the basal levels of activity seen in untreated, control cells. The observation that NF-κB activity fully recoverd after replacement of the media without *E. faecium* SF68 lysates further supported the conclusion that the observed NF-κB inhibition was not due to host cell killing effects, at least not for the duration of the experiments (48 h).

Arginine deiminase (ADI) is the immuno-modulatory factor of E. faecium SF68

To determine the nature of the NF-κB inhibitory factor(s), we subjected the cell-free bacterial lysates to either proteinase K treatment or heat-treatment at 100°C for 10 min. prior to performing the NF-κB activation assays. In both cases, we observed a complete loss of inhibitory activity by the lysates, indicating that the immuno-modulatory factor was likely proteinaceous in nature (see supplementary Figure S4).

As both heat inactivation and proteinase K digestions suggested the NF-κB inhibitory factor(s) was likely a protein, total protein of the *E. faecium* SF68 lysates were subsequently fractionated by ammonium sulfate (AS) precipitation, followed by assays of the various protein fractions for NF-κB inhibitory activities. As shown in Figure 6(a), only proteins present in the 100% saturated AS fraction of *E. faecium* SF68 showed the same inhibition of NF-κB activation as the whole-cell lysates. Ammonium sulfate fractionation of lysates of *E. avium* performed in the same manner showed no inhibitory effects for any of the AS fractions, consistent with the absence of inhibitory effects on NF-κB activation seen with whole cell-free

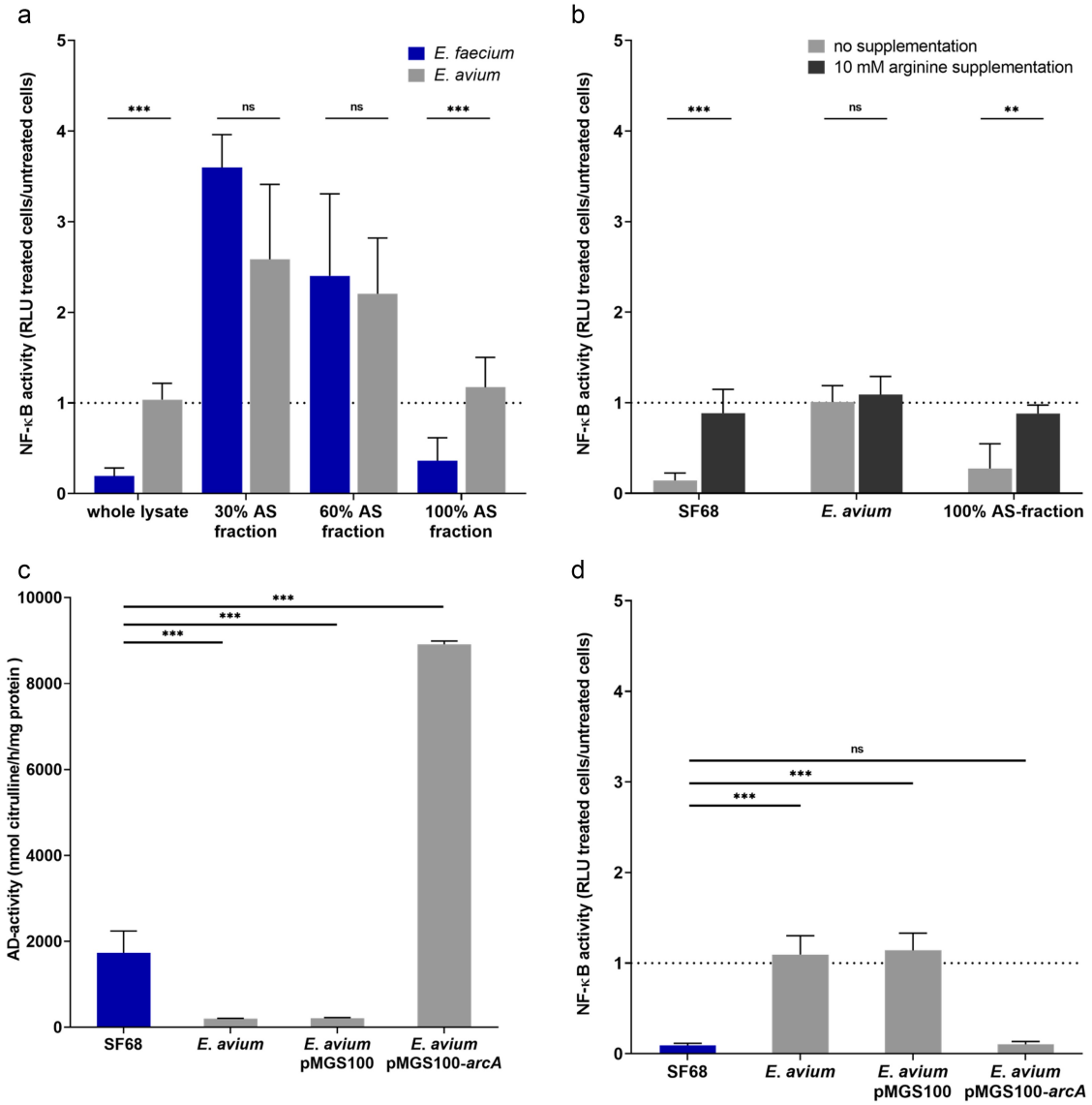

Figure 6. Protein fractions of *E. faecium* SF68 lysates containing arginine deiminase (ADI) inhibit NF-κB activity. (a) Total protein of cell-free, bacterial lysates or ammonium sulfate protein fractions lysates of *E. faecium* SF68 (blue bars) or *E. avium* (gray bars) were screened for effects on NF-κB activity in the IPEC-J2/K6 cell line. A total of 5 µg of total protein from either whole lysates, or the ammonium sulfate fractions as indicated in the figure, were added to confluent monolayers and incubated for 24 h prior to determination of NF-κB activity. The values shown are the relative ratios of treated cells normalized to the values for untreated, control cells (dotted line). The results shown are the averages of at least three, independent assays, and at least two, independent ammonium sulfate preparations. (b) NF-κB activity was determined for cells treated for 24 h with either cell-free, bacterial lysates of *E. faecium* SF68, *E. avium* IMT39925, or the 100% AS fractions of *E. faecium* SF68 lysates in the absence (gray bars) or presence of 10 mM L-arginine in the cell culture medium (black bars). The dotted line indicates the normalized NF-κB activity of untreated, control cells. (c) Arginine deiminase (ADI) activity determined for equivalent total protein (50 µg) of bacterial lysates of *E. faecium* SF68, *E. avium* IMT39925 (UW11197), *E. avium* harboring the vector plasmid pMGS100 (vector), or *E. avium* harboring the cloned *E. faecium* SF68 *arcA* gene in plasmid pMGS100-$arcA_{SF68}^{+}$ ($arcA^{+}$). Assays were performed in reaction buffer containing 10 mM L-arginine for 2 h. (d) Total protein samples (5 µg) of bacterial lysates of *E. faecium* SF68, *E. avium* IMT39925, and *E. avium* harboring either the vector *plasmid* pMGS100 (vector) or the cloned *E. faecium* SF68 *arcA* gene in plasmid pMGS100-$arcA_{SF68}^{+}$ ($arcA^{+}$) were used to treat IPEC-J2/K6 cells for 24 h prior to determination of NF-κB activities. Shown are the relative ratios of NF-κB activity of treated cells normalized to the values for untreated, control cells (dotted line). The results shown in all panels are the averages of at least three, independent assays.

lysates of *E. avium* (Figures 3 and 4). Furthermore, the observations with the *E. avium* AS fractions indicated that the addition of ammonium sulfate was not responsible for the reduced NF-κB activity seen for the *E. faecium* SF68 AS fractions (Figure 6(a)). Interestingly, both the 30% and 60% *E. faecium* SF68

AS fractions had a stimulatory effect on NF-κB activity in these assays, but this effect was not significantly different from fractions of the *E. avium* whole-cell lysates.

In an effort to identify proteins specific for *E. faecium* SF68, we compared the protein banding patterns of the 100% AS fractions of *E. faecium* SF68 and *E. avium* after separation on denaturing gels. A total of 12 prominent proteins appeared to be present in the 100% AS fraction of *E. faecium* SF68 but which were not present in *E. avium* 100% AS fractions (data not shown). Samples of these proteins were excised from SDS-PAGE gels and subjected to protein identification by MALDI-TOF. Interestingly, a number of the proteins identified in the active fraction of *E. faecium* SF68 lysates which were not present in *E. avium* are involved in arginine metabolism, including arginine deiminase and ornithine carbamoyltransferase. These results were of particular interest as arginine deiminase, and arginine catabolism in general, has previously been shown to play a role in the inhibition of human peripheral blood mononuclear cell proliferation and in the virulence of *Streptococcus pyogenes*,[38–40] and arginine deiminase of *E. faecium* GR7 has been found to inhibit the proliferation of various cancer-derived cell lines.[41] Indeed, arginine deprivation of cancer cells by recombinant and modified arginine deiminase of *Mycoplasma arginini* has long been considered as a potential means of inhibiting cancer cell growth and proliferation.[42,43]

Arginine deiminase is responsible for NF-kB inhibition by E. faecium SF68 lysates

To determine whether arginine catabolism and subsequent arginine depletion of the cell culture medium was responsible for the inhibitory effects on NF-κB activation levels, we performed the NF-κB assays with the *E. faecium* SF68 lysates, the 100% AS-fractions, or *E. avium* lysates in the presence or absence of excess arginine in the cell culture medium. As seen in Figure 6(b), excess arginine abolished the inhibition of NF-κB activation of cells treated with both the *E. faecium* SF68 lysates and the 100% AS fractions. Control treatments using *E. avium* lysates showed no significant changes in NF-κB activity, indicating that arginine supplementation alone did not have an intrinsic stimulatory effect on NF-κB activity that might have obscured the inhibition due to the *E. faecium* SF68 lysate treatment. These results suggested the active factor present in the 100% AS fractions was indeed the arginine deiminase.

Repeated attempts to inactivate the *arcA* gene encoding arginine deiminase in *E. faecium* SF68 were unsuccessful, owing in part to an endogenous erythromycin resistance that prevented the use of a number of common streptococcal suicide vectors. In order to verify the role of the *E. faecium* SF68 arginine deiminase in NF-κB inhibition, the *arcA* gene, encoding arginine deiminase (ADI) of *E. faecium* SF68, was therefore cloned into the *E. coli-Enterococcus* shuttle vector pMGS100, and introduced into *E. avium* by electroporation. As shown in Figure 6(c, e). *avium* harboring the pMGS100 plasmid vector showed no ADI activity, whereas *E. avium* harboring the cloned *arcA* gene of *E. faecium* SF68 exhibited high, constitutive ADI activity. Furthermore, as shown in Figure 6(d), cell-free lysates of the transformed *E. avium* isolate were also found to inhibit NF-κB activation in IPEC-J2/K6 cells to the same degree as *E. faecium* SF68 lysates. In contrast, lysates derived from *E. avium* harboring the empty pMGS100 vector showed no inhibitory effects on NF-κB activation.

These results indicated that the inhibition of NF-κB activity by *E. faecium* SF68 and other *Enterococcus* strains and isolates was likely due to ADI expression. To determine whether there was a correlation between arginine deiminase activity and NF-κB inhibition, we screened all enterococcal isolates that showed inhibition of NF-κB activity, both commensal and clinical isolates, for ADI activity. All isolates that showed NF-κB inhibition also tested positive for ADI activity. In contrast, enterococcal isolates with no inhibitory effects on NF-κB activity were also found to be negative for ADI activity, except for isolates of *E. gallinarum* and *E. casseliflavus*, which were positive for ADI activity (supplementary Table S2). Notably, *E. gallinarum* and *E. casseliflavus* are exceptional among the Enterococci, as both are flagellated and motile.[44] As *E. gallinarum* flagellin is a strong stimulator of NF-κB activation,[45] the apparent lack of NF-κ

B inhibition was likely due to the presence of flagella in these isolates, which could lead to rapid, high levels of activation, masking the inhibition observed in our standard 24 h assays. These results therefore indicated that *E. faecium* SF68 arginine deiminase was most likely responsible for the observed NF-κB inhibition in both the cell-free bacterial lysates, and the 100% AS fractions of *E. faecium* SF68 containing ADI. In other experiments, we also

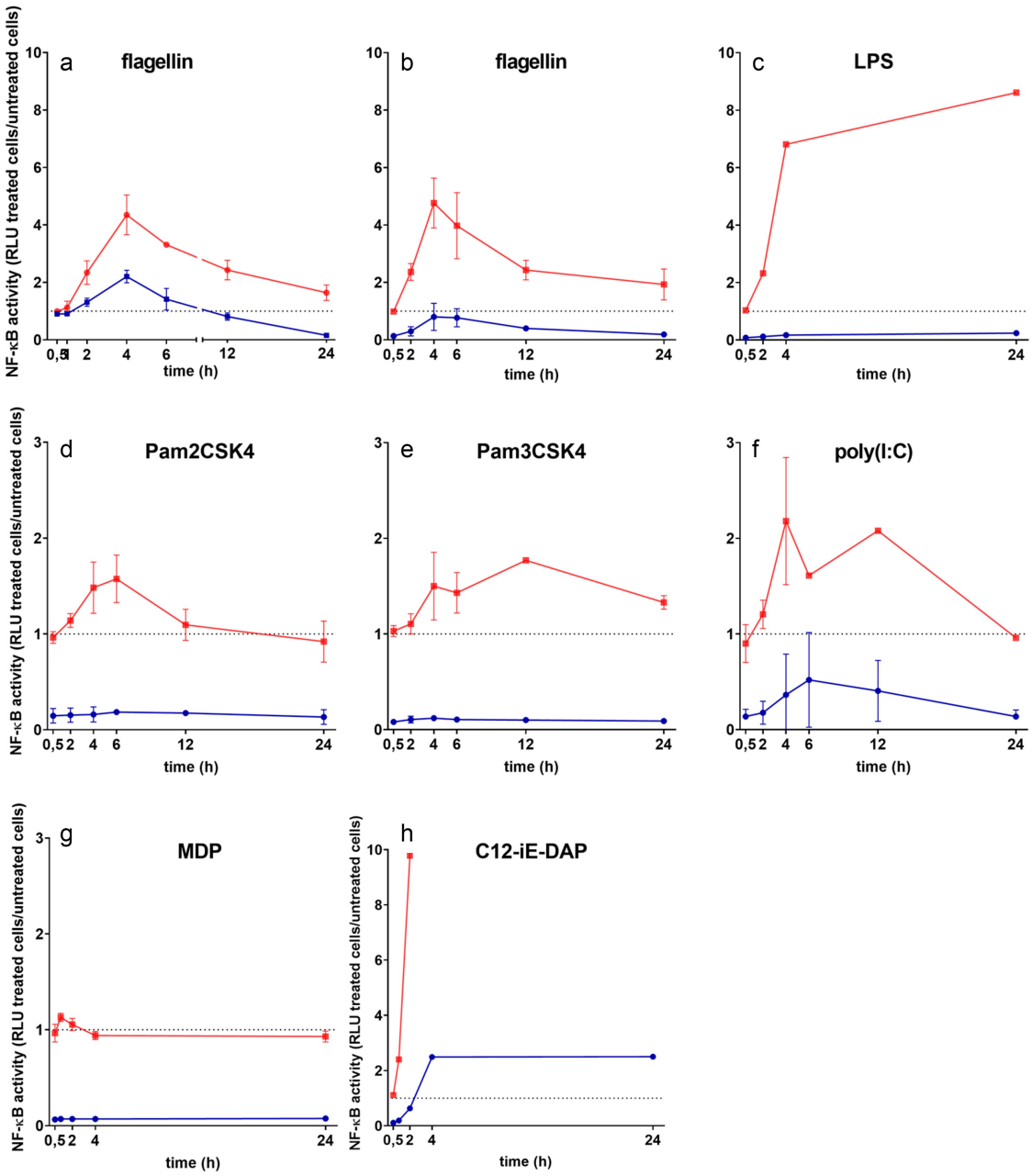

Figure 7. *E. faecium* SF68 inhibits host cell responses to TLR and NOD protein ligands. (a) Confluent monolayers of the IPEC-J2/K6 reporter cell line were treated with either 5 µg of total protein from bacterial lysates of *E. faecium* SF68 (blue line) or purified *Salmonella* flagellin (1.5 µg), and at the times indicated, replicate wells were sampled for NF-κB activity starting at 30 min. post-challenge. In panels B-H, cells were pre-treated for 24 h with 5 µg of *E. faecium* SF68 cell-free lysate (blue line) or left untreated (red line) prior to addition of the indicated TLR or NOD agonists, and NF-κB activity was determined for replicate wells at the times indicated in the figures. (b) TLR5 ligand, flagellin (1.5 µg). (c) TLR4 ligand, purified *E. coli* LPS (50 µg). (d) TLR2 ligand, Pam2CSK4 (1 µg). (e) TLR1/TLR2 ligand, Pam3CSK4 (1 µg). (f) 10 µg of the TLR3 ligand, poly(I:C). (g) 10 µg of the NOD2 ligand, muramyl dipeptide, (MDP). (h) 50 µg of the NOD1 ligand, acylated iE-DAP (C12-iE-DAP). In all panels, the dotted line indicates the normalized NF-κB activity of untreated, control cells. The results shown in all panels are the averages of at least two, independent assays.

verified that gentamicin-killed, intact *E. faecium* SF68 which also showed NF-κB inhibition (Figure 1(a)) also retained ADI activity (data not shown).

E. faecium SF68 inhibits NF-$_k$B activation by TLR and NOD ligands

A challenge of host cells with total bacterial lysates would be expected to result in activation of intestinal epithelial innate immune responses through the NF-κB signaling pathway *via* Toll-like receptor (TLR) or other receptor pathways.[45–48] While the earliest time point (4 h post-treatment) in Figure 2 (a) suggested the host cells were capable of responding with increased NF-κB activation for a short time post-challenge with the *E. faecium* lysates, we were interested to know how treatment of cells with *E. faecium* SF68 would compare to NF-κB activation in response to other, non-*E. faecium* related ligands. As seen in Figure 7(a), when challenged with *E. faecium* SF68 lysates, cells responded with a peak in NF-κB activation within the first 4 h post-challenge. However, rather than returning to either the basal level or a new, elevated activation level, NF-κB activity continued to decline over the entire 24 h period of the assay to around only 10% of the basal, pre-challenge level of activation seen in untreated cells, consistent with the results shown in Figure 2(a). Whereas a decline in NF-κB activity following an initial stimulation would be expected due to the NF-κB-dependent expression of its own inhibitors,[49,50] the reduction in activity to levels below the basal, maintenance activity was unlikely to be a normal response. NF-κB is known to regulate the expression of a large number of genes involved in host cell growth, metabolism, and proliferation in addition to its role as a central regulator of immune and inflammatory responses.[23,29] The regulation of metabolic, house-keeping gene expression explains the low, but non-zero levels of activation seen in even resting cells.[51]

To determine whether these results were a result of the treatment or a characteristic of the IPEC-J2/K6 reporter cell line, we performed the same experiment using the TLR5 ligand, flagellin. As seen in Figure 7(a), there was a rapid activation of NF-κB at early times post-challenge, with a peak around 4 h post-challenge, similar to the kinetics observed for the *E. faecium* SF68 cell lysates. However, the kinetics of the decline phase of NF-κB activation were very different, remaining at all times above the pre-challenge basal level, with an apparent slow decline toward the basal level of NF-κB activity present in untreated, resting cells. Similar results for flagellin were observed for incubation times of up to 48 h (data not shown), suggesting that the lower levels of NF-κB activity seen in *E. faecium* SF68 treated cells was not simply a matter of the initial magnitude of activation. The final levels of NF-κB activity were below that of both untreated cells and at late times post-challenge of TLR ligand-activated cells, both of which showed a plateau representing NF-κB activity consistent with levels required for growth, proliferation, and host cell maintenance. This is also supported by the NF-κB activation kinetics for the TLR2 ligand, Pam2CSK4, which showed a comparable magnitude of NF-κB activation 4 h to 6 h post-challenge as the *E. faecium* SF68 treatment (compare Figure 7 (a) and (d)), yet the TLR2-mediated activation declined to normal, basal levels by 24 h post-challenge, whereas the *E. faecium* SF68 treated cells showed only 10% the basal NF-κB activity at the same time post-challenge. These results indicated that the severe inhibition of NF-κB activity was a phenomenon beyond the normal, self-regulatory, down-regulation of NF-κB on its own activity. Furthermore, the results indicated this effect was a characteristic of the *E. faecium* SF68 lysates, and not a peculiarity of either the host cell line or the NF-κB reporter fusion.

To determine whether the inhibitory effects of *E. faecium* SF68 on NF-κB activation would interfere with host cell responses to other immunostimulatory ligands, we also performed challenge studies with host cells pre-treated with lysates followed 24 h later by a challenge with different TLR- and NOD protein-ligands. As shown in Figure 7(b), pre-treatment of the host cells with *E. faecium* SF68 severely reduced the ability of host cells to respond with NF-κB activation to flagellin, the TLR5 ligand. While there appeared to be a low-level capacity for NF-κB activation in such pre-treated cells, the peak of NF-κB activation did not rise above the basal level of activity of untreated cells. Similar results were observed for the TLR4, TLR2/TLR6, and TLR1/TLR2 ligands LPS, Pam2CSK4, and Pam3CSK4, respectively (Figure 7(c–e)). Interestingly, poly(I:C), a TLR3 ligand, also showed a severely reduced NF-κB activation response in cells pre-

treated with *E. faecium* SF68 (figure 7(f)). Cells pre-treated with *E. faecium* SF68 cell-free lysates showed no response to the NOD2 ligand, muramyldipeptide (MurNAc-L-Ala-D-isoGln, MDP); however, the IPEC-J2/K6 cell line also showed only a minimal response to MDP alone (Figure 7(g)). In contrast, the IPEC-J2/K6 cell line responded strongly to a challenge with the NOD1 agonist, acylated γ-D-glutamyl-*meso*-DAP (C12-iE-DAP), and cells pre-treated wth *E. faecium* SF68 remained partially capable of NF-κB activation in response to C12-iE-DAP, although far below the levels of NF-κB activation seen for untreated cells (Figure 7(h)).

E. faecium SF68 treatment interferes with phosphorylation of NF-κB-p65 at serine 536

The preceding results indicated that host cells challenged with *E. faecium* SF68 lysates were severely compromised in their ability to respond with activation of NF-κB to immunostimulatory signals. As noted above, the activation of NF-κB in the cytosol requires phosphorylation of the inhibitor, IκBα, to release NF-κB for translocation into the cell nucleus. However, phosphorylation is also known to be important for the regulation and selectivity of gene expression by NF-κB itself, and the RelA(p65) subunit is one of the most studied NF-κB phosphorylation targets.[28] We were therefore interested to know the phosphorylation status of NF-κB, in particular phosphorylation at serine residue 536 (S536), located in the transactivation domain of RelA(p65), a modification known to play a role in both the turnover and activity of RelA (p65) as well as providing an alternative means of activation independent of IκBα.[28,52–54]

A Western blot analysis of cells treated with *E. faecium* SF68 lysates (Figure 8(a)) showed similar levels of total NF-κB(p65) compared to untreated, control cells, but reduced levels of phospho-NF-κB (p65) (Figure 8(b)). In contrast, host cells treated with *E. avium* lysates contained similar total NF-κB (p65) and phospho-NF-κB(p65) levels compared to untreated cells, as expected. Immunofluorescence microscopy images of IPEC-J2 cells stained for total NF-κB(p65) indicated qualitatively higher levels of NF-κB present in the cytoplasm of cells treated with *E. faecium* SF68 lysates compared to untreated cells and cells treated with flagellin for 4 h (Figure 8(c)). Furthermore, NF-κB(p65) of cells treated with *E. faecium* SF68 lysates appeared to accumulate in the cytoplasm as aggregates, obscuring the cell nucleus. In contrast, in flagellin-treated cells, there was a clear, nuclear accumulation of NF-κB visible as puncta within the nucleus in treated cells compared to untreated cells.

E. faecium SF68 inhibits activation of the JNK (AP-1) pathway and inflammatory gene expression

In addition to NF-κB, the c-Jun-N-terminal kinase (JNK) signaling pathway is also involved in host cell responses to a large variety of signals, including TLR ligands and bacterial pathogens.[55] The JNK pathway itself is activated by phosphorylation from any of a number of different MAP kinases, including TAK1, an MAP kinase kinase (MAP3K) also involved in the signaling pathway leading to NF-κB activation. However, the downstream activation of gene expression following JNK activation is mediated by the transcription factor activator protein-1 family of proteins, AP-1.[55] The observation that the NF-κB response to the TLR3 agonist, poly(I:C), was also inhibited (figure 7(f)) was not necessarily unexpected, as TLR3 signaling activates the JNK(AP-1) pathway in addition to NF-κB, therefore NF-κB activation may not have been expected. On the other hand, LPS is generally known to lead to NF-κB activation through TLR4 signaling, but can also activate the JNK(AP-1) pathway.[46] It was therefore possible that the severely reduced NF-kB activation in response to the TLR4 and TLR3 agonists LPS and poly(I:C) seen in Figure 7 (c) and (d), respectively, may have nevertheless resulted in activation of the JNK(AP-1) pathway.

To determine whether additional host cell signaling pathways might be affected by *E. faecium* SF68, IPEC-J2 cells harboring a JNK(AP-1) luciferase reporter fusion (IPEC-J2/D6) were treated with either *E. faecium* SF68 lysates or the 100% AS-fraction of the *E. faecium* lysates in the same manner as in the NF-κB assays. As shown in Figure 9(a), host cells treated for 24 h showed a significant reduction in JNK activation levels with the *E. faecium* SF68 cell-free lysates and clear reductions with the 100% AS-fractions, similar to the inhibition observed for NF-κB activity. In contrast, no inhibition was observed for whole-cell lysates of the *E. avium*

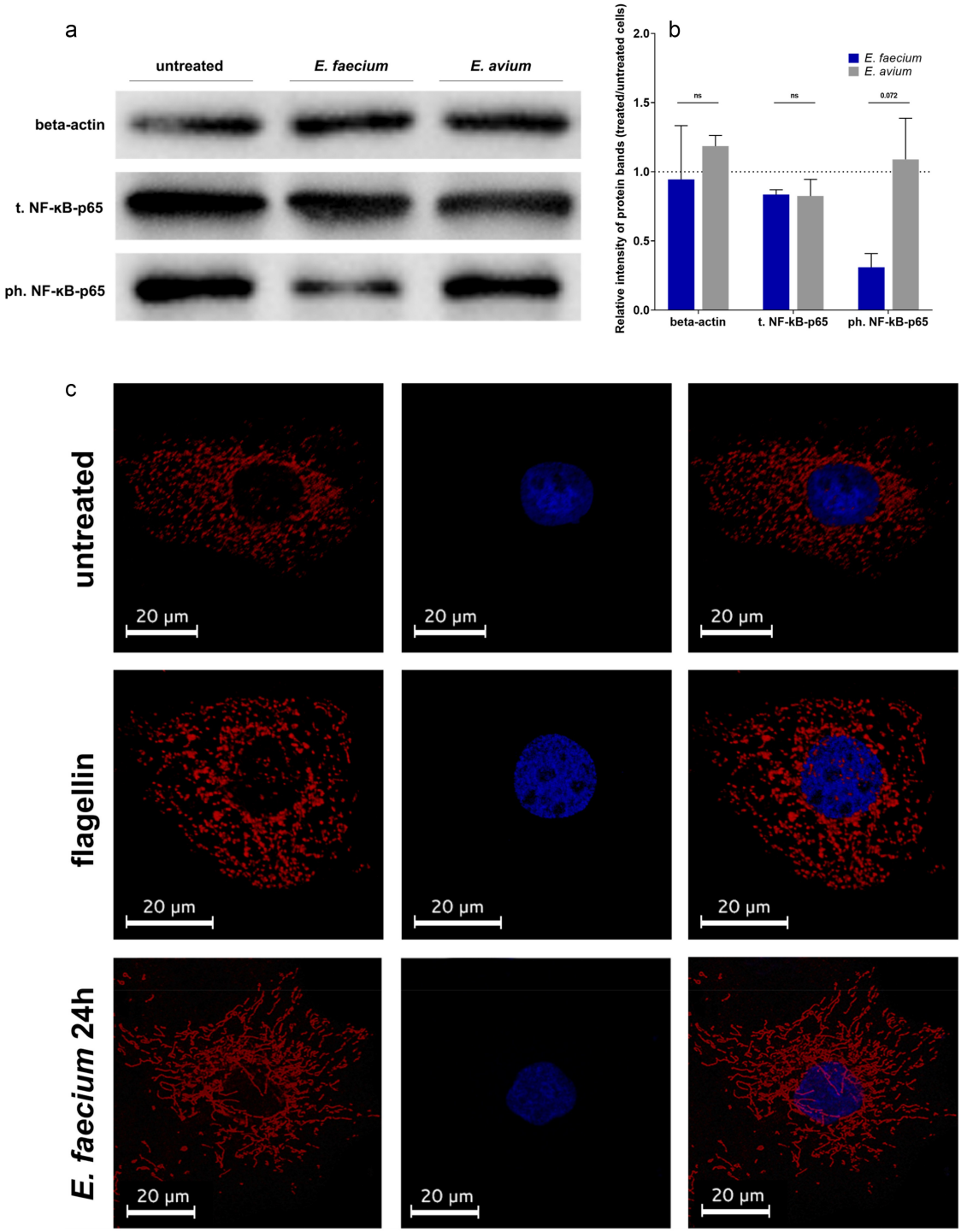

Figure 8. *E. faecium* SF68 inhibits NF-κB phosphorylation at Serine 536. (a) Western blot analyses of total NF-κB(p65) (t. NF-κB-p65) and its Ser536 phosphorylated form (ph.-NF-κB-p65) in IPEC-J2 cells either untreated or treated with 5 µg of total protein of cell-free lysates of either *E. faecium* SF68 or *E. avium* IMT39925 for 24 h as indicated above the blots. The results shown are representative of two, independent determinations. (b) β-actin (upper row in A, beta-actin) was used as a normalization housekeeping protein for determination of the relative levels of NF-κB(p65) and phospho-NF-κB(p65) levels in cells treated with 5 µg of total protein of cells pre-treated with either *E. faecium* SF68 (blue bars) or *E. avium* IMT39925 (gray bars). The dotted line indicates the normalized expression level of the same genes in untreated, control cells. (c) Immunofluorescence micrographs of IPEC-J2 cells after 24 h of incubation either untreated (upper row), in the presence of flagellin (1.5 µg, middle row), or 5 µg of total lysate protein of *E. faecium* SF68. After 24 h incubation, cells were fixed, and stained for total NF-κB(p65) with primary anti-NF-κB(p65) antibody and secondary Cy5-labeled antibody (red fluorescence) and DAPI for cell nuclei (blue fluorescence). The focus depth of the micrographs were chosen to allow visualization of the cell nucleus. The results shown are representative immunofluorescence micrographs from at least two, independent experiments.

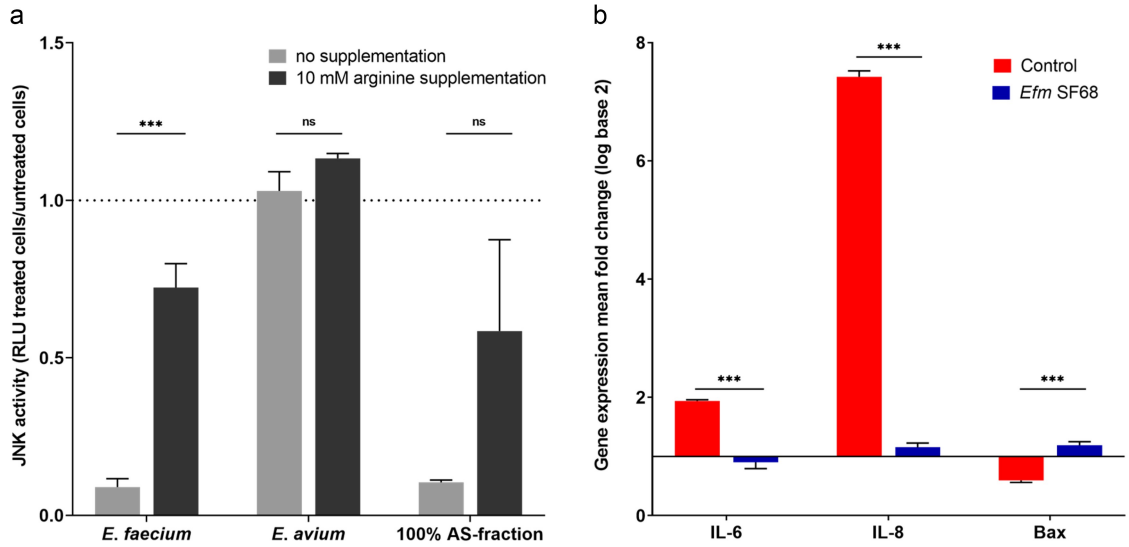

Figure 9. *E. faecium* SF68 inhibits the host cell JNK(AP-1) signaling pathway and gene expression responses to flagellin. (a) Confluent monolayers of the IPEC-J2/D6 cell line harboring a JNK(AP-1) luciferase reporter were treated with 5 µg of protein of either *E. faecium* SF68 or *E. avium* IMT39925 bacterial lysates, or 5 µg of total protein of the 100% AS fraction of *E. faecium* SF68 in the presence (filled bars) or absence (gray bars) of 10 mM arginine. After treatment for 24 h, the JNK(AP-1) activity was determined relative to untreated, control cells (dotted line) determined in parallel and for the same incubation times. (b) Gene expression levels for the pro-inflammatory cytokine IL-6, chemokine IL-8 (CXCL8), and apoptosis regulatory protein Bax genes in IPEC-J2 cells treated with (blue bars) or without (red bars) pre-treatment of *E. faecium* SF68 lysate for 24 h followed by a challenge with flagellin for 4 h. Differential expression was determined using the $2^{-\Delta\Delta Ct}$ method. The data shown are the averages of three, independent assays and are expressed as the mean fold-change relative to untreated cells.

strain. Supplementation of the cell cultures with excess arginine also restored the JNK activity in treated cells in the same manner as for NF-κB, indicating that inhibition of the JNK(AP-1) signaling pathway was also due to the ADI activity of the *E. faecium* SF68 bacterial lysates and 100% AS fractions.

To confirm that the inhibition of NF-κB activity of cells treated with *E. faecium* SF68 lysates would also interfere with downstream responses of immune-associated gene expression, we also determined the effects of pre-treatment with *E. faecium* SF68 lysates on NF-κB-dependent genes. As shown in Figure 9(b), we observed a significantly attenuated upregulation in gene expression levels in response to flagellin for the pro-inflammatory cytokine IL-6 and the neutrophil chemoattractant chemokine IL-8 genes in IPEC-J2 cells pre-treated with *E. faecium* SF68 compared to untreated cells. Notably, little or no change in gene expression was observed for the pro-apoptotic Bcl-2 homologue Bax gene, consistent with the observations shown in supplementary Figure S2C, indicating no activation of apoptosis pathways in cells treated with *E. faecium* SF68 lysates.

Discussion

E. faecium SF68 is a probiotic, bacterial strain primarily used in therapeutic applications for acute diarrhea and enteritis in humans and animals.[10–13] As noted in the introduction, we had previously observed an apparent general reduction in immune-associated gene expression in intestinal and associated lymphoid tissues in *in vivo* feeding trials with *E. faecium* SF68 in otherwise healthy, weaning piglets as well as infection challenge studies.[21] These findings led to the suggestion that *E. faecium* SF68 has a direct anti-inflammatory or immune-suppression effect on local innate immune responses of intestinal epithelial and lymphoid tissues. Here, we show that the prior observations of immuno-modulatory effects of *E. faecium* SF68 are most likely due to expression of arginine deiminase (ADI), resulting in arginine deprivation of host cells with subsequent loss of NF-κB and JNK(AP-1) signaling pathway functions.

Arginine metabolism has become an increasingly important focus of research in the fields of cancer therapy, innate immune responses, and bacterial pathogenesis. A wide variety of human cancers are

known to be auxotrophic for arginine, which explained the strong inhibition of tumor growth by the arginine deiminase (ADI) of *Mycoplasma arginini* and led to clinical trials using ADI as an anti-cancer therapy.[42,56–59] Arginine also plays a key role as a signal regulating the activity of mTORC1 (mechanistic target of rapamycin complex 1), a central regulator of host cell metabolism and autophagy.[60–65] The arginine pools are also involved in host cell innate immune responses where they can serve as a source of antimicrobial reactive nitrogen species. On the other hand, ADI has also been shown to play a large role in the pathogenesis of various bacterial species, supporting their survival within the host.[38–40,66–68] Interestingly, mTORC1 is also a target for a number of bacterial pathogens as a means of immune evasion.[69–71]

In this study, we show that the arginine deiminase (ADI) of the probiotic strain *E. faecium* SF68 inhibits the activation of the NF-κB pathway in intestinal epithelial cells of human, porcine, and murine origins, as well as the JNK(AP-1) pathway in the IPEC-J2 cell line background. The inhibition results in severe reductions in innate immune signaling responses to a variety of TLR and NOD agonists, as shown in Figure 7. Previous studies have also reported effects on NF-κB, MAPK, and JNK(AP-1) signaling pathways by *Enterococcus* spp. or their products. However, the reported effects have ranged from activation to attenuation, required either contact with host cells or involved secreted bacterial products.[72–76] Here, we show that all *E. faecium* and *E. faecalis* isolates, regardless of the source, show the same NF-κB inhibition, correlating with the presence of ADI, a part of the core genome of these and other *Enterococcus* spp. (Figures 2 and 3, and supplementary Figure S3). We suggest that a possible explanation for the often contradictory results of the *in vitro* studies is likely due to the duration of the treatments. As shown in Figures 1, 2 and 7, there is clearly an initial host cell response to *E. faecium* SF68 treatments 4 h post-treatment, as expected, but the basal activity levels of both NF-κB and JNK(AP-1) in resting cells is inhibited up to 90% by 24 h post-treatment. While a given isolate may express additional, variable cell-wall-bound or secreted products affecting host cell signaling pathways, our results indicate that all *Enterococcus* spp. capable of ADI expression will show long-term inhibition of NF-κB and JNK(AP-1) activation.

This suggestion has implications for observations from prior *in vivo* studies. As noted in the introduction, clinical trials in humans and animal studies have shown significant, anti-inflammatory effects of *E. faecium* SF68 treatments *in vivo*.[12,13] However, in two, independent *Salmonella* challenge studies with weaned piglets, higher pathogen loads were found at systemic sites in piglets treated with *E. faecium* SF68 compared to the control groups.[19,20] In addition, we found significantly delayed immune cell proliferative responses to both mitogen and UV-killed *Salmonella* antigens in the *E. faecium* SF68-treated animal groups,[21] an observation consistent with an earlier report with purified *Streptococcus pyogenes* ADI and human PBMC preparations.[38] In our previous animal study, we found significant reductions in immune-associated gene expression in intestinal tissues, mesenteric lymph nodes and spleen. Whereas reductions in pro-inflammatory gene expression (IL-8) was expected based on previous clinical studies on the anti-inflammatory effects of *E. faecium* SF68 in human and animal trials, there were also significant reductions in expression of IL-10, and T-cell co-activator CD86(B7.2) genes as well, *i.e.* reductions in both anti-inflammatory and adaptive immune response gene expression, suggesting a general suppression of immune-associated genes.[21] As all of these genes have been shown to be NF-κB-dependent,[29] the results of the current study suggest an explanation for the prior *in vivo* studies.

The arginine deiminase of *E. faecium* SF68 is clearly not secreted, as the ADI activity was not reproducibly apparent in bacterial culture supernatants (data not shown), although it may be surface bound as is the case in *Streptococcus pyogenes* and *S. suis*.[38,39,77] This raises the question as to how ADI activity is able to affect host cell signaling pathways in intestinal epithelial and gut-associated lymphoid tissues *in vivo* when present as either a bacterial cytosolic or cell wall-bound protein factor. The observation that the ADI activity is present in killed, intact bacteria and cell-free, whole bacterial lysates of *E. faecium* SF68 as well as ammonium sulfate protein fractions indicates that ADI does not require active bacterial metabolism for activity. In addition, ADI from a number of bacterial species is known to be active under highly acidic conditions, which has led to it being regarded as a virulence factor, involved in bacterial survival in low pH environments, including

the lysosome/phagolysosome when internalized by host cells.[38–40,66–68] These observations suggest that *E. faecium* SF68 need not be present *in vivo* as a viable, probiotic strain in order to show the effects on host cell signaling we have demonstrated here. Internalization by host cells could well result in bacterial killing within the lysosome/phagolysosome, but the presence of cell wall-bound or intracellular ADI in killed bacteria, or release of internal ADI by lysozyme digestion within the lysosome would allow ADI to deplete the lysosome of arginine, and raise the pH of the lysosome/phagolysosome through release of ammonia, inactivating many of the hydrolytic, proteolytic, and lipolytic enzymes of the lysosome. In other words, even if the probiotic *E. faecium* SF68 is killed by the host cells, the activity or release of ADI would nevertheless lead to arginine depletion and neutralization of the acidic pH of the lysosomal compartments, the latter effect of which would eliminate an important antimicrobial defense mechanism of host cells.

In addition to neutralization of the lysosomal pH, we suggest an additional effect of internalized *E. faecium* SF68 and/or ADI which would explain the severe inhibition of NF-kB and JNK(AP-1) signaling pathways observed in this study. The observation that both signaling pathways showed severe reductions in activation was somewhat unexpected, as the pathways are largely independent of one another, although they share a number of upstream regulators, such as TAK1, involved in recruitment of IKK kinase.[23] As noted above, mTORC1 is a central regulator of host cell metabolism, growth, and autophagy, and the activity of mTORC1 is dependent on arginine, among other signals. Furthermore, the lysosomal arginine pools play a key role in the regulation (activation) of mTORC1.[65,78,79] An early study found that mTORC1 is involved in the regulation of NF-κB through interaction with the IKK complex which in turn is responsible for phosphorylation and initiation of degradation of the NF-κB inhibitor, IκB, explaining how arginine depletion would affect NF-κB activity.[80] Likewise, defects in lysosome function and mTORC1 activation have been reported to be essential for the phosphorylation (activation) of JNK.[81] More recently, innate immune signaling through TLR4, the LPS receptor, has also been found to involve mTORC1/2 and the JNK and MAPK pathways.[82] We suggest that all of the observations from both this study and prior *in vitro* and *in vivo* studies can be explained by ADI-dependent depletion of arginine pools and inactivation of mTORC1 of host cells, with the possible qualification that it would likely be a localized effect *in vivo, i.e.* affecting intestinal epithelia and gut-associated immune cell populations, rather than systemic effects. This would be consistent with the results from our own[21] and other studies.

Finally, the results of our study raises serious questions regarding the use of *Enterococcus* strains as probiotics for therapeutic purposes. While the use of *E. faecium* SF68 as a therapy for symptoms of intestinal inflammation has yielded encouraging results in the past,[10–13] if one of the major mechanisms of action is a general inhibition of innate immune signaling by ADI in intestinal tissues, including immune cell populations, this would be expected to have consequences in the event of secondary bacterial infections during the treatment period, particularly for invasive pathogens. That ADI alone can inhibit the proliferation of host cells *in vivo* has been the basis for clinical trials in humans for certain forms of cancers. Our group and others have also noted deleterious effects in challenge experiments with *Salmonella* in animal studies with post-weaning piglets, where pre-treatment with *E. faecium* SF68 resulted in higher bacterial loads at systemic organ sites such as tonsils and spleen.[19–21] We suggest the latter observations reflected a dampened, intestinal innate immune response which led to elevated rates of intestinal tissue invasion by the facultative intracellular pathogen *S.* Typhimurium and subsequent spread to systemic sites. Notably, in those studies the humoral immune response reflected in *Salmonella*-specific antibody titers showed elevations; however, the antibody titers were consistent with the higher bacterial loads at systemic sites.[19,20] Whereas Enterococcal probiotics such as *E. faecium* SF68 clearly have applications in alleviating intestinal inflammation, we suggest that where the clinical benefits are based on what is arguably a bacterial virulence factor with such wide-ranging inhibitory effects on innate immune responses, the decision for administration of *E. faecium* SF68 and other Enterococcal probiotics for prophylactic and therapeutic purposes should be made cautiously.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The probiotic *Enterococcus faecium* SF68 (NCIMB 10415, Cernelle SF68, Cylactin) strain was chosen as a representative, probiotic *E. faecium* strain as it is well-characterized, and has been used in a number of prior human and animal clinical trials.[10–13] *E. faecium* SF68 was obtained directly from the proprietary company, Cerbios-Pharma SA, Lugano, Switzerland. Additional clinical and commensal strains of *E. faecium, E. avium, E. casseliflavus, E. cecorum, E. durans, E. faecalis, E. gallinarum, E. hirae*, and *E. raffinosus* are listed in supplementary Tables S1 and S2. The *E. avium* strain IMT39925 (UW11197) used as a host for the heterologous expression of the *E. faecium* SF68 arginine deiminase was provided by Dr. Guido Werner, National Reference Laboratory (NRZ) for *Staphylococcus* and *Enterococcus*, Robert Koch Institute, Wernigerode, Germany. Enterococcal isolates were grown on blood agar plates overnight at 37°C. *E. coli* strain DH5α harboring the vector pMGS100, was generously provided by Dr. Shuhei Fujimoto, Department of Microbiology, Gunma University School of Medicine, Maebashi, Japan. *E. coli* strains were grown in Luria-Bertani broth at 37°C and subcultured on Luria-Bertani agar plates. *Enterococcus* strains were routinely grown on brain heart infusion (BHI) or Todd Hewitt agar plates and liquid cultures. Where appropriate, ampicillin (100 µg/ml), kanamycin (25 µg/ml), or chloramphenicol (20 µg/ml) were added to the plates for selection. Additional references and source information regarding the bacterial strains and plasmids are found in supplementary Tables S1 and S2.

Cell lines and cell culture conditions

The IPEC-J2 cell line is a well-characterized, non-transformed, intestinal epithelial cell line derived from jejunal epithelia of a neonatal piglet. Cells were maintained in Dulbecco's modified Eagle medium (DMEM)/Ham's F-12 medium (1:1) (Biochrom) supplemented with 10% inactivated fetal bovine serum (Biochrom). Cells were grown at 37°C in a humidified incubator, at 5% CO_2. Cell lines harboring NF-κB or JNK(AP-1) chromosomal reporter fusions received 5 µg/ml puromycin (Carl Roth, Germany) as a selective agent in the medium. One day prior to experiments, the cell culture medium was removed, replaced with 1X PBS, and subsequently replaced with full medium without puromycin. The Caco-2 cell line is a human epithelial intestinal cell line, derived from a colorectal adenocarcinoma. The murine intestinal epithelial MODE-K cell line, was derived by transformation of intestinal epithelial cells of C3H/HeJ mice with the SV40 large T antigen. All cell lines were maintained under the same conditions as described for IPEC-J2 cell line. Additional references and source information regarding the cell lines are found in supplementary Table S1.

Construction of NF-κB and JNK/AP-1 luciferase reporter cell lines

NF-κB-luciferase reporter derivatives of porcine (IPEC-J2/K6), human (Caco-2/C6), and mouse (MODE-K/H8) intestinal epithelial and IPEC-J2 MAPK/JNK(AP-1)-luciferase cell lines (IPEC-J2 /D6) were constructed by infection of the cell lines with prepackaged, lentiviral vectors harboring either NF-κB- or JNK(AP-1)-luciferase reporter fusions with selection for puromycin resistance according to the manufactuerer´s instructions (Cignal Lenti Reporters, SA Biosciences, CLS-013 L and CLS-011 L, resp.). These luciferase fusion constructs encode a minimal promoter element (TATA box) preceded by a transcriptional response element specific for either NF-kB or AP-1. The luciferase is a mammalian codon-optimized, non-secreted form of the firefly luciferase gene, carrying a protein-destabilizing sequence to minimize long-term accumulation of the luciferase. After removal of dead, non-adherent cells, puromycin-resistant cells were allowed to form microcolonies, then pooled and diluted in fresh cell culture medium containing puromycin to a concentration of approximately ten cells/ml, and 100 µl of the suspension was used to seed each well of a 96-well plate. Clones derived from single cells under selection with puromycin were grown to monolayers, harvested and used to seed 25 cm^2 flasks (Corning). All clones isolated in this manner were screened for their responses to TLR ligands and those showing the best background/induction ratios and dose responses were retained for further assays.

Preparation of killed, intact bacteria and cell-free, bacterial lysates for co-culture assays

For preparation of killed but intact *E. faecium* SF68, the strain was grown overnight on blood or BHI agar plates at 37°C, followed by resuspension of colonies in phosphate-buffered saline (PBS) which was then adjusted to an optical density at 600 nm (OD_{600}) of 1 (approximately 10^9 CFU/ml). The bacterial suspension was then diluted 1:100 in cell culture medium containing 500 µg/ml gentamicin and incubated at 37°C for 4 h. The total killed bacterial suspensions were subsequently collected by centrifugation and concentrated by resuspension in a final total volume of 0.5 ml of cell culture medium containing 100 µg/ml gentamicin and 100 µg/ml streptomycin. The optical density was again determined and adjusted to an OD_{600} of 1. Appropriate dilutions of the bacterial suspensions were then performed to yield different multiplicities of infection (MOI) in a final volume of 0.1 ml to 96-well plates containing confluent monolayers of host cells (approximately 10^5 cells/well). Controls included plating of different dilutions of the initial and post-gentamicin treatment suspensions to determine the input CFU/ml and efficiency of gentamicin killing.

Cell-free, whole-cell lysates of bacterial isolates were prepared using a FastPrep 24 homogenizer and 0.1 mm silica beads for lysis of Gram-positive and -negative bacteria in 2 ml tubes (Lysing Matrix B, MP Biomedicals). Approximately 10^9/ml bacteria in 1 ml of deionized, distilled water were added to the lysis tubes, and lysed by homogenization (shaking) using a setting of 6 m/s at three bursts of 40s duration each run. The resulting lysates were sterile-filtered by passage through a 0.22 µm PVDF filter (Carl Roth, Germany) to remove debris and non-lysed bacteria. Controls for the efficiency of lysis was generally around 70–80%, indicating the lysates represented an average total protein content of 7.5×10^8 CFU/lysate. The total protein concentration of the filtered lysates was determined using a bicinchoninic acid (BCA) assay, with BSA determinations in parallel for generation of standard curves, as per the manufacturer ´s instructions (Thermo Scientific). The total protein concentration of these lysates, and the average total protein concentration of the lysates was between 100 and 150 µg, corresponding to total protein concentrations equivalent to that of about 6×10^6 bacteria/µg. As indicated in the Figure legends, 5 µg of total protein of the cell-free lysates were used in the cell culture assays, corresponding to approximately 3×10^7 CFU equivalents of total bacterial protein.

NF-$_k$B and JNK(AP-1) activation assays

The NF-κB- and JNK(AP-1)-luciferase reporter assays were performed with cells seeded onto white, flat-bottom, 96-well plates (Corning), grown to near confluency (approximately 3.2×10^4 cells per well) at 37°C, and 5% CO_2. The standard assays consisted of addition of 5 µg of total protein of the cell-free, bacterial lysates to replicate wells, and further incubation of the cells for the times indicated in the figures. Additional wells with no additions served as background (negative) controls. In co-incubation studies for host cell responses to different TLR- and NOD protein-ligands, cells were first pre-treated by addition of 5 µg of *E. faecium* SF68 cell-free lysate for 24 h, followed by addition of the TLR-ligands Pam2CSK4, Pam3CSK4, LPS, Poly (I:C) and flagellin (InvivoGen) at final concentrations of 500 ng/ml, 500 ng/ml, 5 µg/ml, 1 µg/ml and 100–150 ng/ml, respectively, unless otherwise indicated. The NOD protein ligands MDP and C12-iE-DAP (InvivoGen) were used at concentrations of 1 µg/ml and 5 µg/ml, respectively. At the times indicated in the figures, the luciferase activity was determined by addition of Bright-Glo™ Luciferase Assay System reagents (Promega) and luminescence was determined using a Synergy HT microplate reader (BioTek). In kinetics experiments, after the luciferase determinations, the plates were placed in the cell culture incubator and further incubated until the next time-point determinations.

Where indicated, cell culture medium was supplemented with an additional 10 mM L-arginine (standard concentration of arginine in DMEM: Ham´s F12 is approx. 850 µM). In other assays, the bacterial lysates of *E. faecium* SF68 strain were inactivated by either pre-heating the bacterial lysate

for 10 min at 90°C or pre-treating the lysates with 1.25 µg of proteinase K (Qiagen) at 37°C for 60 min. prior to addition to the wells of growing cells. At the end of the experiment, the NF-κB or JNK(AP-1) activities were determined by addition of the Bright-Glo™ Luciferase reagent and luciferase activities determined as above (see supplementary Figure S4).

Cytotoxicity and viability assays

Determination of the effects of cell-free, bacterial lysates and recombinant arginine deiminase on cell cytotoxicity was performed using CytoTox-ONE™ Homogeneous Membrane Integrity assays, performed according to the manufacturer's instructions (Promega). Where indicated, catalase (C1345, Sigma-Aldrich) was included at 2500 U. The cell permeable, pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone in DMSO (Z-VAD-FMK; InvivoGen) was included at a concentration of 10 µg/ml, with DMSO at the same final concentration serving as a control.

Heterologous expression of E. faecium SF68 arginine deiminase in E. avium

The *arcA* gene of *E. faecium* SF68 was amplified by PCR from chromosomal DNA using primers containing restriction sites (underlined) for *Eag*I, primer arcAEagI (5′-TTTTTCGGCCGAACATGGATAAACCTATTC ACGTTTTC-3′) and *Nru*I, primer arcANruI (5′-ATTTTTCGCGAGAGGAAATCCTGACGACAGC-3′). The PCR product was digested with *Eag*I and *Nru*I (Promega) and ligated with plasmid pMGS100 also digested with *Eag*I and *Nru*I. The resulting ligation reactions were used to transform electrocompetent *E. coli* K-12 strain DH5α by electroporation, with selection for chloramphenicol resistance. Plasmid preparations of putative clones were performed using QIAprep Spin Miniprep kits (Qiagen), and positive clones were verified by PCR and sequencing using the primers Bapro-for-01 (5′-AAAATAGTCGACTGATTGAAAC TCAAGAT-3′) and NruI-seq-rev-01 (5′-GCAACGC GGGCATCCCGAT-3′). The resulting plasmid, (pMG S100-$arcA_{SF68}$), harboring the *E. faecium* SF68 *arcA* gene under transcriptional control of the constitutive *E. faecalis* bacteriocin 21 *bacA* gene promoter, and the vector plasmid, pMGS100, were introduced into *E. avium* by electroporation with selecton for chloramphnicol resistance. Transformants were verified by PCR amplification and re-sequencing of putative pMGS100-$arcA_{SF68}$ clones, as above, and screening for arginine deiminase (ADI) activity. For additional information regarding the electroporation of *E. avium*, and ADI assays, see supplementary Methods.

Ammonium sulfate protein fractionation

Ammonium sulfate precipitation/fractionation of *E. faecium* SF68 and *E. avium* bacterial lysates were performed with strains grown in duplicate, 300 ml BHI broth cultures at 37°C with aeration, using standard protocols. Bacteria were collected and concentrated in two centrifugations with resuspension both times in 1X phosphate buffered saline (PBS). The concentrated bacterial suspensions were lysed using a French press at 18000 psi, with five passages. The resulting bacterial cell lysates were then cleared by centrifugation for 30 min at 4°C, 11000 x *g*. The supernatants were collected, and 10 ml was subjected to ammonium sulfate (AS) fractionation in steps of 30%, 60% and 100% (w/v) ammonium sulfate solutions by addition of the appropriate amounts of solid ammonium sulfate with stirring on ice for approximately 30 min., followed by an additional 90 min. on ice. Precipitated proteins from each fractionation step were collected by centrifugation for 30 min., at 4°C, at 11000 x *g*, and resuspended in 1X PBS. Protein concentrations of the initial French press lysates and subsequent supernatant and pellet resuspensions were performed using Micro BCA assays (Interchim). Initial characterization of the AS fractions was performed by screening a total of 5 µg of total protein present in the 30%, 60% and 100% AS fractions of *E. faecium* or *E. avium* in luminescence assays after treatment of the IPEC-J2/K6 NF-κB-luciferase reporter cell line for 24 h, as described above. For additional detailed protocols and references, see supplementary Methods.

Determination of arginine deiminase (ADI) activity

Arginine deiminase (ADI) activities in the AS fractions of *E. faecium* SF68, *E. avium*, and *E. avium* transformants harboring plasmids

pMGS100 or pMGS100-*arcA*$_{SF68}$, were determined from 1:5 dilutions of protein preparations in water in a total of 100 µl added to 400 µl of 0.1 M potassium phosphate buffer, pH 6.5, and 10 mM L-arginine, and the reactions were incubated at 37°C for 2 h. At the end of the incubation, the reactions were terminated by addition of 250 µl of a sulfuric acid/orthophosphoric acid stop solution and 31.3 µL of 3% diacetyl monoxime, and the samples were boiled for 15 min. at 100°C in the dark. Reactions were allowed to cool to room temperature in the dark for 10 min. and the absorption at 440 nm was determined. Reactions without addition of bacterial lysate or AS fractions served as negative (background) controls. The ADI activity of the samples was determined from a standard curve of 0 to 100 µg of citrulline performed in parallel. The final ADI activity was calculated as the as nmol citrulline/h/mg protein. For addtional details of the ADI enzymatic assays and references, see the supplementary Methods.

Screening for ADI activity of additional *Enterococcus* isolates was also determined using arginine dihydrolase (ADI or ADH) tablets (Rosco Diagnostica, Taastrup, Denmark). Bacterial suspensions were adjusted to 4.0 McFarland in 250 µl of 0.85% NaCl solution. An ADH diagnostic tablet was added to the suspension, and 3 drops of sterile paraffin oil were overlayed to provide anaerobic conditions. The tubes were incubated at 37°C and the results were recorded at 4 h and 24 h after incubation. Positive results are indicated by a strong red color resulting from ammonia production and an alkalinization of the medium in the presence of a pH indicator, methyl red. Negative results showed either a yellow to light orange color change.

MALDI-TOF identification of ammonium sulfate fraction proteins

Proteins present in the different AS fractions of *E. faecium* and *E. avium* lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining. The protein bands present in the active, 100% ammonium sulfate (AS) fraction of *E. faecium* SF68 lysate, which were not present in other fractions of the *E. faecium* SF68 lysate or the same AS fraction of *E. avium* lysates, were excised from the gel, destained and digested with sequencing grade trypsin at 100 µg/ml (Promega). Digested peptides were spotted onto a ground steel MTP 384 MALDI target plate (Bruker Daltonics, Germany), using the dried-droplet technique and α-Cyano -4-hydroxycinnamicacid (HCCA) (Sigma-Aldrich, Germany) matrix. Protein identification was carried out using matrix-assisted laser desorption ionization with a time-of-flight mass spectrometer (MALDI-TOF MS) (Ultraflex II TOF/TOF, Bruker Daltonics). For additional information and references regarding protein preparation and analysis for MALDI-TOF, see the supplementary Methods.

Western blot analysis

Confluent monolayers of IPEC-J2 cells were treated with either *E. faecium* SF68 cell-free lysates, flagellin at 100 ng/ml, or cell culture medium only (untreated) for 24 h. Cells were washed with ice-cold PBS and directly lysed with Laemmli sodium dodecyl sulfate sample buffer and 1% phosphatase inhibitor cocktail 3 (Sigma-Aldrich, USA). Western blotting was performed using standard protocols. NF-κB p65 antibody was used at a dilution of 1:2000 (10745-1-AP, Proteintech Group), p-NF-κB p65 antibody (27.Ser536) at 1:300 (sc-136548, Santa Cruz Biotechnology), β-actin antibody at 1:5000 (66009-1-Ig, Proteintech Group). Secondary antibodies included goat anti-mouse IgG coupled to horseradish peroxidase (HRP) at 1:2000 (ab97040, Abcam) and goat anti-rabbit IgG (HRP) antibody at 1:1000 (A0545, Sigma-Aldrich). Quantification of the images was performed using ECL-visualizing kit (GE Healthcare Life Sciences) and Image Lab Touch software.

Immunofluorescence (IF) staining

Immunofluorescent staining for the proliferation marker Ki67 was performed with IPEC-J2 cells seeded onto 12 mm glass cover slips (Carl Roth) and grown to semi-confluency, and either treated with 5 µg of total protein of *E. faecium* SF68 cell-free, bacterial lysates,

or left untreated. The following day, cells were washed with 1X PBS, and fixed with ice-cold methanol for 10 min., followed by a wash with cold 1X PBS. Cells were permeabilized with 1% digitonin in PBS for 10 min., followed by two to three washes with cold, 1X PBS. The fixed cells were then incubated with mouse, monoclonal IgG1κ anti-human Ki67 antibodies (M7240, Dako Omnis) in 1% BSA overnight at 4°C. The following day, the cells were washed three times with 1X PBS, and incubated with AlexaFluor-568 labeled, goat anti-mouse IgG secondary antibodies (A11004, Invitrogen) diluted 1:1000 in 1% BSA for 30 min. at room temperature in the dark. The cover slips were washed again, twice with 1X PBS, and visualized using a Leica TCS SP-2 confocal laser scanning microscope.

For immunofluorescent staining of NF-κB, IPEC-J2 cells were treated with either 5 µg of total protein of cell-free lysates of *E. faecium* SF68 or 100 ng/ml of flagellin for 4 and 24 h. At the end of incubation, cells were fixed with 4% PFA (Carl Roth), and permeabilized for 10 min at RT with 0.1% Triton X-100 (Carl Roth). Permeabilized cells were blocked with 5% donkey serum in PBS for 1 h at room temperature. Immunostaining was conducted overnight using primary NF-κB p65 antibody at 1:300 (10745-1-AP; Proteintech) followed by a 1 h incubation with a 1:50 dilution of MFP-DY -490-Phalloidin (MFP-D490-33; MoBiTec) to label actin, and a 1:1000 dilution of donkey, anti-rabbit antibodies conjugated with the fluorophore Cy5 (711–175-152, Jackson ImmunoResearch). Cover slides were incubated with DAPI (H-1200, Vector Laboratories) for 5 min and mounted with mounting medium (P36961, Thermo Fisher) and visualized using a Leica SP8 confocal laser scanning microscope.

Quantitative real-time PCR

Cells were seeded onto 6-well plates and grown to a confluency of approximately 90% and treated with *E. faecium* SF68 lysates or cell culture medium for 24 h followed by a 4 h treatment with flagellin at 100 ng/ml. Cells treated with only *E. faecium* SF68 lysates or cell culture medium (untreated) served as controls. RNA extraction was performed with a combination of TRIzol (Ambion, USA) and RNeasy Plus Mini Kit (Qiagen) standard extraction protocols and RNase-free DNase treatment (Promega) according to the manufacturer's recommendations. RNA concentrations and purity were determined using NanoDrop Spectrophotemeter (Thermo Fisher) with the criteria of $A_{260}/A_{280} \geq 1.9$, $A_{260}/A_{230} \geq 1.9$ and by gel electrophoresis for the absence of RNA degradation. Complementary DNA (cDNA) was synthetized using 5 µg of purified total RNA, Revert Aid reverse transcriptase and Oligo(dT)18 primers according to the manufacturer's instructions (Thermo Fisher).

Real-time PCRs for cytokine/chemokine (IL-6, IL-8), and apoptosis regulatory (Bax) gene expression were performed using SYBR Green SensiFAST Probe Lo-ROX Master Mix (Bioline, UK) and a -StepOnePlus™ Real-time PCR System (Applied Biosystems). 20 µL of final volume including 2 µL of cDNA (1:10 dilution) template was added for each sample to a MicroAmp™ 96-Well Reaction Plate (Thermo Fisher) and amplified in duplicate using gene-specific primer pairs for porcine β-actin, IL-6, IL-8, and Bax. Results were normalized to the housekeeping gene for β-actin. The relative changes in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method, with relative gene expression indicated as -fold change. For additional information and oligonucleotide sequences, see supplementary Methods and supplementary Table S3.

Statistical analyses

Statistical analyses were performed using the SPSS software, version 25.0 (IBM). The normal distribution of data was evaluated by a 1-sample Kolmogorov–Smirnov test. Significance between the two groups were calculated by an independent, unpaired Student´s t-test. P values of ≤ 0.05 were considered statistically significant (95% confidence intervals). In the figures, statistical significance is indicated as: n.s., $P > .05$; *, $P \leq .05$; **, $P \leq .01$; ***, $P \leq .001$.

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Disclosure statement

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Data availability statement

Supplementary data supporting the findings of this study are openly available at https://figshare.com/ at the doi address listed below.

Supplementary Figures: http://doi.org/10.6084/m9.figshare.20115950

Supplementary Methods: http://doi.org/10.6084/m9.figshare.20115902

Supplementary Table S1. Strains and Plasmids. http://doi.org/10.6084/m9.figshare.20115908

Supplementary Table S2. Enterococcus spp. http://doi.org/10.6084/m9.figshare.20115905

Supplementary Table S3. Oligonucleotides http://doi.org/10.6084/m9.figshare.20115911

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3.2 Publication II

Phenotypic zinc resistance does not correlate with antimicrobial multi-resistance in fecal *E. coli* isolates of piglets

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Authors' contributions:

LHW and SG provided funding and supervision of this study. FG, LHW and SG developed the design and concept of the study. FG, LC and CB obtained the data**.** FG, VJ, LP and KT were involved in analysis and interpretation of the data. FG, LC, CB, VJ, KT, LHW and SG have drafted the work. All authors read and approved the final manuscript.

Gut Pathogens

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Phenotypic zinc resistance does not correlate with antimicrobial multi-resistance in fecal *E. coli* isolates of piglets

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Abstract

Background: Following the ban on antimicrobial usage for growth promotion in animal husbandry in the EU, non-antimicrobial agents including heavy metal ions (e.g. zinc and copper), prebiotics or probiotics have been suggested as alternatives. Zinc has extensively been used in pig farming, particularly during weaning of piglets to improve animal health and growth rates. Recent studies, however, have suggested that high dietary zinc feeding during weaning of piglets increases the proportion of multi-drug resistant *E. coli* in the gut, contraindicating the appropriateness of zinc as an alternative. The underlying mechanisms of zinc effects on resistant bacteria remains unclear, but co-selection processes could be involved. In this study, we determined whether *E. coli* isolates from intestinal contents of piglets that had been supplemented with high concentrations of zinc acquired a higher tolerance towards zinc, and whether multi-drug resistant isolates tolerated higher zinc concentrations. In addition, we compared phenotypic zinc and copper resistance of *E. coli* isolates for possible correlation between phenotypic resistance/tolerance to different bivalent ionic metals.

Results: We screened phenotypic zinc/copper tolerance of 210 isolates (including antimicrobial resistant, multi-drug resistant, and non-resistant *E. coli*) selected from two, independent zinc-feeding animal trials by determining a zinc/copper minimal inhibitory concentration (Merlin, Bornheim-Hersel, Germany). In both trials, groups of piglets were supplemented either with high dietary zinc (> 2000 ppm) or control (50–70 ppm, background) concentrations. Our observations showed that high concentration zinc exposure did not have an effect on either zinc or copper phenotypic tolerance of *E. coli* isolates from the animals. No significant association was found between antimicrobial resistance and phenotypic zinc/copper tolerance of the same isolates.

Conclusion: Our findings argue against a co-selection mechanism of antimicrobial drug-resistance and zinc tolerance after dietary zinc supplementation in weaning piglets. An explanation for an increase in multi-drug resistant isolates from piglets with high zinc dietary feeding could be that resistant bacteria to antimicrobial agents are more persistent to stresses such as zinc or copper exposure.

Keywords: Antimicrobial resistance, Zinc, Co-selection, *E. coli*, Feed supplementation, Pigs

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Background

The administration of antimicrobial growth promoters in animal husbandry has been prohibited in the EU since 2006 [1]. As alternatives to the application of antimicrobials, non-antimicrobial substances including heavy metal ions like zinc and copper, prebiotics or probiotics

have been suggested to improve animal health and growth rates [2–5]. Zinc is one of the compounds widely used in the pig farming industry to overcome problems during weaning of piglets, including infections caused by pathogenic *E. coli* [6–9]. The essential trace elements zinc and copper are both involved in numerous physiological and cellular functions in all organisms [10–12]. Zinc concentrations and resistance are highly regulated through uptake and efflux mechanisms in different organisms [11, 13]. However, recent studies have suggested that feeding zinc in high concentrations during weaning of piglets increases the proportion of multi-drug resistant *E. coli* in the gut of the piglets [14–19]. The enhancement in the spread of antimicrobial resistance by the use of zinc confounds the usefulness of zinc supplementation in piglets and raises the question as to the underlying mechanisms of this observation.

One possible mechanism could be co-selection for both heavy metal/biocide and antimicrobial resistance, either in the form of co- or cross-resistance [16, 20–22]. Cross-resistance occurs as a result of physiological adaptations and affects susceptibility to different compounds, for example through efflux pump regulation or changes in cell wall permeability [23]. Co-resistance phenomena include changes involving genetic linkage of different genes encoding resistance to different classes of antimicrobials [20, 24]. A number of different studies have described possible mechanisms for co-selection of antimicrobial and heavy metal (zinc) resistance [16, 25–29]. Physiological coupling, genetic coupling and linked/co-localized resistance genes on mobile genetic elements have been suggested as possible mechanisms of both cross- and co-resistance [19, 23, 28, 30–32]. Zinc dependent beta-lactamases, effects of zinc on ampicillin stability or bacterial conjugation rates, and class 1 integrons (involved in co-selection) proximity to genes coding the efflux pump CzcA have been proposed as mechanisms involved in simultaneous reduction of susceptibility to antimicrobials and zinc/copper [19, 33–37]. Both intrinsic and acquired resistance mechanisms including efflux pumps and cellular detoxification of high concentrations of copper in bacteria have been reported in different studies [10, 38–40]. In addition to zinc, copper has also been suggested to contribute to antibiotic resistance in gram-negative and positive bacteria [28, 41, 42].

In this study, we tested the hypothesis that the increased antimicrobial resistance of *E. coli* isolates observed in weaning piglets fed with high zinc concentrations is caused by co-selection via phenotypic zinc tolerance. For this purpose, we used selected isolates [including antimicrobial resistant, multi-drug resistant (MDR), and non-resistant/susceptible (S) *E. coli*] and screened the level of their phenotypic zinc tolerance by determining a zinc minimal inhibitory concentration. Isolates originated from two, independent zinc-feeding trials of piglets with two different sampling schemes performed by our group over a period of 5 years. In both trials, groups of piglets were administered either high concentrations of zinc (> 2000 ppm) or a background control (50–70 ppm). From both feeding groups, we determined whether feeding of zinc resulted in higher proportions of phenotypically zinc resistant *E. coli*, and whether multi-drug resistant isolates also tolerated higher zinc concentrations, indicative of a co-selection process. In addition, we also compared phenotypic zinc resistance of these isolates with their phenotypic copper resistance values to determine whether there is a correlation between phenotypic resistance/tolerance to different bivalent ionic metals.

Results

1. Phenotypic antimicrobial resistance

Out of 210 preselected isolates collected during two, independent zinc feeding trials of piglets, 114 isolates belonged to zinc feeding groups (54.3%) and 96 isolates were from control feeding groups (45.7%). From the total number of tested *E. coli*, 63 isolates (30%) were found to be multi-drug resistant (MDR). The resistance pattern of MDR isolates always was a combination of beta lactamases (ampicillin or cefotaxime), tetracyclines (tetracycline), aminoglycosides (streptomycin) and sulfonamides (sulfamethoxazole/trimethoprim). There was no significant difference in the number of MDR isolates between the selected isolates from zinc and control groups of the feeding trials using chi-square test (Fig. 1; P-value = 0.586). Likewise, there was no significant difference in the number of resistant isolates (R) und susceptible (S) in zinc and control groups (P-value = 0.299). The number of resistant isolates to at least one antimicrobial agent was 124 (59%) of all 210 tested isolates.

2. Zinc tolerance (MIC)

All 210 *E. coli* isolates examined in our study were tolerant to 64 µg/ml zinc chloride (break point 128 µg/ml–1 mM) (lower cut-off). The highest tolerated zinc chloride concentration was 256 µg/ml (break point 512 µg/ml–3.7 mM). This includes only 33.3% of isolates (n = 70) (upper cutoff). The largest proportion of isolates (64.3%) showed a medium level of tolerance to zinc chloride at 128 µg/ml (break point 256 µg/ml–1.9 mM) which comprises 135 isolates.

The zinc tolerance data was not normally distributed (Kolmogorov–Smirnov test, P < 0.001). As shown in Fig. 2, there was no significant difference for

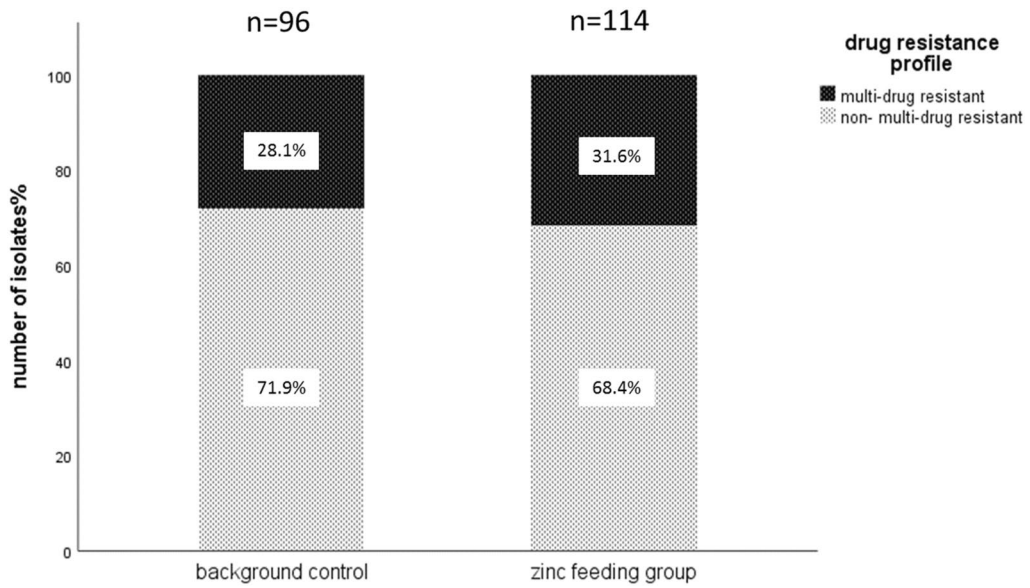

Fig. 1 Distribution of selected multi-drug resistant (MDR) and non-multi-drug resistant (NMDR) isolates in zinc and control groups. Out of a total of 210 isolates from both zinc trials, 36/114 (31.6%) multi-drug resistant (MDR) isolates were isolated in the zinc supplemented group (54.3% of total isolates), and 27/96 (28.1%) were found in the control group (45.7% of total isolates)

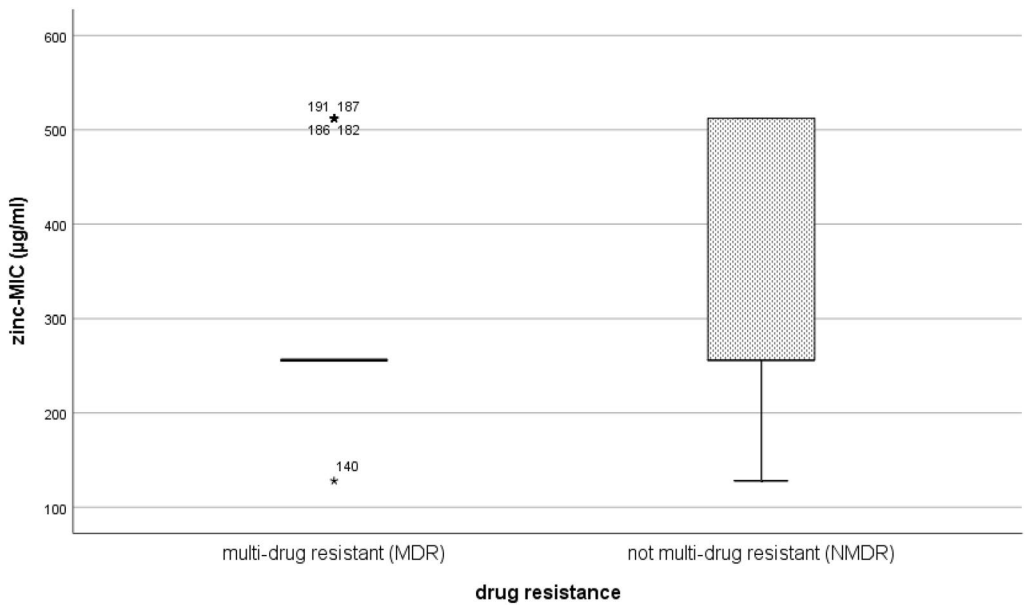

Fig. 2 Comparison of MIC values for zinc; multi-drug resistant and not-multi-drug resistant isolates of both trials. A Mann–Whitney U test comparing 63 MDR isolates and 147 NMDR isolates (overall 210 isolates) showed no significant difference of zinc-MIC value between considered groups (P = 0.085)

the MIC of zinc between MDR and NMDR isolates ($median_{MDR} = 256$ µg/ml, $median_{not\text{-}MDR} = 256$ µg/ml P = 0.085).

There was also no significant difference MIC values towards zinc of resistant isolates (R) compared to susceptible isolates (S) ($median_{resistant} = 256$ µg/ml, $median_{susceptible} = 256$ µg/ml, P = 0.107) (Fig. 3).

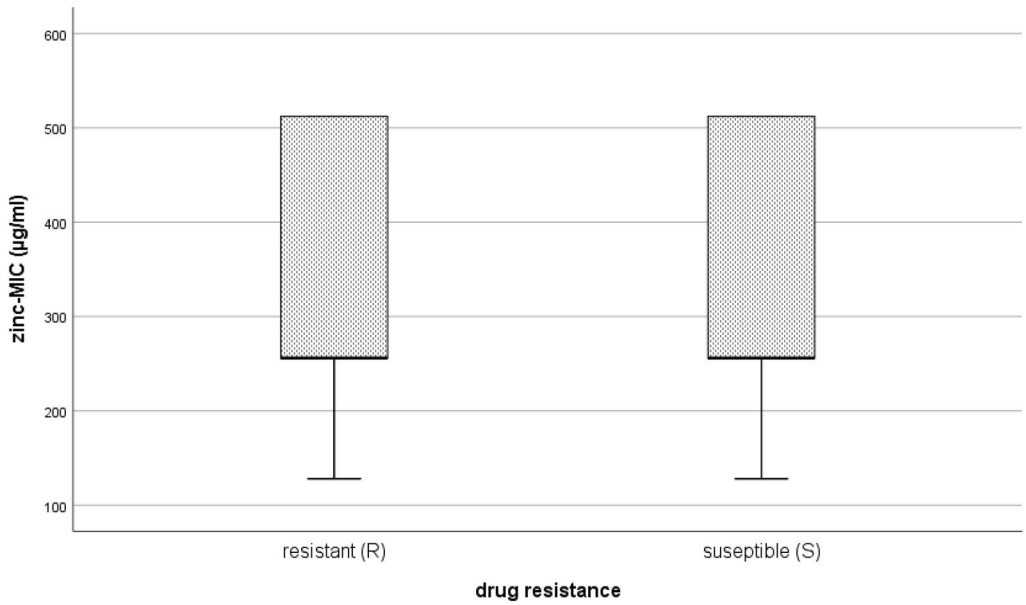

Fig. 3 Comparison of MIC values for antimicrobial resistant (R) and -susceptible isolates (S). A Mann–Whitney U test comparing 124 resistant isolates and 86 susceptible isolates (overall 210 isolates) showed no significant difference of zinc-MIC value between considered groups (P = 0.107)

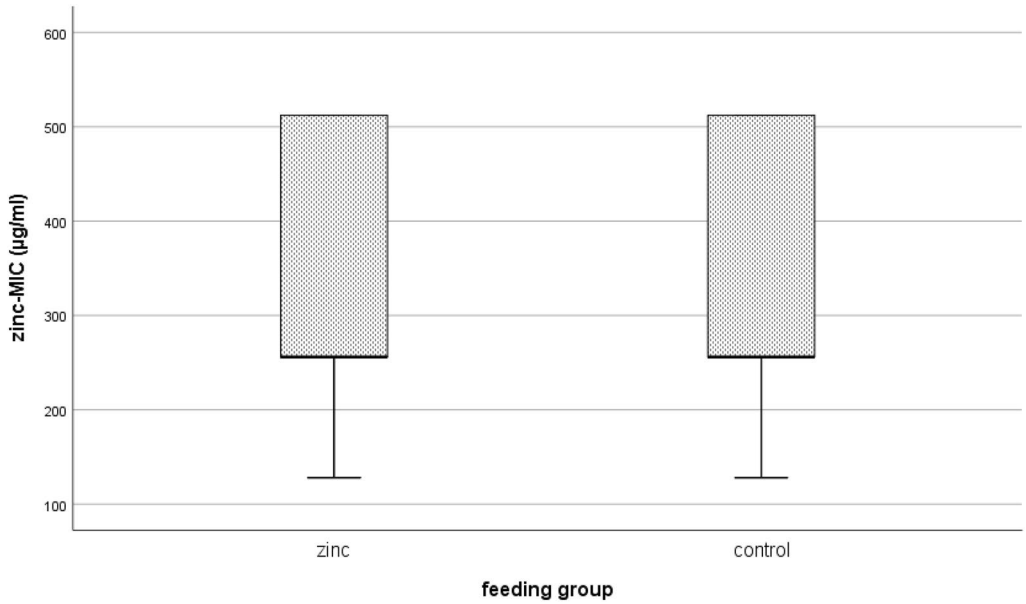

Fig. 4 Comparison of MIC values for zinc; high-zinc supplementation group (zinc) and the background control (control) isolates from both trials. A Mann–Whitney U test comparing 114 isolates from zinc group and 96 isolates from control group (overall 210 isolates) showed no significant difference of zinc-MIC value between considered groups (P = 0.146)

Interestingly, as shown in Fig. 4, there was also no significant difference in the MIC values for zinc comparing isolates from the high-zinc supplementation group ($median_{zinc} = 256$ µg/ml) or control group ($median_{control} = 256$ µg/ml, P = 0.146).

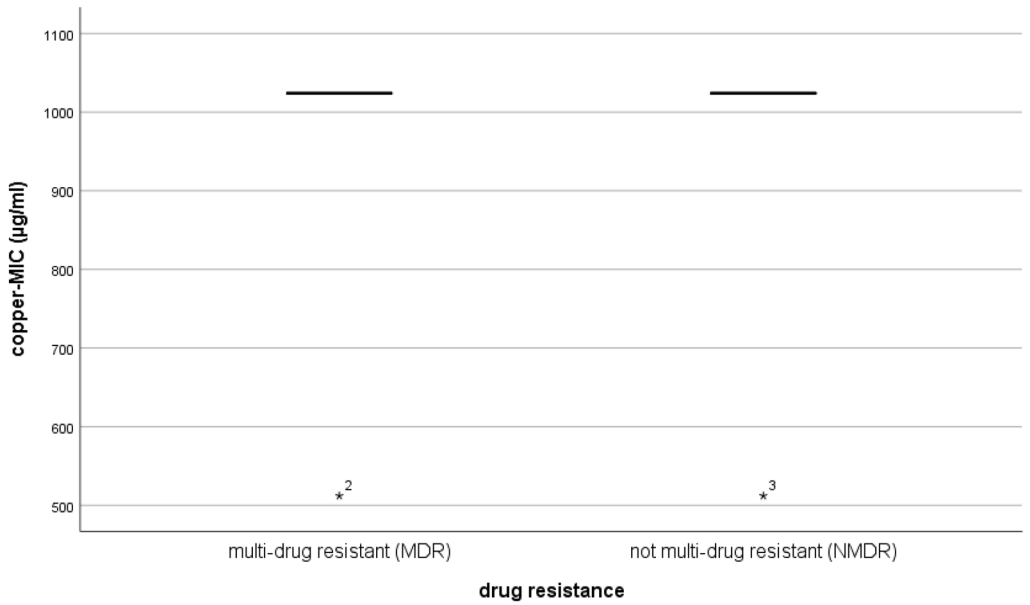

Fig. 5 Comparison of MIC values for copper; multi-drug resistant and not multi-drug resistant isolates of both trials. A Mann–Whitney U test comparing 63 MDR isolates and 147 NMDR isolates (overall 210 isolates) showed no significant difference in the copper-MIC value between groups (P = 0.540)

3. Copper tolerance (MIC)

All tested isolates in our experiment, with two exceptions, had MICs of 1024 µg/ml (~ 6.4 mM) for copper sulphate. No statistically significant difference in the MIC values towards copper comparing MDR ($median_{MDR} = 1024$ µg/ml) and NMDR ($median_{NMDR} = 1024$ µg/ml) isolates was observed (P = 0.540) (Fig. 5). There was also no significant difference in the MIC values for copper between resistant (R) and susceptible isolates, or isolates from the high-zinc supplementation group and control group (data not shown). There was no correlation between the zinc-MIC values and copper-MIC values (P = 0.593, correlation coefficient = − 0.037).

Discussion

During two, independent animal trials, we observed an increase in multi-drug resistant (MDR) *E. coli* in isolates of piglets when fed with high concentrations of zinc. One possible explanation for this effect is a co-selection for heavy metal and antimicrobial resistance, as has been previously suggested [16, 19, 20, 25, 43]. To determine whether there is an association between MDR phenotype and phenotypic zinc tolerance, we screened both MDR and non-MDR (NMDR) isolates for the level of phenotypic zinc tolerance. Out of a total of 210 isolates selected from both zinc supplementation trials, 63 isolates (30%) were multi-drug resistant.

In this study, we determined two different classifications of antibiotic resistance. We compared multi-drug resistant (MDR) to non-multi-drug resistant isolates (NMDR) according to the definition of Schwarz et al. [44], as well as resistant isolates (R), defined as resistance to at least one antimicrobial agent, and susceptible (S) isolates, defined as not resistant to any antimicrobial agents. For both definitions of antimicrobial resistance, we obtained the same result. Isolates tested in this study are not the whole set of isolates derived from two previous studies. We also did not want to show differences in the number of multi-drug resistant strains. In contrary, we chose almost identical number of strains for this experiment to compare their zinc resistance and whether it correlates with their original MDR phenotype. Therefore, it should not necessarily be a significant difference between the number of MDR isolates from zinc and control-feeding groups as was determined in our previous studies.

When comparing susceptible isolates (S) to isolates harboring at least one (or more) resistances (R), we observed no significant difference (P = 0.107) in their zinc MIC values. In addition, the zinc MIC values for zinc of MDR *E. coli* and NMDR isolates also showed no significant difference, suggesting that there is no association between antimicrobial resistance and phenotypic zinc tolerance of these isolates.

The observed increase in MDR—*E. coli* during the zinc feeding trials is therefore not likely a result of

co-selection of zinc and antimicrobial resistance. As proposed by Ciesinski et al. [18], the increase of multi-drug resistant isolates in swine treated with a high dietary zinc, is likely due to formation of a persistent population of resistant bacteria already present in the gut. Furthermore, we found no difference in zinc tolerance levels of isolates from zinc-treated groups in comparison to the control groups, suggesting that the overall zinc tolerance of *E. coli* in the gut of piglets therefore seems not be affected by zinc feeding.

There are no universal interpretative criteria for classification of *E. coli* resistance towards zinc, and studies determining the MIC values for heavy metal ions are scarce [45–48]. In this study, we used a custom-made plate for phenotypic zinc tolerance levels in *E. coli* and which include all inhibitory concentrations mentioned in previous studies in MIC assays [45].

Despite variations in the testing methods used in previous studies and our study, such as use of agar plates or broth micro-dilution, the medium used for growing bacteria, and the formulation of zinc used in the experiments, the biological upper cut-off of phenotypic zinc tolerance for the most of *E. coli* isolates in these studies were around 2–2.5 mM of zinc ion. This is in accordance with the reported MIC of 2.2 mM Zn 2+ for *E. coli* TG1 in LB-medium [47]. The highest reported concentration of zinc (Zn 2+) which could be tolerated by *E. coli* isolates in the literature was 5 mM [48].

To determine whether our findings were similar for other heavy metals, we also compared our isolates for copper tolerance. All tested isolates in our experiment, with two exceptions, had the same MIC values of 1024 μg/ml for copper sulphate (~6.4 mM). The highest MIC concentration of copper (Cu 2+) detected for *E. coli* isolates in prior studies was 10.5 mM. Our results indicated no difference between copper MIC values of MDR and NMDR isolates, suggesting that there is no association between phenotypic antimicrobial resistance and phenotypic copper tolerance of the isolates. Interestingly, we also observed no correlation between the zinc and copper MIC values of the same isolates.

Co-selection for antimicrobial and metal-resistance has been suggested in many studies [11, 39–41]. In many of these studies, co-resistance was not shown, but a co-existence of resistance was reported in the same bacteria. Nevertheless, it is believed that some metal and antimicrobial resistance genes are linked and co-resistance of antimicrobial and heavy metal resistant bacteria have been discussed in several studies as likely to arise through co-selection [22, 29–34].

These studies are mostly on genome level and several of studied antimicrobial and metal ion resistance genes are on plasmids [16, 32, 43, 49–51]. For example, the plasmids of *Salmonella abortus equi* were found to co-transfer antimicrobial resistance (ampicillin-resistance) and heavy metal resistance (As, Cr, Cd, Hg) genes in mating experiments with *E. coli* strains. *Salmonella* strains cured of the plasmids were found to be sensitive towards ampicillin and heavy metals [32]. In a genomic transcriptional study, Lee et al. [30] found up-regulation of the *mdtABC* operon after exposure to high levels of zinc which suggested a potential influence of metal stresses on bacterial resistance to antibiotics.

In a recent genomic study by Pal et al. [28], a total of 2522 fully sequenced bacterial genomes and 4582 plasmids were analyzed. The authors concluded from their large-scale study that plasmids have only a limited potential for horizontal transfer of biocides and metals resistance by co-selection.

Prior studies have also tested isolates at both the genomic and phenotypic levels. One such study showed co-regulation of resistance to heavy metals and carbapenems through the CzcR–CzcS system in *Pseudomonas aeruginosa* strain PT5. In that study, it was shown that a mutation in the CzcS sensor protein found in zinc and imipenem resistant isolates led to efflux pump CzcCBA overexpression and down-regulation of the OprD porin resulting in a co-selection for both increased zinc and carbapenem resistance [52]. In a series of retrospective studies screening *E. faecium* isolated from different species, it was found that *tcrB* (transfer copper resistance) and *ermB* (transfer macrolide resistance) genes were present on the same conjugative plasmid. However, the data did not demonstrate a co-selection between these two phenomena and the strong correlation between copper and macrolide resistance was found only in pig isolates. In addition, while the prevalence of macrolide resistance in isolates decreased during the years covered in the study, the prevalence of copper resistance among pig *E. faecium* isolates remained more or less the same. The authors argued that the reduction in the antimicrobial usage during this period lead to a decrease in antimicrobial resistance, whereas in the same time period the use of copper derivatives remained unchanged. Therefore, they concluded that copper exposure might not alone be sufficient to induce antimicrobial resistance and a strong selective pressure of macrolide administration should be present to select the antimicrobial resistance [40, 53, 54].

There are few experimental studies available evaluating the induction of antimicrobial resistance following metal exposure. Peltier et al. [55] investigated antimicrobial resistance to ciprofloxacin, oxytetracycline, and tylosin in zinc-activated sludge bioreactors. Zinc application alone did not affect zinc and antimicrobial resistance to ciprofloxacin and oxytetracycline. Increased antimicrobial resistance could be the result of co-exposure of zinc and

antimicrobial agents. Berg et al. [56] found that strains isolated from soil treated with copper for 21 months were more resistant to both copper and indirectly antimicrobials compared to control plots.

In contrast to the above-mentioned studies in which co-selection was the subject of discussion, there are also studies reporting counter-selection of heavy metal and antimicrobial resistance [57]. Hölzel et al. [26] found that while exposure to zinc and copper increased the rate of β-lactam resistance in *E. coli*, the presence of mercury was associated with a lower rate of antimicrobial resistance.

Conclusions

In summary, our results do not indicate a co-selection process of antimicrobial resistance and higher zinc tolerance in the MDR isolates of our feeding trials. An increase of *E. coli* more tolerant to zinc due to the feeding of high zinc concentrations as an explanation for the increase of multi-drug resistant isolates via co-selection can therefore be excluded. This seems to be also true for copper tolerance levels. These results would appear to argue against a co-selection mechanism for drug-resistance after zinc supplementation, since we did not find an association between antimicrobial resistance and phenotypic zinc/copper tolerance for the same isolates. We also found that zinc exposure did not have an effect on either zinc or copper phenotypic tolerance of the isolates.

An explanation for an increase in MDR isolates from piglets with high zinc dietary feeding in our previous studies could be that resistant bacteria to antimicrobial agents are more persistent to stresses such as zinc or copper exposure. Ciesinski et al. have argued that the increase in multi-drug resistant *E. coli* populations is associated with persistence of the resistant population under the influence of high dietary zinc, while in that study the total number of *E. coli* population had been decreased.

Another explanation might be that in the zinc-fed groups, zinc activates genes involved in metal ion resistance to deal with the metal ion load, and which might also be involved in antibiotic resistance, but this is a transient phenotypic zinc resistance. In accordance to this argumentation, Peltier et al. also found that zinc exposure increases resistance to antibiotics but had a minimal effect on zinc resistance [55]. In addition, the duration of experiments, co-exposure to both metal and antimicrobial agents and concentration of the substances could play role in either in vivo or in vitro-resistance studies.

Interpretation and analysis of resistance data based only on genetic data should be made carefully, a combination of both genetic and phenotypic resistance determinations is required, and it will also be important to show whether resistance could be developed in non-resistant isolates. The result of these types of studies could have implications for the prophylactic use of zinc in the field, i.e. pigs daily fed zinc to prevent infections.

Methods

Sample origin

A total of 210 *E. coli* isolates originally collected during two independent zinc feeding trials (S_1 and S_2) in 36 and 32 piglets respectively were used in this study ($S_1 = 105$, $S_2 = 105$) [18, 58]. All the experimental trials of these studies were approved by the local state office of occupational health and technical safety 'Landesamt für Gesundheit und Soziales, Berlin' (LaGeSo Reg. Nr. 0347/09 and LaGeSo Reg. Nr. 0296/13). The *E. coli* isolates were isolated from intestinal contents (digesta) on the 1st, 2nd and 4th weeks of both feeding trials. The first trial (S_1) was a clonal study concentrating on the diversity of the *E. coli* analyzed via PFGE, which identified 105 clones from 1481 isolates in either only control or only zinc feeding groups independent of sampling time. In this study, one isolate from each of the 105 clones was tested. To obtain a comparable number of samples from the second feeding trial (S_2), we randomly chose 105 isolates using representative random sampling method out of a total of 550 samples isolated from digesta [59]. The second feeding trial was performed with a selective culturing approach using CHROMagar Orientation plates supplemented with one of nine different antimicrobials as well as CHROMagar Orientation plates without supplementation to select resistant *E. coli* populations during the zinc treatment. Antibiotic concentrations in media plates were adapted from Guenther et al. [60] or are derived from the breakpoint concentrations of the Clinical and Laboratory Standards Institute [61, 62]. The schematic workflow of *E. coli* analyses (Fig. 6) shows the study design of previous and current studies.

In both trials, zinc oxide (Sigma Aldrich, Taufkirchen, Germany) was applied as a feed supplement to a high zinc feeding group (2000–2500 ppm) and background control (50–70 ppm). Further details of the animal trials can be found in the original publications [18, 58].

Phenotypic antimicrobial resistance

All isolates were initially screened for their resistance profiles against ampicillin, chloramphenicol, gentamicin, streptomycin, tetracycline, cefotaxime, enrofloxacin, sulfamethoxazole/trimethoprim and imipenem (BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs, Becton-Dickinson, United States) according to the standards of the Clinical and Laboratory Standards Institute [63]. The results from the agar disc diffusion tests were confirmed using minimum inhibitory concentration

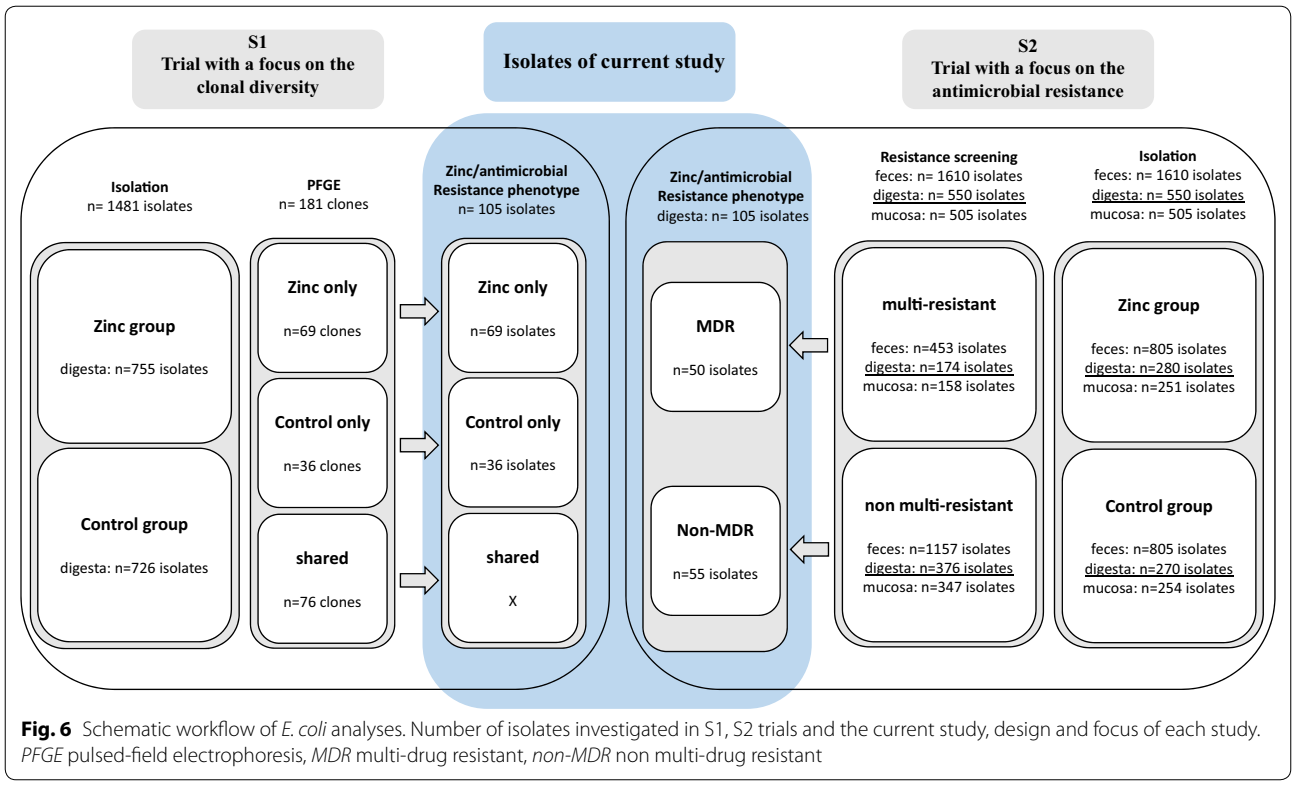

Fig. 6 Schematic workflow of *E. coli* analyses. Number of isolates investigated in S1, S2 trials and the current study, design and focus of each study. *PFGE* pulsed-field electrophoresis, *MDR* multi-drug resistant, *non-MDR* non multi-drug resistant

(MIC) microdilution using cation adjusted Mueller Hinton II medium (Micronaut breakpoint plates, Genzyme Diagnostics, Rüsselsheim, Germany) according to CLSI standards (CLSI, 2008). Based on their resistance patterns these strains were stratified as multi-drug resistant or non-multi-drug resistant according to the definition of Schwarz et al. [44], as resistant (resistant to at least one antimicrobial agent) or susceptible (completely sensitive to the tested antimicrobials).

Phenotypic zinc/copper resistance testing

Overnight cultures of all *E. coli* isolates were adjusted to McFarland Standard 0.5 (1.5×10^8 CFU) and 50 µl of 1:200 dilution of adjusted suspensions in Mueller–Hinton broth (Roth, Karlsruhe, Germany) were used as inocula for incubations for 16 to 20 h at 35 °C in biocide and heavy metal microtiter-plates (Merlin, Bornheim-Hersel, Germany). The plates contained a wide range of concentrations of biocides/heavy metals in twofold dilution steps including 32 to 8192 µg/ml copper sulfate (COP) and 4 to 8192 µg/ml zinc chloride (ZKC) [45]. In our study, the minimal inhibitory concentration data of two heavy metals including copper sulfate and zinc chloride were collected. To prevent drying of the plates during incubation a sealing tape was used to seal the surface of the plate. After the incubation, the MIC for zinc was determined visually and reported as the growth breakpoint. *E. coli* ATCC25922 and ATCC10536 strains were used as reference strains for internal quality control.

Statistical analysis

Statistical analysis was performed based on the combined datasets from both zinc feeding trials. Isolates were stratified irrespective of the zinc feeding either as multi-drug resistant (MDR) or non- multi-drug resistant (NMDR) isolates, as well as resistant (R) (at least one resistance) or susceptible (S) isolates. In addition, the isolates were subsequently grouped based on their origin from either high-zinc supplementation group (zinc) or the background control (control). Statistical analyses were performed using the SPSS software, version 25.0 (IBM, New York, NY, USA). The normal distribution of data was evaluated by a 1-sample Kolmogorov–Smirnov test. Mann–Whitney (non-parametric test) and chi-square tests were used for the analysis of data [64, 65]. The correlation between zinc tolerance and copper tolerance was calculated using Spearman rank correlation test (non-parametric correlation) [66]. The non-normally distributed data are shown as the median ± standard deviation (SD), and $P < 0.05$ was considered statistically significant.

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Authors' contributions
LHW and SG provided funding and supervision of this study. FG, LHW and SG developed the design and concept of the study. FG, LC and CB obtained the data**.** FG, VJ, LP and KT were involved in analysis and interpretation of the data. FG, LC, CB, VJ, KT, LHW and SG have drafted the work. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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4 Discussion

4.1 Host-microbe interaction in *in vitro* model

In vitro studies are appropriate models for investigating the nature of physiological and pathological conditions (Schierack et al. 2006; Kauffman et al. 2013). Regarding host-microbe interactions, *in vitro* studies can assist to precisely explore the communication strategies of both sides and their mechanisms of action. Moreover, active molecules contributing to reciprocal interaction of host and microbes can be well studied (Li et al. 2019b). In the first part of the discussion, our results obtained from the *in vitro* study on the effects of *E. faecium* SF68 lysates on intestinal epithelial cells of different species will be discussed.

4.1.1 *E. faecium* SF68: NF-κB inhibition

NF-κB is involved in many physiological processes and is one of the main triggers of innate immune responses in host cells, particularly a key regulator factor of intestinal epithelial cells encountering invasive enteric pathogens (Elewaut et al. 1999; Pasparakis 2009; Baltimore 2011). Our observations showed that treatment of different intestinal epithelial cells, including IPEC-J2 (porcine), Caco-2 (human) and MODE-K (murine) cells, with the cell-free whole lysate of probiotic *E. faecium* SF68 leads to an inhibitory effect on NF-κB activation level of these cells within 24 h.

Many studies have demonstrated the inhibitory/stimulatory effect of microbial molecules on the metabolism and immune responses of host cells (Ruiz et al. 2005; Fukuda et al. 2011; Plaza-Díaz et al. 2017; Liu et al. 2018). In the case of pathogenic microorganisms, the purpose of microbial properties and metabolites are to deteriorate the function of the immune system and to evade it (Jensen et al. 1998; Ruiz et al. 2005; Groschwitz and Hogan 2009; Fukuda et al. 2011; Plaza-Díaz et al. 2017; Liu et al. 2018). Contrarily, non-invasive microorganisms such as gut microbiota and beneficial microbes exert positive anti- or pro-inflammatory effects on the epithelial and immune cells, in a mutualistic approach (Kelly et al. 2004; Ukena et al. 2005; Avram-Hananel et al. 2010; Plaza-Díaz et al. 2017). Moreover, they may protect the host by taking direct actions against harmful pathogens in a competitive manner (Gueimonde et al. 2007; Fukuda et al. 2011). In this regard, one of the well-known anti-inflammatory strategies of beneficial microbes is to inhibit activation of the main inflammatory signaling pathways in cells, including NF-κB, JAK/STAT and MAPK (Plaza-Díaz et al. 2017; Kanmani and Kim 2018; Bhardwaj et al. 2020), thereby deleterious inflammation and damages caused by pathogens or during the course of chronic non-infectious inflammatory conditions can be alleviated (Campana et al. 2017). The perturbation of inflammatory signaling pathways can occur at

different steps of these signaling cascades. Regarding NF-κB as one of the main inflammatory signaling pathways, TLRs-induction, phosphorylation, ubiquitination, and proteasomal degradation of IκBα, nuclear translocation, DNA-binding activity, transcriptional activation, and mRNA expression of phosphorylated NF-κB can be targeted as intervention points by beneficial microbes (Ma et al. 2004; Yan and Polk 2010; Kaci et al. 2011; Li et al. 2016; Bhardwaj et al. 2020).

For instance, in a well-structured study on a selection of beneficial lactic acid bacteria, attenuation of LPS-induced inflammation at different steps of NF-κB and MAPK signaling pathways in HepG2 cells treated with whole cellular fluid of these bacterial strains has been reported. These immunoregulatory effects included modulation of TLRs and TLR negative regulators at the mRNA level, and p38 MAPK and p65 subunit of NF-κB at the protein level. As a result, following LPS stimulation, production of inflammatory cytokines including IL-6, CXCL8, CCL2, and TNF-α were reduced in cells pretreated with bacterial fluid (Kanmani and Kim 2018). Similarly, we observed that pre-treatment of porcine intestinal epithelial cells (IPEC-J2) with cell-free whole lysate of *E. faecium* SF68 drastically decreases the expression of IL-8 in these cells following flagellin stimulation. In accordance with our results, the findings of Klingspor et al. indicated that pre- or co-incubation of IPEC-J2 and Caco-2 cells with the same probiotic strain decreases the upregulation of pro-inflammatory IL-8 at the mRNA and protein levels in response to pathogenic ETEC. Moreover, they reported an improvement in terms of TEER decrease and the heat shock protein 70 increment following ETEC challenge. However, they did not detect the same pattern of changes in cells treated with *E. faecium* SF68 after EPEC infection (Klingspor et al. 2013). The same inhibitory effect on ETEC-induced IL-8 production of IPEC-J2 cells has been demonstrated in an *in vitro* co-incubation study model with a different strain of the same bacterial species, *E. faecium* HDRsEf1, where both *E. faecium* and its cell-free supernatant showed immunomodulatory effects on intestinal epithelial cells (Tian et al. 2016). In another study, administration of *Streptococcus salivarius* strains, a commensal species from genus *Streptococcus*, led to NF-κB downregulation in intestinal epithelial cells (HT-29) and monocytes. The active metabolite accountable for this anti-inflammatory effect was less than 3 kDa and present in the supernatant. This immunomodulatory molecule was able to minimize the production of pro-inflammatory IL-8 following TNF-α induction. The authors postulated that the target of inhibition is located downstream of the NF-κB signaling pathway, since the inhibition is independent of induced receptor, e.g. TNF-α, IL-1β, or flagellin (Kaci et al. 2011). Our data showed that only inflammatory ligands with strong stimulatory potentials in IPEC-J2 cells such as flagellin (MyD88-dependent) or poly(I:C) (MyD88-dependent) can promote NF-κB activation in cells treated with the lysate for 24 h. Therefore, we also suggest that the inhibitory effect of *E. faecium* SF68 in intestinal epithelial cells is independent of TLRs or MyD88.

To have a better understanding of the mechanism behind the observed inhibitory effect, we determined phosphorylation of p65 at serine 536. RelA/p65 is an important NF-κB subunit, mainly involved in transcription activation (Hayden and Ghosh 2008; Zhang et al. 2017). Serine 536 on the C-terminal TAD of p65 is one of the foremost studied phosphorylation sites of this subunit and contributes to the transactivation of NF-κB. This phosphorylation site is frequently inducible in response to inflammatory stimulation (Hu et al. 2004; Christian et al. 2016). Our western blot analysis indicated a reduced level of phosphorylation at serine 536 of RelA/p65 subunit in IPEC-J2 cells treated with the *E. faecium* SF68 lysate for 24 h compared to untreated cells. It has been shown that IKKβ plays a critical role in serine 536 phosphorylation of p65 in monocytes and macrophages after LPS stimulation, contributing to the NF-κB inflammatory transcriptional activity (Yang et al. 2003). Additionally, cyclin-dependent kinase 6 (CKD6) has been identified as another NF-κB p65 Ser536 kinase contributing to both basal and TNFα-inducible p65 serine 536 phosphorylation. This enzyme plays an important role in both inflammation and tumor progression. An increase in CKD6 expression, p65 Ser536 phosphorylation, and subsequent upregulation of NF-κB target cell cycle-regulatory protein, namely cyclin D3, have been shown to be linked with tumor development (Buss et al. 2012). Moreover, cytokine-independent phosphorylation of p65 subunit at serine 536 by IKKε in cancer cells has been shown to contribute to cell proliferation and cancer development (Adli and Baldwin 2006). However, Pradère et al. found that phosphorylation of p65 subunit at serine 536 is essential for sending a negative signal to interrupt further activation of NF-κB, but it is not crucial for NF-κB nuclear translocation (Pradère et al. 2016). Altogether, independent of the cause behind the activation of inflammation machinery and the specific kinases accountable for phosphorylation of p65 subunit at serine 536, the findings of different studies imply an important role of this phosphorylation site downstream of NF-κB activation and its mediated transcription alterations. The inhibitory effect we observed on NF-κB activation in intestinal epithelial cells treated with *E. faecium* SF68 lysate for 24 h was also accompanied with a considerable decrease in phosphorylated p65 subunit at serine 536 compared to untreated cells as well as decreased flagellin-induced upregulation of IL-8 expression. In addition, our fluorescence microscopy observations suggest that the inhibitory mechanism of *E. faecium* SF68 on NF-κB activation is due to hindered NF-κB trafficking between cytoplasm and nucleus. These findings comprised more prenuclear and cytoplasmic accumulation of NF-κB in cells treated with the lysate compared to intestinal epithelial cells treated with flagellin or even untreated cells. Other beneficial microbes have been shown to have anti-inflammatory effects via disturbing NF-κB trafficking, such as *Bacteroides thetaiotaomicron*, which has been shown to enhance the nuclear export of RelA via a PPAR-gamma dependent mechanism (Kelly et al. 2004).

Unlike the great number of studies explaining the mechanism behind the immunomodulatory effects of beneficial microbes, in most of these studies, exact molecules responsible for the reported effects in different host cells or *in vivo* models have remained unknown. A good example in this regard is a study performed by Petrof et al. on a well-known probiotic mixture, namely VSL# 3, comprised of *Streptococcus thermophilus* and several species of *Lactobacillus* and *Bifidobacteria*. The authors demonstrated that the soluble factors of the probiotic blend impede the NF-κB pathway via proteosome inhibition, but they could not detect the exact factor(s) involved in this observation (Petrof et al. 2004). In another study, *Lactobacillus fermentum* supernatant has been shown to antagonize inflammatory alterations induced by *Yersinia enterocolitica* in HeLa cells. The authors suggested a secreted phospholipid (<10 kDa) from *L. fermentum* as the accountable factor for decrease of NF-κB activation and subsequent IL-8 secretion in the cells infected with *Y. enterocolitica*, but they could not identify the exact factor (Frick et al. 2007).

Anti-inflammatory effects of beneficial microbes, including probiotics, on NF-κB signaling have been shown in *in vivo* study models as well. Li et al. demonstrated that *Lactobacillus acidophilus* minimizes the adverse inflammatory effects of ETEC in piglets, by impeding NF-κB and MAPK signaling cascades in the spleen and MALT tissues (Li et al. 2016). In another study, it has been shown that clonal deletion of lymphocytes due to an early inflammatory response induced by *Salmonella enterica* can be prevented by a mixture of probiotic bacteria. This preventive effect emanated from the modulation of immune responses of cells in MALT of mice treated with the probiotic blend. The immune-associated alterations of cells included upregulation of mRNA expression levels of *Cd2*, *Ptprc* and *Tlr5* genes. Moreover, the downregulation of mRNA expression levels of NF-κB-dependent inflammatory and cytokine genes (*RelB*, *Myd88*, *Ikka*, *Jun* and *Irak2*) and apoptotic genes (*Casp2*, *Casp12*, *Dad1*, *Akt1* and *Bad*) were observed (Wagner and Johnson 2017). In another experiment, a combination of several probiotic *Lactobacillus* and *Bifidobacterium* strains exhibited anti-inflammatory activity on colonic tissues with dextran sulfate sodium (DSS)-induced injury. The immunomodulatory effects of these bacteria included an attenuation in the expression levels of TLR4, iNOS, and NF-κB. Additionally, the plasma levels of NO and INF were decreased. As a result, these anti-inflammatory modulations alleviated inflammation in mice with colitis (Toumi et al. 2014). In an ETEC challenge in piglets, administration of *E. faecium* SF68 reduced the inflammatory increase of TLR2 and NF-κB mRNA expression as well as expression of their associated cytokines in the ileum of animals (Peng et al. 2019). In another example, soluble factors of *Bifidobacterium breve* have been shown to alleviate intestinal inflammation via anti-inflammatory effects on immune and epithelial cells. The authors demonstrated that *Bifidobacterium breve* and its soluble factors inhibited CXCL8 secretion induced by NF-κB and AP-1 pathways in mice with TNBS-induced colitis (Heuvelin et al. 2009). Overall, since

impaired intestinal barrier integrity and diarrhea are mainly caused by activation of the inflammatory TLR-MyD88-NF-κB axis, the anti-inflammatory effects of beneficial microbes can ameliorate damages in intestinal inflammatory conditions.

Other beneficial non-bacterial microorganisms can also exert immunomodulatory effects on host cells. For instance, *Saccharomyces boulardii*, a non-pathogenic yeast with healing effect on intestinal injuries, has been shown to hamper NF-κB activation and subsequent upregulation of IL-8 expression at mRNA and protein levels, in THP-1 and HT-29 cells induced with different stimulants including TNF-α, IL-1β, or LPS (Sougioultzis et al. 2006).

However, immunomodulatory effects of beneficial microbes can be both inhibitory and stimulatory. Regarding the immunostimulatory effects of beneficial or commensal microbes, *Bifidobacterium lactis* has been reported to enhance TLR2 mediated IL-6 gene expression via NF-κB and p38-MAPK signaling pathways. The authors of this study concluded that early pro-inflammatory stimulation of intestinal epithelial cells by commensal bacteria plays a pivotal immunoregulatory role at early stages of bacterial colonization and assists in maintenance of mucosal homeostasis (Ruiz et al. 2005). In another case, *E. faecalis* CECT7121 has been shown to robustly activate dendritic cells and alter their cytokine production profile. These alterations manipulate T-cell populations to have higher IFNɣ production. The same research group also showed that *E. faecalis* CECT7121 can remain in the murine gastrointestinal tract and shifts T-cell populations towards Th1(Molina et al. 2015). Similarly, *E. coli* Nissle 1917, a well-studied probiotic, has been demonstrated to increase mRNA expression levels of pro-inflammatory genes MCP-1 and MIP-2α in Caco-2 and LoVo cells. MCP-1 was also elevated at the protein level in Caco-2 cells (Ukena et al. 2005).

To further investigate the nature of the observed inhibitory effect of the *E. faecium* SF68 lysate on NF-κB activity of intestinal epithelial cells, we used cell viability and cytotoxicity assays. We measured lactate dehydrogenase (LDH), which is normally released from damaged and necrosed cells and indicates cell death (Shi et al. 2003). LDH levels of cells treated with the probiotic lysate were the same as untreated cells. Thus, cells exposed to the bacterial lysate of *E. faecium* SF68 were not dying at increased rates. Nevertheless, using Ki67 staining we determined that cells treated with the lysate were not proliferative compared to untreated cells, suggesting an apoptotic state of the cells. This is consistent with the fact that apoptotic cells do not release LDH as they do in necrosis. However, generally distinguishing apoptosis from necrosis can be difficult, since necrosis can also later be induced in apoptotic cells (Lekshmi et al. 2017). Moreover, in our setting cells treated with the lysate had lower expression levels of IL-8 (NF-κB target gene) and slightly higher expression levels of Bax (pro-apoptotic regulator) compared to untreated cells following the flagellin stimulation. However,

the upregulation of Bax was negligible. Pro-apoptotic effects of beneficial microbes have been shown in previous studies as well. For instance, human-derived *Lactobacillus reuteri* ATCC PTA 6475 has been shown to inhibit TNF-induced NF-κB activation and concomitantly to enhance pro-apoptotic MAPK signaling (Iyer et al. 2008). Thus, despite our observations in favor of apoptosis, our findings included contrary evidence as well. Firstly, the inhibitory effect of the lysate on the NF-κB activity levels of the cells was reversible and host cells can recover their NF-κB activity by adding fresh medium. Moreover, JNK-AP-1 signaling which can induce apoptosis was also impeded by lysate treatment. However, JNK-AP-1 has been shown to be involved in both cell proliferation and apoptosis (Ameyar et al. 2003). Later, we identified arginine deiminase as the active factor responsible for the observed inhibitory effect on intestinal epithelial cells. Additionally, we showed that arginine supplementation can eliminate the inhibitory effect. Altogether, our results imply that nutrient depletion and unavailability of arginine hinder NF-κB activity in intestinal epithelial cells treated with *E. faecium* SF68 lysate. It has been shown that nutrient depletion activates AMPK signaling, followed by inhibition of the mTOR pathway contributing to a general attenuation in mRNA translation rate. Moreover, distinct cell processes and enzymatic reactions such as protein modifications (e.g. phosphorylation, ubiquitination, etc.) will be affected. These events also lead to an induction of autophagy (Finch and Ruvkun 2001). Autophagy induction has been reported in cells facing arginine depletion. These cells can recover if they receive arginine (Savaraj et al. 2010). Nevertheless, it should be noted that maintaining cells in arginine deprivation conditions leads to cell cycle arrest and apoptosis (Patil et al. 2016).

4.1.2 *E. faecium* SF68: arginine catabolism and arginine deiminase

To identify the precise immunomodulatory factor in the lysate, the lysate was fractioned with ammonium sulfate (AS). Proteins present in the active fraction of the *E. faecium* SF68 lysate and not present in the equivalent AS-fraction of *E. avium* lysate, were subjected to MALDI-TOF for protein identification. Among identified proteins of *E. faecium* SF68 lysate, we detected several enzymes involved in arginine metabolism including arginine-tRNA ligase, arginine deiminase, and ornithine carbamoyltransferase. An increasing number of studies have demonstrated that arginine and its metabolic products play a pivotal role in gut hemostasis and immune responses (Morris 2010; Quirino et al. 2013). Therefore, the role of arginine supplementation as immunonutrition (De Jonge et al. 2002b; Tan et al. 2009) and arginine-consuming enzymes (e.g. arginine deiminase, the first enzyme in the arginine deiminase pathway) as anti-tumor and immunomodulatory agents, have been considered in various studies (Ni et al. 2008; Brin et al. 2017; Kim et al. 2018). Arginine plays an essential role in production of nitric oxide (NO) and proliferative polyamines. NO produced by nitric oxide synthases is a main regulator of immunity and inflammation particularly at early stage of

infections, cancers, or injuries. Conversely, polyamines are important for cell growth, homeostasis, and late-stage repair (Satriano 2004; Keshet and Erez 2018). Hence, according to the published literature and our data we focused on arginine deiminase for further investigations on the nature of the immunomodulatory factor responsible for inhibitory effect of *E. faecium* SF68 lysate on NF-κB activity levels of intestinal epithelial cells.

We used different approaches to determine whether arginine deiminase is the factor we are searching for. Firstly, we demonstrated that supplementation of the cell culture medium with L-arginine can abolish the observed inhibitory effect of the lysate. In a similar study, addition of L-arginine has been shown to restore the growth of mouse hepatoma cells treated with arginine deiminase. Their experiments demonstrated that arginine deiminase purified from two *Mycoplasma* species impedes the growth of MH134 cells. The authors concluded that AD elicits a growth inhibitory effect on tumor cells via arginine deprivation and hindrance of polyamines biosynthesis (Takaku et al. 1995). In another example, arginine supplementation has been demonstrated to increase the concentration of polyamines and maintain intestinal permeability in a murine model of intestinal obstruction (Quirino et al. 2013). Xia et al. determined that IPEC-J2 cells can tolerate a reduction in arginine concentration from 0.7 to 0.2 mM. Their result showed that cationic amino acid transporter (*CAT1*), excitatory amino acid transporter (*EAAT3*), and alanine/serine/cysteine transporter (*ASCT1*) were upregulated in IPEC-J2 cells in presence of low concentration of arginine. The authors concluded that the increased absorbance efficiency of amino acids in the arginine deprivation condition is inevitable for maintenance of intestinal integrity and biological functions (Xia et al. 2016). Regarding the importance of arginine in hemostasis and immune responses of host cells, interfering with arginine metabolism is a known strategy often used by pathogens (e.g. *Salmonella*, *Giardia*, etc.) to overcome host defense responses such as NO production and also to impair mucosal barrier integrity (Madhuchhanda et al. 2009; Stadelmann et al. 2012; Stadelmann et al. 2013). For instance, arginine depletion induced by *Giardia intestinalis* attenuated the proliferation of the intestinal epithelial cells and led to cell cycle arrest in HCT-8 and Caco-2 cells. This halt in the cell cycle was temporary and arginine supplementation could reverse the proliferation inhibitory effect (Stadelmann et al. 2012). In another example, consumption of arginine by *Giardia intestinalis* contributed to suppression of NO production in Caco-2 cells. Moreover, arginine deiminase enzyme derived from this intestinal pathogen directly reduced T-cell specific PBMCs proliferative response (Stadelmann et al. 2013). *Helicobacter pylori*-mediated arginine depletion and arginase II induction in host macrophages led to activation of SMO, induction of oxidative stress, and enhanced DNA damage in gastric epithelial cells. These cells undergo apoptosis via the ERK, c-Fos/c-Jun, and c-Myc pathway (Chaturvedi et al. 2012).

The mechanisms proposed in the literature as to how arginine depletion leads to growth inhibition, cell cycle arrest, and eventually apoptosis have been investigated in different studies. As mentioned, autophagy is induced at early stages of arginine depletion. At this point, the effects are still reversible in normal cells, which can restore their proliferation and growth upon receiving arginine (Kim et al. 2009a; Savaraj et al. 2010). Contrarily, cancer cells are exceedingly sensitive, and unavailability of arginine promotes apoptosis. Therefore, enzymes such as arginine deiminase have been assessed, which is being used in cancer therapy in the last decade. However, arginine-depletion induces upregulation of arginine-producing ASS expression in tumor cells. Thus, cancer cells can also resist arginine-depleted conditions (Kim et al. 2009a; Kim et al. 2009b; Savaraj et al. 2010; Phillips et al. 2013; Patil et al. 2016). One of the revealed mechanisms for induction of autophagy in arginine-depleted conditions is mTOR inhibition via activation of MEK/ERK. Cells sensing arginine deficiency caused by AD activate AMPK signaling, which modulates mTOR activity (Savaraj et al. 2010). Additionally, Bauchart-Thevret et al. corroborated that arginine-induced protein synthesis and survival of IPEC-J2 cells is mTOR-dependent. They also indicated that NO and insulin are not involved in these processes. Furthermore, an increase in p70 S6 kinase and 4E-BP1 activation was reported by the authors of the study in cells supplemented with arginine. They showed that IPEC-J2 cells hold lower potentials to synthetize arginine compared to primary porcine IECs because they exhibit considerable arginase activity, which converts produced arginine to ornithine. Therefore, they are more dependent on arginine availability for their survival (Bauchart-Thevret et al. 2010). This finding may also provide an explanation why IPEC-J2 cells were excessively sensitive to *E. faecium* SF68 lysate treatment compared to other intestinal epithelial cell lines screened in our experiments. The reduction in the NF-κB activity levels of IPEC-J2 cells after treatment with the lysate for 24 h was down to the 10% of NF-κB activation levels of untreated cells. At advanced stages, arginine depletion induces mitochondrial outer membrane permeabilization and release of apoptotic effector proteins such as AIF, EndoG, and cytochrome c. Release of these inter-membrane space proteins induces apoptosis in the cells via both caspase-dependent and -independent pathways (Kim et al. 2009a; Kim et al. 2009b; Patil et al. 2016).

Inhibition of *de novo* protein synthesis is one other suggested mechanism, whereby arginine depletion promoted by arginine deiminase inhibits cell proliferation (Shen et al. 2006). Protein synthesis requires arginine from intracellular pools or the citrulline-arginine regeneration pathway, and arginine deprivation leads to different modulation of both protein and polyamines synthesis (Patil et al. 2016). Moreover, lack of arginine can be sensed by the amino acid response (AAR) pathway. Subsequently, there will be an increase in the uncharged t-RNA pool and a general inhibition of translational initiation (Kilberg et al. 2012).

Except for the indirect and general inhibitory effect of arginine depletion on physiological activities and hemostatic processes in host cells, deprivation of arginine has been shown to particularly inhibit NF-κB. In a study on the synergistic effects between arginine deiminase and chemotherapeutic gemcitabine to suppress argininosuccinate synthetase-deficient pancreatic cancer cells, an AD-mediated decrease in phosphorylation of p65 at serine 536 and nuclear translocation of activated NF-κB was observed. AD hindered phosphorylation of the p65 subunit at serine 536 via inactivation of PI3K/Akt survival signaling. Moreover, arginine deiminase enhanced cell cycle arrest and upregulation of apoptotic caspases in these cells (Liu et al. 2014). Interestingly, Kundu et al. demonstrated a non-enzymatic anti-tumor activity of arginine deiminase from *Pseudomonas aeruginosa*. The authors of this study reported that this bacterial arginine deiminase possesses a CARD-domain (Pa-CARD) at its N-terminal, thereby inducing apoptosis in cancer cells. The cell cycle arrest induced by Pa-CARD was even greater than by Pa-AD itself. Moreover, the growth inhibitory effect was only induced in cancer cells while normal HOSE6-3 cells did not undergo caspase activation and apoptosis. However, their findings showed that truncated AD containing only Pa-CARD upregulates NF-κB target genes expression, such as GM-CSF, IL-2, IL-8, IL10, IL-1α, and IL-1β, in ovarian cancer SKOV-3 cells. They suggested that the upregulation of cytokines by induction of CARD-domain enhances the recruitment of immune cells and removal of cancer cells (Madhuchhanda et al. 2009).

Knowing that the inhibitory effect of *E. faecium* SF68 lysate is associated with arginine depletion, we further determined if arginine deiminase is directly involved in deprivation of arginine. Therefore, in the next step we cloned the arginine deiminase gene of *E. faecium* SF68 into our *E. avium* strain, which does not possess enzymes of the ADI pathway. The results showed that the transformed *E. avium* isolate constitutively expressing arginine deiminase can inhibit NF-κB activity in IPEC-J2 cells. Additionally, lysate of isolates from other enterococcal species with AD-activity, including *E. faecalis*, *E. durans*, and *E. hirae*, had the same inhibitory effect. Nevertheless, isolates from *E. gallinarum* and *E. casseliflavus* species did not impede NF-κB activity of cells even though they possess AD-enzymes as well. *E. gallinarum* and *E. casseliflavus* are motile species of the genus *Enterococcus* and express flagellin. Bacterial flagellin is a strong immunostimulatory ligand which can induce pro-inflammatory responses in both immune and non-immune cells. Moreover, it has a key role in the shaping innate and adaptive immune responses to invading pathogens, particularly in the gut (Fulde et al. 2018). In this regard, our data from co-incubation experiments of IPEC-J2 cells treated with the probiotic lysate and distinct immunostimulatory ligands also showed that only a strong inflammatory stimulant like flagellin can re-activate NF-κB in the affected cells. Therefore, we suggest that the immunostimulatory potential of flagellin from *E. gallinarum* and *E. casseliflavus* overcame the inhibitory effect of arginine deiminase on the NF-κB activity of the

cells. An example of the immunostimulatory role of flagellin is the case of *Enterococcus gallinarum* MRx0518, a human commensal strain, proposed as a biotherapeutic strain for its anti-tumor effects. It has been shown that this commensal strain strongly activates NF-κB via TLR5 induction and enhances production of IL-8 in HT29-MTX cells. The authors of this study postulated an association between the robust immunostimulatory effect of bacterial flagellin and its anti-tumor properties (Lauté-Caly et al. 2019). However, we assume that the anti-tumor effect observed in *Enterococcus gallinarum* MRx0518 by Lauté-Caly et al. can be due to AD-activity of this strain and the profound pro-inflammatory response to flagellin is a completely independent observation.

Finally, we also screened lysate of *Streptococcus suis* 10 strain to determine if bacterial strains from other genera expressing AD elicit the same inhibitory effect on NF-κB activation levels in IPEC-J2 cells. Arginine deiminase is a surface protein expressed by different *Streptococcus* species such as *S. suis*, *S. gordonii*, and *S. pyogenes* and it has been identified as a putative virulence factor in these species (Gruening et al. 2006; Fulde et al. 2014; Sakanaka et al. 2015). AD-activity of arginine deiminase of *S. suis* and *S. gordonii*, or its homolog streptococcal acid glycoprotein (SAGP) from *S. pyogenes* contributes to the survival of these bacteria at lower pH and enhances their biofilm-forming ability (Curran et al. 1995; Sakanaka et al. 2015). Our results showed that *S. suis* 10 was able to inhibit NF-κB activity of IPEC-J2 cells in the same concentration and time dependent manner as *E. faecium* SF68. Moreover, we screened an arginine deiminase knock-out mutant of *S. suis* 10 (Gruening et al. 2006) in reporter cells and showed that in this case there was no inhibitory effect on intestinal epithelial cells. Furthermore, we screened the overexpressed and purified recombinant arginine deiminase protein of *S. suis* 10 and observed that recombinant arginine deiminase hampers NF-κB activation in IPEC-J2 cells within 24 h. Altogether, our experiments confirmed that arginine deiminase is the factor directly involved in the inhibition of NF-κB activation in our *in vitro* model of intestinal epithelial cells via arginine depletion. The main purpose of this doctoral study was to reveal more detailed information on local innate immunomodulatory effects of *E. faecium* SF68 in intestinal epithelial cells to explain *in vivo* observations of previous studies (Scharek et al. 2005; Szabó et al. 2009; Mafamane et al. 2011; Siepert et al. 2014). Therefore, in the next part, the significance of arginine deiminase in observed *in vivo* effects of probiotic *E. faecium* SF68 will be discussed.

4.2 Host-Microbe interaction in the gut

Gut microbiota and host epithelial and immune cells in the gut communicate with each other through vast numbers of molecules and metabolites in a mutualistic relationship. Moreover, this complex interacts with harmful and beneficial microbes (Geva-Zatorsky et al. 2017). On

the bacterial side, fine differences at the strain level decide whether bacteria of the same species are pathogens/pathobionts or probiotics (Marchesi et al. 2016). Moreover, species, age, immune status, and health condition of the host can play a role in the host-bacteria equilibrium. *E. faecium* SF68, the probiotic strain investigated in the present doctoral study, is a commensal strain isolated from a healthy infant (Holzapfel et al. 2018). In this section, regarding the observations made in the current study, the possible mechanisms by which this beneficial probiotic delivers its effects *in vivo* will be discussed.

4.2.1 *E. faecium* SF68: gut microbiome composition and alteration

Probiotic strains are intended to constructively modulate the composition of microbiota by enhancing the growth of beneficial microbes and confining invading pathogens. This aim can be achieved via alteration of the gut environment conditions such as modulating the available nutrients and pH as well as improving the intestinal epithelial barrier and mucus layer (Campana et al. 2017). Moreover, production of bacteriocins and metabolites which directly targets other microorganisms and inhibits the growth of competitors or stimulates expansion of accomplices have been determined as probiotic strategies to shift the structure of the gut flora and confer health benefits to the host (Ljungh and Wadström 2006; Kawai et al. 2018).

E. faecium SF68 is a lactic acid producing enterococcal probiotic strain. In the current study, the comparison of the protein content of the cell-free whole lysate of this probiotic and its ammonium sulfate (AS) fractions to the lysate and equivalent AS-fractions of *E. avium* by MALDI-TOF confirmed that *E. faecium* SF68 possess the enzymes of arginine deiminase pathway including arginine deiminase, and ornithine transcarbamylase. L-arginine conversion to citrulline and L-ornithine is accompanied by the generation of ATP, ammonia, and CO_2. This chain of events mainly occurs in the presence of arginine supplementation in the growth environment of *Enterococcus* and under anaerobic conditions (Abdelal 1979; Champomier Verges et al. 1999), both of which are found in the gut environment (Albenberg et al. 2014; Xia et al. 2016). Therefore, it is highly probable that following the entrance of *E. faecium* SF68 into the gut its ADI pathway gets induced. Our *in vitro* observations on AD-activity of *E. faecium* SF68 by measuring citrulline production showed AD-activity of this strain. pH alteration of the medium to a very strong basic pH value was also detected. Several strains from various LAB, including different *Lactobacillus*, *Streptococcus*, and *Enterococcus* strains, possess ADI enzymes. Ammonia synthesis via the ADI pathway increases both cytoplasmic and environmental pH and contributes to regulation of pH homeostasis (Champomier Verges et al. 1999; Lindgren et al. 2014). Therefore, ADI system aids its producer strain and other LAB strains to survive their own acidifying metabolic activities as well as environmental low pH and hence facilitates the expansion and further beneficial activity of gut lactic acid bacteria in a

mutualistic symbiosis (Xie et al. 2018). ADI-dependent adaptation to acidification has been shown to protect streptococci in other environments such as in oral cavity (Marquis et al. 1987; Curran et al. 1995).

Several *in vitro* and *in vivo* studies with *E. faecium* SF68 indicated that this probiotic enhances the growth of other LAB strains including *Enterococcus* and *Lactobacilli* bacteria and modifies the microbiota composition in favor of these beneficial microorganisms. Shifts in the gut microbiota composition towards beneficial LAB strains and increased lactic acid production decrease the pH of the gut lumen. Consistently, a recent study on the effect of *E. faecium* SF68 by Xie et al. indicated that probiotic supplementation increases the count of *Lactobacillus* strains and concurrently decreases the pH in different parts of the gastrointestinal tract including duodenum, jejunum, and cecum in piglets (Xie et al. 2018). In another experiment, supplementation of turkeys with *E. faecium* SF68 has been shown to be associated with a higher lactate concentration in the small intestine and an increase in the metabolic activity of lactic acid bacteria and *Lactobacilli* in particular (Vahjen et al. 2002). Furthermore, broilers that received the same probiotic strain showed enhanced performance, had longer villi in the ileum, and carried a higher number of lactic acid bacteria in their ileal content and excreta (Samli et al. 2007). *E. faecium* SF68-induced pH modification via the ADI pathway may support higher metabolic activity and lactic acid production by *Lactobacillus* strains and hence opens an opportunity for *Lactobacilli* to shift the gut niche in their own favor and eliminate their opponents. Starke et al. investigated the possible direct inhibitory or stimulatory effects of *E. faecium* SF68 on the growth of other indigenous or pathogenic bacteria to have a better understanding of the mechanism of action of this probiotic in modifying the gut microbiota (Starke et al. 2015). They showed that the probiotic *E. faecium* SF68 has a strain-specific effect on the growth of other bacteria. They reported enhanced growth of beneficial *Lactobacillus* strains and decline in the number of one pathogenic *E. coli* strain co-cultured with of *E. faecium* SF68. These results were reflected in an *in vivo* study from the same group in sows and their offspring. In this study, probiotic supplementation modified the composition of fecal microbiota of sows, especially with an increase in the number of *Lactobacilli*. Additionally, modification of intestinal flora affected suckling piglets of sows receiving probiotic supplementation as well (Starke et al. 2013). Furthermore, it has been shown that changes in the pH by LAB bacteria can inhibit the growth of enteric pathogens such as *Salmonella* and *E. coli* strains by increasing the permeability of their outer membrane (Alakomi et al. 2000). Therefore, *E. faecium* SF68 can indirectly inhibit the growth of pathogenic bacteria as well. Nevertheless, *in vivo* challenge studies on effects of *E. faecium* SF68 supplementation during *Salmonella* infection have even shown adverse effects of this probiotic on colonization and shedding of *Salmonella* (Szabó et al. 2009; Kreuzer et al. 2012).

Metabolites of beneficial microbes, aside from simple changes in the lumen environment, can affect the gut microbiota as well as invading pathogens by metabolic cross-feeding and by directly enhancing or inhibiting their growth and metabolism (O'toole and Cooney 2008; O'shea et al. 2012; Bäumler and Sperandio 2016; Campana et al. 2017). Among studied metabolites of this kind, intermediate- and end-products of arginine metabolism pathways are particularly interesting (Beckmann et al. 2013; Keogh et al. 2016; Herrero Del Valle et al. 2020). In this regard, one of the well-known strategies is the link between arginine metabolism and iron acquisition (Beckmann et al. 2013). Recent studies have shown that iron plays a pivotal role in niche selection and virulence of pathogenic and pathobiont bacteria of Proteobacteria and Firmicutes phyla at different body sites, such as the gut, gingiva, and skin. In fact, iron directly and indirectly contributes to enhanced metabolism, aggregation, biofilm formation, and adhesion of several bacterial species (Dogan et al. 2014; Buret et al. 2019). Therefore, augmenting the iron uptake from host cells and iron acquisition by microorganisms can accelerate dysbiosis or alleviate infection and inflammation in the gut. It has been shown in different microorganisms that L-ornithine from arginine metabolism plays a role in enhanced iron acquisition, particularly under iron limitation (Beckmann et al. 2013; Sakanaka et al. 2015; Keogh et al. 2016). For instance, *E. faecalis*, an enterococcal species possessing enzymes of ADI pathway has been shown to support *E. coli* in a wound co-infection study model under iron limitation. L-ornithine generated from *E. faecalis* induced biosynthesis of siderophores in *E. coli* and hence, indirectly enhanced the growth and biofilm formation ability of this *Enterobacterales* member (Keogh et al. 2016). Meanwhile, LAB e.g. *Enterococcus* and *Lactobacillus* strains with AD-activity themselves are needless of iron uptake and siderophore production (Pandey et al. 1994; Keogh et al. 2016). In another instance, arginine-ornithine transporter (ArcD) of *Streptococcus gordondii* enhanced biofilm formation of *Fusobacterium nucleatum* via metabolic cross-feeding (Sakanaka et al. 2015). Metabolic cross-feeding mechanisms may provide an explanation for enhanced translocation and colonization of *Salmonella* in weaned piglets receiving *E. faecium* SF68 in earlier *in vivo* observations as well (Szabó et al. 2009; Kreuzer et al. 2012; Siepert et al. 2014). In other words, ornithine may induce siderophore production and iron acquisition in a part of commensal microbiota residing healthy gut lumen, which can use it to enhance their growth, but in an infection or dysbiosis situation, pathogenic or pathobiont Enterobacteriaceae can take over the iron. It should also be noted that the kinetics of arginine conversion to intermediate- or end-products of the ADI pathway depends on pH-conditions. In an optimal pH-condition citrulline is the major product of the ADI pathway, while in a sub-optimal pH-condition arginine will be converted mainly to ornithine (Vrancken et al. 2009). As mentioned, *E. faecium* SF68 promotes a shift in gut microbiota composition in favor of LAB, higher production of lactate, and an overall decrease

in the pH of different parts of the intestine. Therefore, in the presence of *E. faecium* SF68 more ornithine will likely be produced.

Another notable example of cooperation between different bacteria using arginine and its metabolites is the teamwork of probiotic *Bifidobacterium* strains and gut bacteria in human intestine for higher production of polyamines (Kitada et al. 2018). Direct enhancement in synthesis of polyamines via arginine metabolism can be exploited by gut microbiota to improve gut health under normal circumstances (Tofalo et al. 2019). Furthermore, polyamines have been shown to alleviate adverse symptoms of enteritis (Quirino et al. 2013). Probiotic *Bifidobacterium* spp. have been shown to increase polyamines in the gut, though they do not possess the necessary enzymes for polyamine production. In fact, *Bifidobacterium* spp. reduce the pH in the gut and in response to lower pH, the acid-tolerance system of *E. coli* employs arginine for survival. Agmatine is a byproduct of this process and its end-product is putrescine. Agmatine can be consumed by *E. faecalis* for energy production via the agmatine deiminase system and in this way even more putrescine will be produced. As a result, levels of putrescine in the gut will be significantly increased by both *E. coli* and *E. faecalis* (Kitada et al. 2018). It should be considered that metabolites driven from the ADI pathway of beneficial microbes such as *E. faecium* SF68 can be similarly employed by other bacteria via a cross-feeding mechanism. However, depending on the health status of the host, microbiota composition, and presence of pathogens or pathobionts in the gut, positive and negative outcomes of probiotic consumption in the host should be regarded.

Production of antimicrobial substances such as bacteriocins is one of the well-known strategies of beneficial microbes to modulate gut microbiota (O'shea et al. 2012). Moreover, these antimicrobial properties can inhibit pathogens and reduce their adhesion to mucosal surface (Vimont et al. 2017). At least one enterocin of 4.48 kDa has been purified from *E. faecium* SF68 and shown to inhibit the growth of different enterococcal strains from *E. hirae*, *E. casseliflavus*, *E. faecium*, and *E. faecalis* species (Foulquie Moreno et al. 2003). Therefore, the modulatory effect of probiotic *E. faecium* SF68 to some extent may be due to its antimicrobial properties.

Regarding the direct effect of *E. faecium* SF68 on microbiota, there are some controversial issues to discuss. This probiotic can survive digestion and transit through the gut in different host species but cannot permanently colonize their gastrointestinal tract (Underdahl et al. 1982; Lund et al. 2002; Macha et al. 2004). Therefore, the long-term mechanism of action of *E. faecium* SF68 cannot be due to the direct production of metabolites or antimicrobial substances, since the detected number of probiotic cells in the gut is negligible when compared to the total number of bacteria present in the gastrointestinal lumen, unless metabolites such

as ammonia or L-ornithine generate lasting impact. This means that the efficacious metabolite has only a short and direct influence on a part of the gut bacterial populations and these altered populations further manipulate the microbiota composition and metabolites production. Otherwise, other conceivable mechanisms for beneficial effects of this strain such as a strong modification of innate immune responses should be considered, which will be discussed later in the next section. However, it should also be considered that probiotics are often preventive agents and are frequently applied in human and animal neonates. Therefore, beneficial microbes as well as harmful pathogens can demonstrate stronger traits in weaning newborns and young recipients, since they do not yet carry a fully shaped gut microbiota with a higher density and more diverse composition (Matamoros et al. 2013). Moreover, weaning stress itself and drastic nutritional changes in this period lead to shifts and alterations in the equilibrium of the gut microbiota of suckling mammals, particularly piglets (Lalles et al. 2007). Additionally, instantaneous loss of maternal immunity transferred by milk make freshly weaned individuals more susceptible to enteric primary and opportunistic pathogens as well as the effects of administered beneficial microbes (Madec et al. 1998; Matamoros et al. 2013). These facts may provide some explanations for the observations made previously on the effects of *E. faecium* SF68. For instance, application of *E. faecium* showed a more significant alteration of gut microbiota composition in suckling piglets compared to weaned piglets and adult sows (Broom et al. 2006; Vahjen et al. 2007; Starke et al. 2013). Furthermore, in *Salmonella* challenge studies in weaning piglets, administration of *E. faecium* SF68 worsened the infection status, shown by increased colonization and shedding of *Salmonella* (Szabó et al. 2009; Mafamane et al. 2011; Siepert et al. 2014). In the next section, *in vivo* immunomodulatory effects of *E. faecium* SF68 regarding its arginine deiminase activity will be presented.

4.2.2 *E. faecium* SF68: modulation of gut immune responses

E. faecium SF68 has been shown to elicit anti-inflammatory effects in the host (Holzapfel et al. 2018) and has successfully been used to treat intestinal inflammation and diarrhea in humans and animals. Most studies on its effectiveness were mainly focused on the alleviation of symptoms and enhancement of performance (Lewenstein et al. 1979; Underdahl et al. 1982; Wunderlich et al. 1989; D'souza et al. 2002; Allen et al. 2004; Taras et al. 2006; Allen et al. 2010).

However, in a number of studies, particularly infection model studies, adverse anti-inflammatory effects of *E. faecium* SF68 were detected. Dysregulation and downregulation of immune-associated genes of intestinal tissues, lower production of immunoglobulins (serum total IgG and fecal IgA), and an attenuation in CD8+ intraepithelial lymphocytes populations have been included in the list of undesirable anti-inflammatory effects of *E. faecium* SF68 in

piglets (Scharek et al. 2005; Scharek et al. 2007; Siepert et al. 2014). In *Salmonella* Typhimurium infection studies in piglets, supplementation of post-weaning piglets with *E. faecium* SF68 exacerbated the course of infection. Greater colonization and translocation of *Salmonella* in different host organs, like tonsils and colon, higher shedding of pathogen and higher blood immunoglobulin levels (IgM and IgA) were determined in these animals (Szabó et al. 2009; Mafamane et al. 2011; Kreuzer et al. 2012). Additionally, peripheral blood mononuclear cells (PBMCs) of supplemented piglets exhibited delayed proliferative responses to *Salmonella* antigens (Mafamane et al. 2011). Nevertheless, controversial immunostimulatory potential of *E. faecium* SF68 has been reported as well. For instance, Benyacoub et al. treated freshly weaned puppies with *E. faecium* SF68 supplementation until the age of one. The result of this study suggests that mechanisms of action of *E. faecium* SF68 are local and systemic immunomodulatory effects rather than direct modification of gut microbiota. In this regard, young dogs supplemented with *E. faecium* SF68 had higher fecal IgA and blood $CD21^+/MHCII^+$ B cells compared to control group. Additionally, specific IgA antibody against *E. faecium* SF68 was detected in treated puppies, demonstrating the immunogenicity of this beneficial microbe. Nevertheless, $CD4^+$ and $CD8^+$ cells remained unaffected (Benyacoub et al. 2003). Though the authors gave a positive weight to these findings, it should be noted that the stated immunostimulatory effects mediated by *E. Faecium* SF68 in young puppies can be the direct results of infection with *E. faecium* SF68 and detection of specific IgA against the probiotic strain is strong evidence for this assumption. Plus, young recipients with underdeveloped immune systems are more sensitive to any kind of external stimuli particularly around weaning time, which reinforces the hypothesis of *E. faecium* SF68 virulence in the puppies.

With the positive and negative immunomodulatory effects of *E. faecium* SF68 reported in the literature and previous studies in our lab, the potential significance of arginine deiminase in these effects will be discussed here. Our results from the current doctoral study demonstrated that arginine deiminase of *E. faecium* SF68 inhibits NF-κB activity of intestinal epithelial cells via arginine-depletion. As mentioned, arginine plays an important role in growth and physiological processes, especially during early life in humans and animals (Satriano 2004; Wu et al. 2004; Xia et al. 2016). Arginine will be metabolized and processed in enterocytes and mediates distinct supportive functions in the gut in health or stress conditions including infection, weaning, and injury (Li et al. 2007; Quirino et al. 2013). For instance, Tan et al. reported an immunosupportive role for arginine in healthy early-weaned piglets. These immune-associated modulations targeted antibody production (e.g. elevated serum IgG and IgM concentrations), cytokine production (e.g. enhanced IL-8 expression level in spleen), and leukocyte populations (e.g. increased granulocytes) (Tan et al. 2009). Moreover, arginine contributes to lymphocyte development, proliferation and activation of T cells, prevents

bacterial translocation, and also promotes mucus layer formation, enterocyte proliferation, and intestinal barrier permeability (Quirino et al. 2007; Tan et al. 2009; Quirino et al. 2013; Geiger et al. 2016). In an experimental setting it has been shown that overexpression of arginase in the small intestine hinders lymphocyte development in mice. The authors demonstrated that the observed dysregulation is arginine-dependent, since the same effect was not determined in NOS- or OTC-deficient mice (De Jonge et al. 2002a). In another study from the same author, a 30-35% decline in tissue and blood arginine of transgenic mice with arginase overexpression impeded early maturation of B cells and IgM production, though T cells remained unaffected (De Jonge et al. 2002b). Furthermore, decreased bacterial translocation and enhanced bacterial killing activity in severe clinical conditions, such as intestinal injury and peritonitis, have been reported following arginine administration (Quirino et al. 2007; Quirino et al. 2013). One of the main arginine-associated immunoregulatory mechanisms is NO production. NO is a signaling mediator, which assists in pathogen killing and cytokine production of immune cells. Three nitric oxide synthase (NOS) isoforms (nNOS, eNOS and iNOS) require arginine for NO production. Activated macrophages generate iNOS to attack pathogens from arginine sources and hence arginine availability is crucial for local host defense mechanisms (Kubes and Mccafferty 2000; Kim et al. 2018). Consequently, one of the strategies often used by allochthonous and pathogenic bacteria is to induce arginine catabolic enzymes like arginase, via TLRs to cut host access to arginine for iNOS production. As a result, depletion of arginine impedes the inflammatory MAPK pathway induced by TLR4 and less iNOS is produced (Morris 2010; Chaturvedi et al. 2012).

In the *in vivo* trials using *E. faecium* SF68 supplementation, PBMCs isolated from piglets that received *E. faecium* SF68 were less able to prevent intracellular growth of *Salmonella* in IPEC-J2 cells (Mafamane et al. 2011). Similarly, it has been shown that AD-activity of SAGP from *Streptococcus pyogenes* inhibits the proliferation response of human PBMCs to phytohemagglutinin stimulation while L-arginine supplementation restored the proliferative response of the T cells (Degnan et al. 1998). Moreover, *Salmonella* is an intracellular pathogen possessing ADI enzymes and utilizes host arginine for its own metabolism and survival. Consumption of arginine from the host pool reduces its availability for production of cytokine-induced NO and leads to downregulation of iNOS expression. Therefore, host defense against invading pathogens is impaired (Gogoi et al. 2016). Downregulation of cytokine-induced iNOS expression were reported in Caco-2 cells infected with Giardia for 6 h as well. Giardia reduced the protective NO production of the host by consumption of arginine and NO destruction via its NO-degrading enzymes (Stadelmann et al. 2013). In the same study, a reduced proliferation of T cells was observed in lymphocytes treated with arginine deiminase of Giardia which match findings of *in vivo* studies in piglets supplemented with *E. faecium* SF68 showing lower CD8+ IELs (Pollmann et al. 2005; Scharek et al. 2005; Mafamane et al. 2011). Additionally, it has

been demonstrated that arginine depletion impairs immunoglobulin production in transgenic mice by disturbing early B cell maturation and lymphoid organ development (De Jonge et al. 2002b). This arginine-dependent perturbation may reflect the underlying cause of lower IgG and IgA in piglets supplemented with *E. faecium* SF68 (Scharek et al. 2005; Broom et al. 2006; Scharek et al. 2007). Overall, these findings may provide an explanation for observations made in *Salmonella* trials in piglets supplemented with *E. faecium* SF68. In young piglets under weaning stress, with high nutrient demands, imbalanced nutrition, and immune properties, concurrent presence of a pathogen (*Salmonella* Typhimurium) and a probiotic (*E. faecium* SF68), both equipped with arginine metabolizing enzymes, can lead to unfavorable outcomes including greater translocation and higher shedding of *Salmonella* (Szabó et al. 2009; Mafamane et al. 2011).

There are some points, which should particularly be considered in the interpretation of the obtained results from *in vivo* studies with *E. faecium* SF68. Neonates have an underdeveloped immune system (Macpherson and Harris 2004). Furthermore, neonates have a rapid growing rate and high metabolic activity (Davis et al. 1993). Therefore, they have higher nutrient requirement and more rapidly encounter nutrient shortages such as arginine deficiency. In addition, generally catabolic conditions, weaning stress, inflammation, and malnutrition lead to depletion of arginine and citrulline in humans and animals (Flynn et al. 2002). Importantly, the host needs to develop communication strategies of tolerance to the gut microbiota, otherwise, there will be an exaggerated inflammatory response towards commensal flora as seen in inflammatory bowel disease (Melmed et al. 2003). At the same time, the intestinal epithelial barrier needs to confine microbiota to the gut and the crosstalk between the local innate and systemic adaptive immune systems and the microbiota restrains the gut flora (Round and Mazmanian 2009). Therefore, any unreasonable anti-inflammatory effect in intestinal epithelial cells, M-cells, and IELs can disturb this balance and lead to a secondary inflammatory response caused by colonizing and translocated gut microbiota. Additionally, if there is an intestinal infection, those anti-inflammatory effects can cause aggravation of the clinical condition. Altogether, anti-inflammatory effects of arginine deiminase of *E. faecium* SF68, either directly by inhibition of NF-κB signaling pathway (non-enzymatic activity) or indirectly by inhibition of arginine-mediated immune modulation (enzymatic activity), can act as a double-edged sword. Therefore, prior to administration of beneficial microbes, health condition, immune status, and age of the recipients should be considered in addition to the nature of the probiotic and its appropriate dose which should cautiously be investigated.

4.3 Concluding remarks

Like other microorganisms, probiotic microbes are complex systems with communication strategies in the body environment, especially in the gut. The metabolites and byproducts, along with their secreted and structural molecules, mediate inhibitory or stimulatory effects on immune responses in the gut. *E. faecium* SF68 is a probiotic strain which has been shown to effectively mitigate inflammatory responses in enteritis and diarrhea in humans and animals. These anti-inflammatory effects mainly appear to deal with the local innate immune responses in the gut. Moreover, this probiotic has been used in animals as a feed additive to enhance their growth and performance. However, *E. faecium* SF68 was not effective in all trials and even unfavorable immunomodulatory effects of this probiotic have been reported. For instance, enhanced colonization and shedding of pathogens were observed in several *Salmonella* challenge studies in piglets supplemented with *E. faecium* SF68.

In this doctoral study, investigation of a direct interaction between *E. faecium* SF68 and intestinal epithelial cells revealed that cell-free, whole bacterial lysates of *E. faecium* SF68 reversibly inhibits NF-κB activation in intestinal epithelial cells of various host species. This inhibitory effect is mediated by the AD-activity of arginine deiminase and associated with arginine depletion. Arginine depletion, via disruption of *de novo* protein synthesis, attenuation of translation rates and activation of autophagy/apoptosis pathways, may cause inhibition of central signaling pathways such as NF-κB. As a result, inflammatory and proliferative responses will be impeded in intestinal epithelial cells. Moreover, activation, differentiation, and proliferation of immune cells such as IELs will be hampered. Nevertheless, the arginine deiminase pathway has byproducts and end products, such as ornithine and ammonia, which can also contribute to the immunomodulatory effects of *E. faecium* SF68. Additionally, arginine and its metabolites can also shift the microbiota populations in the gut and thereby, modulate intestinal immune responses and alter gut homeostasis.

The anti-inflammatory effects of this probiotic strain can be beneficial in recipients with healthy immune system. Moreover, the anti-inflammatory effects of *E. faecium* SF68 can be applicable for amelioration of inflammation and reduction of diarrhea in non-infectious enteritis. In contrast, individuals with underdeveloped or disrupted immunity, such as neonates and immunocompromised patients, could develop complications such as dysbiosis, secondary infections or inflammation by inappropriate utilization of beneficial microbes like *E. faecium* SF68 as preventive or therapeutic agents. Furthermore, in cases of infectious enteritis, suppression of innate immune responses can exacerbate the clinical conditions. Therefore, the administration of *E. faecium* SF68, should be evaluated cautiously and based on the health status of the recipients, their age, immune and metabolic function to achieve the expected

outcomes. Certainly, there are more factors in addition to arginine deiminase playing a role in immunomodulatory effects of *E. faecium* SF68, which should be investigated further. Additionally, it is necessary to determine the effects of AD-knockout *E. faecium* SF68 *in vivo*. In conclusion, more knowledge about the immunomodulatory properties of beneficial microbes and their mechanisms of action will help to make the application of these microorganisms more efficient and predictable.

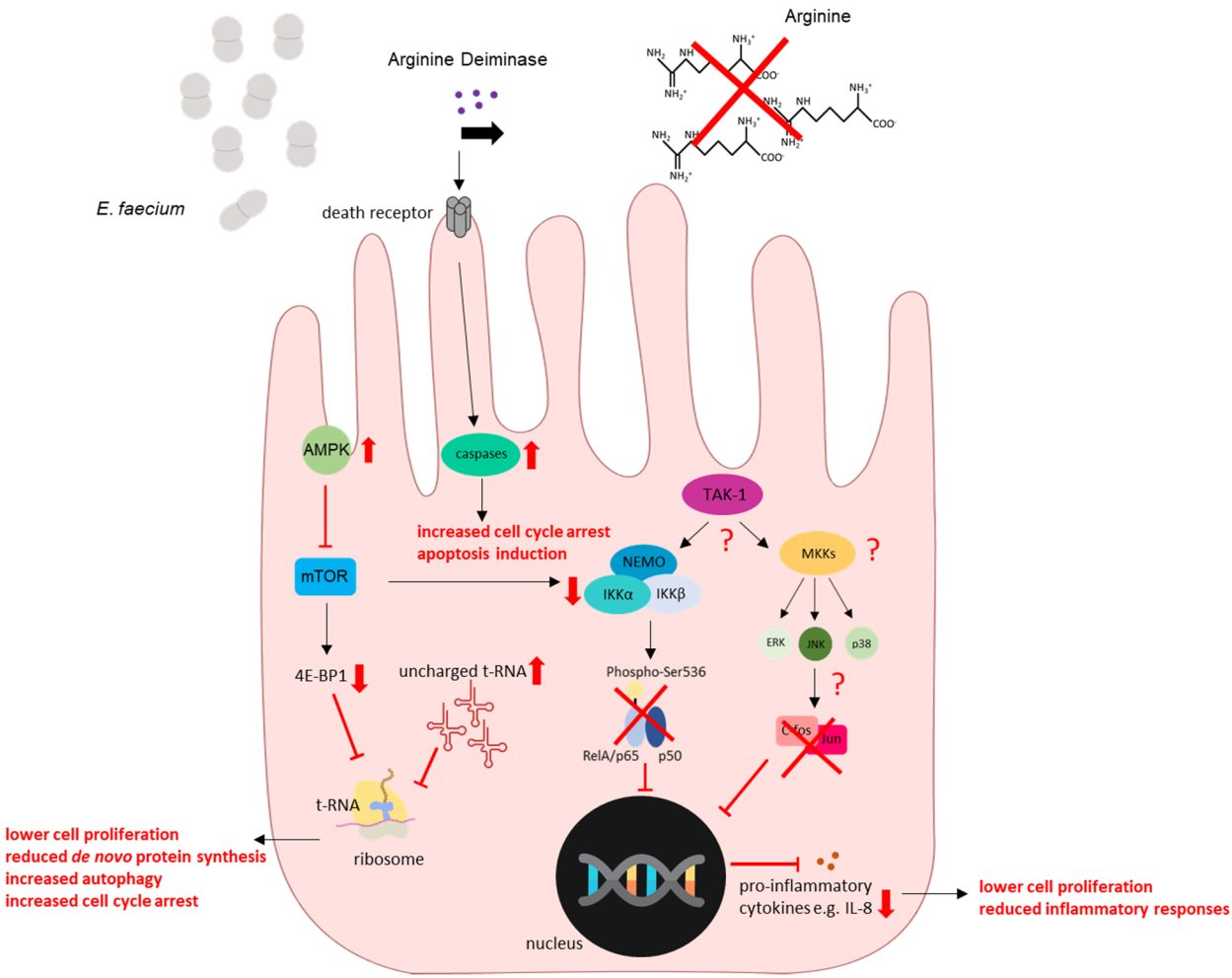

Figure 6: Proposed model for anti-inflammatory effect of probiotic *E. faecium* SF68 promoted by arginine deiminase in intestinal epithelial cells.

E. faecium SF68 possess enzymes of arginine deiminase pathway. Arginine deiminase converts L-arginine to L-citrulline and ammonia. Therefore, enzymatic AD-activity in the presence of arginine and anaerobic condition leads to arginine depletion in the microenvironment of the intestinal epithelial cells exposed to *E. faecium* SF68 *in vitro* and possibly *in vivo*. Arginine deprivation enhances the accumulation of uncharged t-RNA and hence an attenuation in translation rate. In addition, nutrients deficiency such as arginine depletion activates AMPK. Activation of AMPK inhibits mTOR mainly via PI3K/AKT pathway. mTOR inhibition reduces activation of 4E-BP1, thereby de novo protein synthesis will be reduced. As a result, autophagy process will be initiated, and cells undergo cell cycle arrest. Moreover, mTOR inhibition may decreases the stimulation of Ikks and therefore, affects activation of NF-κB and its phosphorylation at serine 536. Arginine unavailability disturb other parallel pathways to NF-κB such as JNK/AP-1 as well. Altogether, hindrance of main inflammatory signaling pathways such as NF-κB and JNK/AP-1 reduces the production of inflammatory cytokines such as IL-8 particularly during cells encounter with pathogens or inflammatory stimulants. Furthermore, CARD-like domain in arginine deiminase has been shown to induce apoptosis in cells via a non-enzymatic mechanism, which should also be considered in anti-inflammatory effects of *E. faecium* SF68 regarding its arginine deiminase.

5 Summary

Identification and characterization of immuno-active factors of *Enterococcus* spp. involved in immunomodulatory effects of probiotic *Enterococcus faecium* SF68

E. faecium SF68 is a licensed probiotic, which has been shown to ameliorate symptoms of inflammation in enteritis and to reduce the incidence and severity of diarrhea in humans and animals. *In vivo* trials with weaning piglets performed in our laboratory or in cooperation with our laboratory, yielded controversial observations on the anti-inflammatory effects of *E. faecium* SF68. Supplementation with the probiotic in animals was reported to contribute to reduced expression of immune-associated genes in intestinal tissues and associated lymphoid organs. Moreover, these animals had lower amounts of serum IgG and fecal IgA, and reduced CD8+ intraepithelial lymphocyte populations. Piglets supplemented with *E. faecium* SF68 showed higher colonization and greater pathogen shedding in *Salmonella* infection studies. Since the alterations observed were mainly associated with local innate immune responses, this doctoral study focused mainly on the direct immunomodulatory effects of *E. faecium* SF68 on intestinal epithelial cells in an *in vitro* model. NF-κB, one of the key initiators of innate immune responses in intestinal epithelial cells encountering microbiota and invasive enteric pathogens, was selected as a reporter for determination of the effects of *E. faecium* SF68 on immune response modulation in intestinal epithelial cells.

Cell-free, whole bacterial lysates of *E. faecium* SF68 inhibited NF-κB activation in intestinal epithelial cells from different host species. This inhibitory effect occurred in a reversible manner; after removal of the lysate and addition of fresh culture medium, cells recovered their NF-κB activity. Cells treated with the bacterial lysates ceased to proliferate, based on the results of host cell cycle protein Ki67 staining. Porcine intestinal epithelial cells treated with the probiotic lysate contained lower levels of phosphorylated p65 subunit of NF-κB at serine 536 compared to untreated cells. Phosphorylation at this amino acid is one of the most studied modifications involved in the transcriptional activity of p65 subunit. Moreover, fluorescence microscopy images of porcine intestinal cells suggested a dysregulation in the trafficking of NF-κB between the cytoplasm and nucleus.

Ammonium sulfate (AS) fractionation of proteins of the *E. faecium* SF68 lysates and analysis of the proteins present in different AS-fractions revealed that several enzymes involved in arginine metabolism are present in the active AS-fraction. Other commensal and clinical *E. faecium* isolates, as well as isolates of other enterococcal species possessing enzymes of arginine deiminase pathway including *E. faecalis*, *E. hirae* and *E. durans*, exhibited the same inhibitory effect on NF-κB activity of IPEC-J2 cells. *E. gallinarum* and *E. casseliflavus*

species were the two exceptions. These two species are the only motile enterococcal species expressing flagellin which is a strong stimulant of inflammatory pathways such as NF-κB, particularly in our *in vitro* cell model. Experiments examining pre- and co-incubation of host cells with *E. faecium* SF68 lysates and different Toll-like receptor (TLR)- and NOD-like receptor (NLR)-ligands showed that cells pre-treated with the *E. faecium* SF68 bacterial lysates were severely impaired in their NF-κB activation responses to these pathogen-associated molecular pattern (PAMP) signals. Lysates from strains of other enterococcal species with no AD pathway activity (*E. avium*, *E. cecorum* and *E. raffinosus*) did not inhibit the NF-κB activity of treated IPEC-J2 cells. The *arcA* gene of *E. faecium* SF68 encoding arginine deiminase was cloned into an *E. coli-Enterococcus* shuttle vector and introduced into an *E. avium* strain which showed no inhibitory effects on NF-κB activity. Transformants of *E. avium* constitutively expressing arginine deiminase of *E. faecium* SF68 showed the same NF-κB-inhibitory effects on porcine and human intestinal epithelial cell lines as *E. faecium* SF68. Comparison of results with the *Streptococcus suis* 10 strain, another bacterial genus possessing AD-pathway enzymes, exhibited the same results. Furthermore, an *arcA* knockout mutant of *S. suis* 10 no longer showed inhibition of NF-κB activity of host cells and purified recombinant arginine deiminase of *S. suis* 10 was shown to have the same inhibitory effects on NF-κB activity as the cell-free, whole bacterial lysates of *S. suis* 10 and *E. faecium* SF68. Finally, arginine supplementation of the cell culture medium eliminated the inhibitory effects on NF-κB activity mediated by arginine deiminase or bacterial lysates containing arginine deiminase.

In summary, we postulate that the reported anti-inflammatory effects of *E. faecium* SF68 *in vitro* and likely *in vivo*, are associated with arginine deiminase-mediated arginine depletion. Beneficial bacteria in general can have a large number of molecules and metabolites, allowing them to interact with intestinal epithelial and immune cells. Therefore, the immunomodulatory effects elicited by beneficial microbes in the gut may not be limited to a single factor. Additionally, the health condition of the recipients, their age and immune status can determine the outcome of beneficial microbe administration. For instance, neonates with underdeveloped immune systems, not fully shaped microbiota and higher metabolic activity are more sensitive to arginine depletion (Flynn et al. 2002). Moreover, the concurrent presence of an intestinal pathogen such as *Salmonella* or *Giardia*, which use arginine depletion as a defensive strategy to hinder the host iNOS production and proliferation of immune cells, can aggravate the clinical condition. These pathogens promote arginine depletion by their own AD-pathway enzymes or induction of arginine consuming enzymes in the host cells. The mechanisms of action of beneficial microbes should be studied well and in detail. More importantly, the assessments and decisions on the administration of a beneficial microbe as therapeutic or preventive measure should be made cautiously and individually for each case to optimize the use of these advantageous biological tools.

6 Zusammenfassung

Identifizierung und Charakterisierung von immunaktiven Faktoren von *Enterococcus* spp., die an den immunmodulatorischen Effekten des Probiotikums *Enterococcus faecium* SF68 beteiligt sind

E. faecium SF68 ist ein lizenziertes Probiotikum, dass nachweislich Entzündungssymptome bei Enteritis lindert und die Häufigkeit und Schwere von Durchfall bei Menschen und Tier reduziert. In einigen *In vivo*-Studien, die in unserem Labor oder in Zusammenarbeit mit unserem Labor durchgeführt wurden, wurden kontroverse Beobachtungen über entzündungshemmende Effekte von *E. faecium* SF68 bei Ferkeln gemacht. Es wurde berichtet, dass die Supplementierung von Tieren mit dem Probiotikum zu einer geringeren Expression von immun-assoziierten Genen des Darmgewebes und der assoziierten lymphoiden Organe beiträgt. Außerdem hatten diese Tiere ein niedrigeres Serum-IgG und fäkales IgA. Darüber hinaus wurden bei diesen Tieren geringere CD8+ intraepitheliale Lymphozyten-populationen nachgewiesen. Ferkel, die gleichzeitig mit Salmonellen infiziert wurden, hatten eine höhere Kolonisierung und eine größere Ausscheidung der Infektionserreger. Da die beobachteten Veränderungen hauptsächlich mit lokalen angeborenen Immunantworten in Verbindung gebracht wurden, lag der Fokus dieser Doktorarbeit auf der Analyse direkter immunmodulatorischer Effekte von *E. faecium* SF68 auf intestinale Epithelzellen in einem *in vitro* Modell. NF-κB, einer der wichtigsten Regulatoren angeborener Immunantworten in Darmepithelzellen, die auf Mikrobiota und invasive enterische Pathogene treffen, wurde als Kriterium für die Bestimmung der Modulationen der Immunantwort in Darmepithelzellen ausgewählt.

Das zellfreie Gesamtlysat von *E. faecium* SF68 hemmte die NF-κB-Aktivierung in intestinalen Epithelzellen, die von verschiedenen Wirtsspezies stammen. Dieser inhibitorische Effekt trat reversibel auf. Durch Entfernen des Lysats und Zugabe von frischem Kulturmedium erlangten die Zellen ihre NF-κB-Aktivität wieder. In Schweine-Darmepithelzellen, die mit dem probiotischen Lysat behandelt wurden, kam die phosphorylierte p65-Untereinheit von NF-κB an Serin 536 im Vergleich zu unbehandelten Zellen in geringerer Menge vor. Darüber hinaus deuteten fluoreszenzmikroskopische Aufnahmen von Schweine-Darmzellen auf eine Behinderung des Trafficking von NF-κB zwischen Zytoplasma und Zellkern hin. Zudem waren die mit dem bakteriellen Lysat behandelten Zellen nicht proliferativ, was mittels Zellproliferationsassay und Ki67-Färbung nachgewiesen wurde.

Die Ammoniumsulfat-Fraktionierung von *E. faecium* SF68-Lysat und die Vergleichsanalyse der in den verschiedenen AS-Fraktionen vorhandenen Proteine ergab, dass mehrere am

Arginin-Stoffwechsel beteiligte Enzyme in der aktiven AS-Fraktion vorhanden waren. Andere kommensale und klinische *E. faecium*-Isolate sowie Isolate anderer Enterokokken-Spezies, die Enzyme des Arginin-Deiminase-Pfads besitzen, einschließlich *E. faecalis*, *E. hirae* und *E. durans*, zeigten die gleiche inhibitorische Wirkung auf die NF-κB-Aktivität von IPEC-J2-Zellen. Die beiden Ausnahmen waren die Spezies *E. gallinarum* und *E. casseliflavus*. Diese beiden Spezies sind die einzigen motilen Enterokokken-Spezies, also Flagellin exprimieren. Flagellen sind ein starker Stimulator von Entzündungswegen, wie z.B. NF-κB, insbesondere in unserem *In vitro* Zellmodell. Darüber hinaus zeigten unsere Co-Inkubationsexperimente von Zellen mit *E. faecium* SF68-Lysat und verschiedenen TLR- und NLR-Liganden, dass die Verabreichung von Flagellin auf Zellen, die mit dem bakteriellen Lysat von *E. faecium* SF68 vorbehandelt wurden, die NF-κB-Aktivierung zusätzlich zu der inhibitorischen Wirkung des Lysats stimuliert. Lysat von Stämmen anderer Enterokokken-Spezies, die keine Enzyme des AD-Wegs enthalten, wie *E. avium*, *E. cecorum* und *E. raffinosus*, hingegen hemmten die NF-κB-Aktivität der behandelten IPEC-J2-Zellen nicht.

Im nächsten Schritt wurde das *arcA*-Gen von *E. faecium* SF68, das Arginin-Deiminase exprimiert, in einen *E. avium*-Stamm kloniert, der keine hemmende Wirkung auf die NF-κB-Aktivität der Zellen besaß. *E. avium*, das die Arginin-Deiminase von *E. faecium* SF68 konstitutiv exprimiert, hatte die gleichen NF-κB-hemmenden Effekte auf porcine und humane intestinale Epithelzelllinien wie *E. faecium* SF68. Darüber hinaus zeigten Tests mit *S. suis* 10-Stamm aus anderen Bakteriengattungen, die AD-Weg-Enzyme besitzt, die gleichen Ergebnisse. Außerdem konnte die *arcA*-Knockout-Mutante von *S. suis* 10 die NF-κB-Aktivität der Zellen nicht beeinträchtigen. Schließlich wurde gezeigt, dass überexprimierte und gereinigte rekombinante Arginin-Deiminase von *S. suis* 10 die gleiche hemmende Wirkung hatte wie das zellfreie Gesamtlysat von *S. suis* 10 und *E. faecium* SF68. Die Arginin-Supplementierung des Kulturmediums wiederrum eliminierte die suppressiven Effekte auf die NF-κB-Aktivität, die durch Arginin-Deiminase oder bakterielle Lysate, die Arginin-Deiminase enthalten, vermittelt werden.

Insgesamt postulieren wir, dass die entzündungshemmenden Effekte von *E. faecium* SF68 *In vitro* und möglicherweise auch *In vivo* mit dem Arginin-Deiminase-vermittelten Arginin-Abbau verbunden sind. Allerdings haben probiotische Bakterien im Allgemeinen eine große, komplizierte Anzahl von Molekülen und Metaboliten, wodurch sie mit intestinalen Epithel- und Immunzellen interagieren können. Daher können die immunmodulatorischen Effekte, die probiotische Mikroben im Darm hervorrufen, nicht auf einen einzigen Faktor zurückgeführt werden. Darüber hinaus können der Gesundheitszustand der Empfänger, ihr Alter und ihr Immunstatus für das Ergebnis der Verabreichung von nützlichen Mikroben ausschlaggebend sein. So sind zum Beispiel Neugeborene mit einem unterentwickelten Immunsystem, einer

nicht vollständig ausgebeildeten Mikrobiota und einer höheren Stoffwechselaktivität empfindlicher gegenüber einer Arginin-Verarmung. Darüber hinaus kann das gleichzeitige Vorhandensein eines Darmpathogens, wie Salmonellen oder Giardien, die die Arginin-Depletion als Verteidigungsstrategie zur Behinderung der iNOS-Produktion und der Proliferation von Immunzellen im Wirt nutzen, den klinischen Zustand verschlimmern. Diese Erreger fördern den Arginin-Abbau durch ihre eigenen AD-Weg-Enzyme oder die Induktion von Arginin-verbrauchenden Enzymen in den Wirtszellen. Die Wirkungsmechanismen probiotischer Mikroben sollten gut und detailliert untersucht werden. Noch wichtiger ist, dass die Bewertungen und Entscheidungen über die Verabreichung einer probiotischen Mikrobe als therapeutische oder präventive Maßnahme in jedem Fall vorsichtig und individuell getroffen werden sollten, um den Einsatz dieser vorteilhaften biologischen Werkzeuge zu optimieren.

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Interessenskonflikte – Conflict of Interest

There is no conflicts of interest to be declared.

Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 10.08.22, Fereshteh Ghazisaeedi