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**Effect of dietary *Enterococcus faecium* NCIMB 10415 and zinc oxide  
on porcine influenza virus infection *in vitro* and *in vivo***

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**Dedicated to my dear parents and wife**

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## LIST OF ABBREVIATIONS

BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
bp	base pairs
BSL	Bio-safety level
CCL	Chemokine ligands
CFU	Colony forming units
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocytes
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dpi	days post infection
<i>E.</i>	<i>Enterococcus</i>
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
FACS	Fluorescence-activated cell sorting
GH	Growth hormone
GIT	Gastrointestinal tract
HA	Hemagglutinin
HE	Hematoxylin and eosin
HI	Hemagglutination inhibition
hpi	hours post infection
HIV	Human immunodeficiency virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
MDBK	Madin-Darby Bovine Kidney

## List of Abbreviations

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MDCK	Madin-Darby Canine Kidney
MOI	Multiplicity of infection
NA	Neuraminidase
NK cells	Natural killer cells
NO	Nitric oxide
NP	Nucleoprotein
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RT	Reverse transcription
RV	Rotavirus
SIV	Swine influenza virus
TCID <sub>50</sub>	50% tissue culture infective doses
TGEV	Transmissible gastroenteritis virus
Th	T-helper
TNF	Tumor necrosis factor
VSV	Vesicular stomatitis virus
VAC	Vaccinated
WHO	World Health Organization
Zn	Zinc
ZnO	Zinc Oxide

## List of Abbreviations

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# 1 INTRODUCTION

## 1. 1 Swine influenza virus

Influenza virus belongs to the family *Orthomyxoviridae* which consists of viruses that have a negative-sense, single-stranded, and segmented RNA genome. Swine influenza virus is common throughout pig populations worldwide and causes significant economic losses in the livestock industries [1]. Three main SIV subtypes H1N1, H1N2 and H3N2 are currently circulating in the swine population despite regular vaccinations, and exchange of influenza viruses between human and swine is common and not a one-way street [2,3].

### 1. 1. 1 Historical overview

Swine influenza virus was first recognized clinically in pigs during the “Spanish influenza” in 1918 and 1919, pigs presented with symptoms similar to those observed in humans, which are nasal discharge, coughing, fever, labored breathing, and conjunctivitis [1]. Phylogenetic analyses indicated that the 1918/1919 human and swine viruses were genetically similar and likely originated from a common ancestor [4,5]. The first swine influenza virus (A/swine/Iowa/15/30 [H1N1]) was isolated by Shope in 1930 [6]. Since then, swine influenza was found to be enzootic and today is the most prevalent respiratory disease in pigs. For the following 40 years, swine influenza strains were almost exclusively H1N1- subtypes [1].

In the mid-1970s, human H3N2 influenza viruses were reported to be transmitted to pigs in Italy for the first time [7]. Reassortant viruses of the H1N2 subtype derived from human and avian viruses in Great Britain and other parts of Europe in the late 1970s [7-10]. Viruses of the H1N2 subtype have also been derived from genetic reassortants which emerged from strains endemic in pigs, and have been established in pigs in Japan since 1978 [1]. Pigs have been firstly regarded as the amalgamation unit for the development of new influenza viruses since swine, avian, and/or human influenza viruses undergo recombination in pigs [11]. It is of high epidemiological and public health significance that swine influenza viruses are able to infect humans, as was shown when a person died of swine influenza infection in New Jersey USA in

1976 [1].

In 1998, an outbreak of swine flu was found in pigs initially in four U.S. states. It had spread through pig populations across the United States and Europe within a year [12,13]. Scientists found this virus had originated from pigs as a recombinant flu strain from birds and humans [14]. This outbreak confirmed that pigs can serve as a “mixing vessel” [15] where novel influenza viruses emerge as a result of reassortment of genes from different strains. It was also confirmed that this strain is much related to the H1N1 strain of classical swine influenza virus which caused the 1918 flu pandemic [14,16]. Genetic information of these 1998 triple-hybrid strains showed six of the eight viral gene segments presented in the 2009 flu outbreak [17,18].

The 2009 flu pandemic (swine-original flu) was the second of the two influenza virus pandemics involving H1N1 influenza virus (the first one being the 1918 flu pandemic). The virus was initially characterized and classified in April 2009, and appeared to be a new strain of H1N1 which apparently emerged when a previous triple reassortant of bird, swine and human flu viruses further combined with an Eurasian pig flu virus [19,20]. Based on these findings, authorities introduced the term "swine flu" for this pandemic (World Health Organization (WHO)). As stated above, the phylogenetic origin of the flu virus that caused the 2009 pandemics can be traced back to 1918 [21]. According to the WHO statistics (July 2010), the virus has killed more than 18,000 people since it appeared in April 2009 [22]. However, experts including the WHO have agreed that an estimated 284,500 people were killed because of this disease by 2012, much higher than the initial death toll [23].

This brief historical view emphasizes that undoubtedly swine influenza viruses play an important and threatening role both for animal health and for public health. Thus, any scientific efforts that aim to controlling the spread of swine influenza virus are of primary public interest.

### **1. 1. 2 Clinical disease in pigs**

Swine influenza virus (SIV) infections can cause respiratory problems characterized by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, difficult breathing, and depressed appetite [1]. In some cases, SIV infections are associated with reproductive disorders such as abortion [24]. Although mortality is usually low (around 1–4%),

the infection can result in weight loss and poor growth especially when accompanied by secondary infections. This causes suffering of the infected animals as well as serious economic losses to the farming industries [1,25].

Two forms of the SIV infection are defined in pigs, the epidemic and the endemic. In the epidemic one, the virus usually quickly infects all parts of the swine production unit with rapid recovery and no complicating factors such as secondary bacterial infections [1]. In the endemic one, clinical signs may be less obvious [1]. However, in this form, the virus prevails in the hosts for longer periods of time and thus allows for both, emergence of new reassortants and a continuous source for further spread.

Clinical signs and nasal shedding of the SIV can occur within 24 hours (h) after infection. Morbidity can reach 100%, while mortality rates are generally low” [1,24]. Combined viral–bacterial pneumonia is quite common and can exacerbate the clinical signs. Microscopic lesions usually consist of airways filled with exudate, widespread alveolar atelectasis, interstitial pneumonia, emphysema, peribronchial and perivascular cellular infiltration [25,26].

A major consequence of swine influenza is the economic loss with retarded growth and prolonged finishing time. It has been documented that these pigs can lose body weight from 5 to 12 pounds over a 3 to 4 week period [24,25]. Human infections with swine-origin influenza virus can occur and a large number of deaths has been reported [23,27].

### **1. 1. 3 Structure and classification**

Swine influenza virus is classified as a type A Orthomyxovirus with a segmental RNA genome. The viral envelope, derived from the host plasma membrane, consists of a lipid bilayer containing viral trans-membrane proteins hemagglutinin and neuraminidase (HA and NA) and two viral matrix proteins (M1 and M2). HA is the major envelope protein above the virion surface [28], it provides the receptor-binding site and elicits neutralizing antibodies. HA binds to host cell receptors that contain terminal -2, 6-linked or -2, 3-linked sialic acid (-2, 6-SA or -2, 3-SA) moieties. Proteolytic cleavage of HA is essential for virus fusion with host cell membranes and infectivity [18]. An important function of neuraminidase, the second major antigenic determinant, is to catalyze the cleavage of glycosidic linkages to sialic acid, thereby

assisting in the release of progeny virions from infected cells [29]. Accordingly, neuraminidase has become an important target for antiviral activity [29]. M2, an ion channel, is crucial during uncoating for dissociating the virus ribonucleocapsids (vRNP) from M1 in the early phase of the infectious cycle [29]. The viral core consists of helical vRNP containing vRNA (negative stranded) and nucleoprotein (NP) along with minor amounts of the nuclear export protein (NEP) (also called non-structural protein NS2) and three polymerase proteins (PB1, PB2, and PA) which form the viral RNA polymerase complex [30-32]

It is world acknowledged so far that three genera of Influenza virus, which are identified by antigenic differences in the nucleoprotein and matrix protein and could be infected vertebrates as follows: Influenza A virus causes all flu pandemics which infects humans, other mammals and birds. Influenza B virus infects humans and seals. Influenza C virus infects humans and pigs [33]. Influenza A virus, subtypes are classified by antigenic and genetic properties of the two surface proteins H and N [18]. Currently, 17 H (H1–H17) (H17 recently described in [34]) and 9 N subtypes (N1–N9) are known. Subtypes of SIV that are most frequently identified in pigs include H1N1, H1N2, and H3N2. Other subtypes that have been identified in pigs include H1N7, H3N1, H4N6 and H9N2 [21,35].

The uses of an internationally accepted naming convention for influenza viruses include the following components (in sequence): the antigenic type, the host of origin, the geographical origin, the strain number, and the year of isolation. Finally, for influenza A viruses, a full description of the hemagglutinin and neuraminidase antigen should be given. However, no host of origin designation is given for human-origin viruses. An appropriate example for this is *A/swine/Iowa/15/1930* [H1N1].

### **1. 1. 4 Virus transmission**

Swine-origin influenza viruses can infect many species of animals, such as birds, horses and humans. In the swine industry, outbreaks are most common in fall and/or winter, often at the onset of cold weather. Usually, an outbreak is preceded by 1 or 2 individual cases and then spreads rapidly within a herd [36]. The direct transfer of the virus probably occurs either by pigs touching noses, or through dried mucus. Transmission through aerosols produced by

coughing or sneezing pigs is regarded as the predominant means of infection [1]. Therefore, transmission mainly occurs through direct contact between infected and uninfected animals. Close contacts are particularly common during animal transport. Intensive farming may also increase the risk of transmission as pigs are raised in production units with high animal densities [1,36]. Transmission may also occur through wild animals, such as wild boar, which can spread the disease between farms [37]. After infection of an individual pig, the virus usually spreads rapidly through a herd, infecting all the pigs within just a few days [24]. However, the virus is unlikely to survive outside living cells more than two weeks except in cold conditions. It can be readily inactivated by disinfectants [37].

A number of findings have suggested a role for pigs in the emergence of pandemic influenza viruses. First of all, epithelial cells in pig trachea contain both human and avian type receptors ( $\alpha 2, 6$ - and  $\alpha 2, 3$ -linked sialic acid, respectively) [38]. Secondly, pigs can be naturally or experimentally [39] infected with avian viruses. Thirdly, interspecies transfer and reassortant events have led to the establishment of two new lineages of pig viruses since 1979, demonstrating that pigs can host genetically diverse viruses [40,41]. Last but not least, swine viruses and avian-human reassortant viruses can infect humans and, in some cases, cause fatal disease [23,42]. These observations support the “mixing vessel” [15] hypothesis that pigs are simultaneously infected with avian and human influenza viruses, which allow for the generation of reassortants capable of causing pandemics. It is noteworthy that, in recent decades, human infections with swine viruses have been self-limiting, which may indicate that mutational changes are required for transmission to and among humans [3,12].

### **1. 1. 5 Prevention and treatment**

Vaccination is the method that is widely employed to control influenza virus infection. Influenza virus vaccines that are commercially available for humans, horses, birds and pigs are in the forms of inactivated, whole virus and subunit vaccines. Although these vaccines may decrease the incidence and severity of clinical disease, they are not sufficient to cause full viral clearance [43]. In a flu vaccine, the adjuvant usually used around world is water-oil mixture. Current swine influenza vaccines are adjuvanted, inactivated, whole-virus vaccines

prepared from virus propagated in embryonated hen eggs [44]. Effective control of SIV infection at present likely requires simultaneous vaccination against both H1 and H3 subtypes. These two subtypes of viruses have been infecting pigs for decades [43]. However, current vaccines still lack full effectivity because it cannot possibly cover all the strains actively infecting pigs around the world.

There is no effective treatment after SIV infection, although antibiotics may reduce secondary bacterial infections. Expectorants may help relieve clinical signs in severely affected herds [1]. Most viruses have sustained antiviral drug development. Thus controlling an ongoing infection with SIV or any other viral infection is a huge challenge at farm situations [45]. At present, partial depopulation, segregation of early weaned piglets, all-in-all-out system and hygiene measures, are considered essential steps to control the spread of the disease around the farm and to minimize the effects of the disease on the farm's economy [45]. Vaccination and strict import controls are the only specific preventive measures.

The history of swine influenza in countries all over the world suggests that the future is not predictable and that continued changes are likely to last forever [21]. Therefore, therapeutic alternatives for preventing infections and maintaining the health of livestock are highly warranted.

### **1. 1. 6 Immune response**

The immune responses to infection with influenza virus have been studied extensively. Once the epithelial cell is infected with influenza virus, a complicated series of intracellular events are triggered [46]. Proinflammatory cytokines, notably interleukin (IL)-6 and IFN- $\alpha$ , are induced when experimentally infected with influenza virus and released into the respiratory tract, reaching peak level by day 2 after experimental infection [47]. The peak release of these two cytokines corresponds with most severe clinical symptom score, highest mucus production, fever, and viral load. Other cytokines either appear later (IL-8 and TNF- $\alpha$ ) or do not increase (IL-1 $\beta$ , IL-2) in the respiratory secretions or serum [48,49]. In a porcine model, infection of alveolar macrophages leads to the induction of tumor necrosis factor alpha (TNF- $\alpha$ ), which has been implicated as being inhibitory to influenza virus [49,50].

The respiratory tract has multiple non-specific protective tissue areas and/or mechanisms against influenza virus infection, including the mucin layer, ciliary action, and protease inhibitors, that may prevent effective cell entry and virus uncoating [46]. The extremely short incubation period between infection and clinical illness implies that innate immunity or preformed cognate recognition components are important contributors to provide defense [46].

Knowledge related to host adaptive immune responses in the SIV-infected pigs is limited. There was a study reported pigs infected with H3N2 and H1N1 viruses increased the frequency of neutrophils, NK cells, and CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the BAL fluid [51]. Another study showed pigs infected with the pandemic H1N1 virus activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the peripheral blood on 6 dpi [52]. Higher frequencies of cytotoxic T lymphocytes,  $\gamma\delta$  T cells, dendritic cells, activated T cells, and CD4<sup>+</sup> and CD8<sup>+</sup> T cells were detected in SwIV-infected pig lungs were also reported [53].

Most of the SIV infections do not show defined clinical signs suggesting that many influenza infections remain subclinical. However, subclinical infections can result in immunity possibly for the rest of the life span [1,54]. Protection by maternally derived antibodies during early infection may have accounted for the absence of clinical signs [1,54]. In another experiment, it was shown that piglets with high maternally derived antibody levels developed weaker immunity than pigs with a low level of maternally derived antibodies [55]. There was also another study which either failed to show any maternal protection or a level of protection depended on the level of maternally derived antibodies [56]. Besides differences in levels of maternally derived antibodies, the age of the host animals at the moment of infection and/or the influenza strain used for experimental infection may have affected the results of the above findings [54].

### 1. 2 Probiotics

#### 1. 2. 1 Overview of probiotics

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the "host" (Food and Agriculture Organization/WHO, 2001). Due to increasing safety concerns about the risk of inducing antibiotic resistance in the environment, and the persistence of chemical residues in animal products, antibiotics have been widely restricted [57], other strategies based on supplementation of more "natural" products such as probiotics, have been developed to improve animal health and productivity [58]. Increasing amounts of scientific data are supporting the view that these products can beneficially affect the balance of gastrointestinal tract (GIT) microbiota and that they have a real benefit in animal nutrition and health [57,58]. It has been recognized that the beneficial effects of probiotics are shared among a vast number of genera and species belonging not only to the human or animal GIT, but also to the respiratory tissues by affecting mucosal immune response [59-61]. Many of them have been utilized for many years without causing any problem [58]. Probiotics are also reported to promote the host defense and to modulate the immune system, supporting the use of probiotic as an alternative to antibiotics in improving animal health and protection against infectious agents [62]. There is evidence that some specific probiotics can modulate monocyte and natural killer cell function [63-65]. Evidence is also accumulating that particular probiotics can boost antibody response to oral and systemically administered vaccines [63,64].

Probiotic activity can be related to genera, species, or strains. The most commonly investigated and commercially available probiotic species are *Lactobacillus*, *Saccharomyces*, and *Bifidobacterium*. In addition, several other species such as *Enterococcus*, *Propionibacterium*, *Peptostreptococcus*, *Clostridium*, *Streptococcus*, *Bacillus*, and yeasts are also used. Among these, *Lactobacillus*, *Enterococcus* and *Bacillus* are actually the most used probiotics in livestock and poultry [58]. An approach in probiotic application could be the use of mixtures of strains belonging to different genera or species. Dose, timing and duration of the administration of probiotics may be a factor affecting efficacy [57,66].

Future research should focus on determining the mechanism of probiotic, elucidating how the genetic and bacterial profiles of the host can influence treatment responsiveness. Better knowledge of the structure and activities of the gut microbiota, functional interactions between gut microbes and interrelationships between microbes and host cells represent a fundamental aspect of probiotic research [57]. The future plan should focus on increasing the genomic information on both probiotic and microbiota activities and improving the understanding of the interactions with specific diseases [58]. The advanced molecular methods, such as microarrays will improve the detection of these multiple characteristics, also allowing the analysis of phenotypic and genetic properties which could be useful for industrial production [58].

### **1. 2. 2 *Enterococcus (E.) faecium* as a probiotic**

Probiotic strains of *Enterococcus faecium* have a long history of apparent safe use in industrial and agricultural applications [58]. *E. faecium* NCIMB 10415 is authorized in the EU as a probiotic feed additive for pigs and seems a suitable probiotic that allows us to study possible anti-viral efficacy. It has been demonstrated that this *E. faecium* strain modulates the intestinal immune system in sows and piglets and that it affects the shedding of porcine enteric viruses [67,68]. *In vitro* experiments also showed direct antiviral effects of *E. faecium* against enteric and non-enteric viruses [69,70]. The potential mechanisms include pathogen exclusion by means of competition for attachment as well the induction of cytokines and signaling molecules which might stimulate host-cell immune defense [69,70].

### **1. 2. 3 Probiotics in virus infections**

Probiotic feed supplementation may benefit an animal host directly. Recent experimental studies *in vitro* suggested that certain strains of probiotics were capable of providing protection against virus infections by combating the causative agent and by stimulating antiviral cytokine and chemokine responses in respiratory and gastrointestinal epithelial cells [71]. In pig alveolar macrophages live or heat-inactivated *Lactobacillus* and *Bifidobacteria* equally increased cell survival against vesicular stomatitis virus (VSV) infection depending on

the strain used [72]. Botić et al. [73] also reported similar results using probiotics to protect a porcine macrophage cell line against VSV disruption. Later on, their group [74] continuously proved a protective effect of lactic acid bacteria against rotavirus (RV) and transmissible gastroenteritis virus (TGEV) on animal and human intestinal and macrophage cell lines.

Animal experiments have also shown that certain strains of probiotics provide protection against respiratory virus [75]. *Lactobacillus* appears to provide significant benefit when administered during vaccination as indicated by the higher body weight gain following PRRS virus infection although there was no effect of this probiotic on the prevalence or duration of viraemia and virus shedding [75]. In addition, oral administration of *lactobacilli* in mice may affect respiratory virus infections (such as influenza) by reducing the virus titer in the lungs, and by increasing the survival rate of the animals via stimulating their innate immune responses [76-81] suggest that probiotics may also be beneficial in the treatment and prevention of respiratory virus infection in clinical studies.

### **1. 2. 4 Mechanisms of probiotics in virus infections**

*In vitro* and *in vivo* studies have demonstrated that specific probiotics are effective in viral infections, and possible antiviral mechanisms of probiotics can be divided to non-immunological (physiological barriers) and immunological defense.

Non-immunological barriers represent an important and often the first line of defense. The intake of probiotics has been suggested to strengthen the intestinal barrier integrity [82] and protect against microbial and viral infection by producing antimicrobial substances, competing with pathogens for adhesion receptors, stimulating mucin production, stabilizing the gut mucosal barrier and improving gut motility [61,64]. Probiotic may bind directly to the virus, and inhibit virus attachment to the host cell receptor through their higher affinity for nutrients or adhesion sites [61]. There is evidence that *in vitro* specific strains of *Lactobacilli* and *Bifidobacteria* are able to bind and inactivate RV [83] and VSV [72,73]. Intestinal mucins may bind to viruses through specific mucin-bacterial/viral interaction, inhibit their adherence to the epithelial cells [84,85], and inhibit virus replication [86] by influencing the regulation of mucin gene expression. There is also reported that the production of organic acids (lactic or acetic

acid) by probiotics can help to decrease the gut pH, create more favorable ecological conditions for the resident microbiota and decrease the risk of pathogen colonization [87]. It is also noteworthy that the induction of reactive nitric oxide ( $\text{NO}^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) release by cell lines co-incubated with probiotics could also contribute to the antiviral activities at the cellular level [69,74].

Probiotics also interact with the host, by influencing the immune response [60,62,64,88-90]. An optimally functioning immune system is fundamental for protection against infectious diseases [71]. Probiotics with proven immunostimulatory properties may be appropriate candidates for the prevention, moderation or treatment of viral infections [61]. One possible mechanism of probiotics against virus infections could be the stimulation of the gut immune system [88,89]. Probiotics may modulate cytokine expression patterns within the intestinal epithelial cell layers [88,89], and in the underlying macrophages and dendritic cells [91-93]. A large body of evidence has proven that probiotics induce the production of proinflammatory cytokine (tumor necrosis factor- $\alpha$ , IL-1, IL-6, and IFN- $\gamma$ ) and chemokine ligands (CCL) [91,93]. Data from animal studies indicate that several specific *Lactobacilli* and *Bifidobacteria* provide protection against respiratory and gastrointestinal virus infections by inducing the synthesis of virus-specific Igs in intestinal and respiratory secretions, in Peyer's patch cells and in serum [79,94,95] and by increasing specific antibody response to vaccination [61,96-98].

### **1. 2. 5 Probiotics and influenza virus infection**

Influenza virus infection is often treated with antibiotics to control secondary bacterial infection [63]. Antibiotic resistance, which is in large part attributed to antibiotic overuse, has encouraged investigators to seek for alternative methods for either preventing or treating the common cold and influenza virus infection (flu) [63,99]. Evidence suggesting that the consumption of certain probiotics could reduce the risk of some common viral infections such as the common cold or influenza draws up great interest [99,100]. Although not obvious on first glance, the respiratory tract is a suitable area for probiotic immune stimulation, because its mucosal surfaces are functionally linked to other mucosal surfaces of the common mucosal-associated lymphoid tissues [100]. Studies of respiratory virus infections in mice

provide strong evidence that certain strains of probiotics such as *Lactobacilli* and *Bifidobacteria* protect from influenza virus infection by reducing virus titers in the lungs and nasal swabs, or increasing body weight and mice survival rates during infection [76-81,94,100]. It seems that the decrease of virus titer in the upper respiratory tract to 1/10 of the control level was an important parameter in preventing death [76-81,94,100]. Inductions of IL-12, IFN- $\gamma$  gamma-interferon, and TNF- $\alpha$ , which are known to counteract influenza virus infection, was evident in mediastinal lymph node cells of the rodent animal model [76-81,94,100]. These findings in the mouse system suggest that administration of probiotics could also enhance cellular immunity in the respiratory tract of other hosts such as swine, thereby protecting the pig against influenza virus infection [76]. No such studies have been conducted hitherto.

### 1. 3 Zinc

#### 1. 3. 1 Overview of zinc

Zinc (Zn) is an essential trace element for humans and animals. It is a cofactor for more than 300 enzymes [101-103]. In some enzymes Zn confers structural integrity, whereas in other enzymes it is the central ion for functional activity, but sometimes it serves both these functions [104]. Zinc is also supposed to be the only metal which appears in all enzyme classes [105]. It plays an important role in antioxidant enzymes, DNA-, RNA polymerases and also in hormones and ionophores. Proteins which carry Zn as a functional component are often involved with enhancing host resistance to inflammatory responses and various viruses' infections [103,105].

Zinc plays a critical role in homeostasis of the immune system. It is widely accepted that zinc deficiency decreases NK cell activity and also reduces phagocytotic function of macrophages and neutrophils. It is further reported that mast cells and the complements are impaired activated in zinc deficiency [106-108]. Zinc deficiency also reduces the B cell antibody response to T cell dependent antigens during an adaptive immune response [106-108].

Zinc deficiency in animals is characterized by decreased growth, low circulating levels of growth hormone (GH) and insulin-like growth factor-I, and decreased hepatic production of insulin-like growth factor-I, GH receptor, and GH binding protein [109]. Therefore, zinc deficiency reduces efficiency of feed utilization and thus leads to growth retardation [109,110]. Because Zn has a protective role on pancreatic tissue against oxidative damage, it may help the pancreas to function properly including secretions of digestive enzymes, thus improving digestibility of nutrients [109,111].

Zn supplementation can reduce the incidence, severity and duration of diarrhea [110,112-115]. It was shown that oral Zn supplementation was able to improve intestinal mucosal integrity as well as absorption of water and electrolytes [115,116]. Furthermore, Zn supplementation may increase feed intake by promoting an increase in the synthesis of ghrelin in the digestive tract [117] and may also reduce the severity of inflammation of the intestinal mucosa [113,118].

Zinc is often used as feed additive in animal production. According to the National Research Council recommendations [119], piglets (<20kg) require 100 mg/kg Zn in the diet and growing pigs require 50 mg/kg Zn. However, the maximum level of Zn allowed in pig diets was set to 150 mg/kg in the European Union irrespective of the source or formulation [120]. In swine nutrition, dietary Zn is frequently added at a high dose of 2,000-3,000 mg/kg to the diet of weaned piglets because this was shown to significantly reduce non-specific post-weaning diarrhea [121] and to improve performance of the immune response [122].

### **1. 3. 2 Zinc oxide**

Zinc oxide (ZnO) is one of the most wide spread feed additive among Zn ions, high dose of ZnO supplementation to the diets of piglets for the first 14-35 days after weaning promoted piglet growth [114,123] and reduced the incidence of diarrhea [121,124]. ZnO improved gut morphology and absorptive capacity to the diet [125] possibly by increasing zonula occludens protein-1 in the ileal mucosa [126]. It was also reported that high dose of ZnO reduced bacterial translocation from the small intestine to the corresponding lymph nodes [127].

### **1. 3. 3 Zinc in virus infections**

Published evidence over the past twenty years indicates that zinc may play a direct role in virus infection and at the same time influences the defense status of infected host animals. A slightly decreased Zn status might be associated initially with retarded immunological effects that could cause an increased number of virus infections [106]. Zinc was reported to show a broad range of antiviral activity against a variety of virus such as human influenza A virus [128], human immunodeficiency virus (HIV) [129], human rhinovirus [130], human herpes simplex-virus [131,132], and equine arteritis virus [104]. Interestingly, high Zn concentrations and the addition of compounds that stimulate the cellular import of Zn ions, such as hinokitol, pyrrolidine dithiocarbamate and pyrithione, were also found to inhibit the replication of various RNA viruses, including human influenza virus [133] respiratory syncytial virus [134] and several picornaviruses [135-138]. In the context of the farming industries, it is clearly essential to investigate Zn in reducing porcine viral infection.

### 1. 3. 4 Role of zinc in virus infections

Zinc has long been known as a crucial structural cofactor for virus propagation during virus infections of host animals. On the other hand, zinc ions are also participating in signal transduction and in the folding and functioning of numerous cellular proteins, so zinc could change the activities of different transcription factors and thus modulate the expression patterns of viral as well as cellular genes [139]. Furthermore, it has been demonstrated that free-Zn ions can serve not only as modulators of signal transduction but also as classical cellular second messengers [140,141] and therefore may trigger apoptosis or a decrease in protein synthesis at elevated concentrations [142,143]

Among the immunomodulatory effects of zinc that could counteract viral infections is its influence on the synthesis of cytokines and thus it could contribute to protect cells against apoptosis [106]. It is widely accepted that zinc induces the production of antiviral interferon (IFN)- $\alpha$  as well as IFN- $\gamma$  and it can potentiate the antiviral action of IFN- $\alpha$  but not of IFN- $\gamma$  [106]. It also has been reported that zinc addition induced the release of IL-1, IL-6, TNF- $\alpha$ , soluble IL-2 receptor and IFN- $\gamma$  in PBMC [144-146]. IL-1, IL-6, and TNF- $\alpha$  are induced in monocytes in the absence of lymphocytes, whereas the induction of IFN- $\gamma$  in lymphocytes is dependent on the presence of monocytes [145-148]. This protective action has been reported in relation to almost all apoptosis inducing factors, including tumor necrosis factor- $\alpha$ , IL-2 and cytotoxic T-cells [149].

Zinc is an important element for all aspects of immunity [150,151] and is critical for the integrity of the cells involved in the immune response [152]. Zn deficiency also affects adaptive immunity and causes a decrease in cellular immunity [153] by affecting thymus [154], spleen [155], and interleukin production [156]. Zinc deficiency causes an imbalance in the functions of T helper-1 and T helper-2 cells [157]. Clearance of viral infections requires T-lymphocytes. Therefore an abnormal T-lymphocyte development is thought to be the primary consequence of Zn deficiency [158]. B cell development and antibody production, particularly that of IgG, is also compromised by Zn deficiency [159,160].

### **1. 3. 5 Zinc and influenza virus infection**

Zinc supplementation has long been considered an effective means of reducing the duration of the common cold and influenza virus infection [161]. Such information has mainly been collected in human medicine research, hardly any data originated from veterinary research [128,133,161-164]. The first report dates back to 1974, describe that zinc could produce clinical benefits in flu patients without increasing adverse effects and the authors claim that this treatment of the influenza virus infection is effective by inhibiting RNA polymerase activity [128]. In a randomized study, 200 healthy children were assigned to receive oral zinc sulfate (15 mg/day elemental zinc) for seven months, the mean number of colds probably caused by influenza virus infection in the zinc group was statistically significantly fewer than in the control group [163].

Pertinent to bacterial pneumonia–complicating influenza, a 1-year study of 420 nursing home patients who daily were administered vitamins and minerals found that participants with plasma higher concentrations of zinc had a significantly lower risk of pneumonia requiring antibiotics than did participants with plasma lower concentrations of zinc [164]

At the practical level, zinc treatment was found to be effective against a variety of viral infections including influenza virus in humans [165,166]. Zinc treatment is believed to be most useful when administered together with a mixture of other micronutrients and is usually recommended with adequate intake of vitamin A and D [167].

### 1. 4 Aim of study

Porcine respiratory tract epithelial cells express sialic acid receptors utilized by both avian and mammalian influenza viruses. Pigs are, therefore, considered “mixing vessels” [15] for new human-avian influenza A virus reassortants with the potential to cause significant respiratory disease or even pandemics in humans [11,53]. Thus, the control of SIV is of concern for the economics of swine production as well as for animal and public health. Since there is no causative treatment for SIV, and no sterile immunity is achieved with current vaccines, a positive effect on prevention and/or course of clinical disease achieved through nutritional supplementation would be highly useful.

Probiotics have been recently shown to mediate antiviral effects against certain viruses *in vitro* and *in vivo* [75,95,168,169] and the effect of various strains of probiotics on the course of virus infections in pigs is being studied intensively. However, while some descriptive information on the effect of probiotics on model viruses such as vesicular stomatitis virus (VSV), transmissible gastroenteritis virus (TGEV) and rotaviruses [75,95,168,169] are available, no such data are yet available for swine influenza viruses which are most important in view of their exquisite zoonotic capacity. It is commonly believed that mammalian influenza viruses are restricted to the respiratory tissue and thus may hardly be affected by probiotics acting in the intestine. However, a recent report on the pathogenesis of seasonal influenza virus H1N1 in ferrets shows that this virus is also present in the intestine [17]. Furthermore it is world acknowledged that avian influenza virus infections frequently initiate in the intestine of the avian host [3]. Therefore it appears justified to include influenza viruses in studies on the probiotic inhibition of virus multiplication both *in vitro* and *in vivo*.

Zn was utilized frequently in attempts to treat various virus infections or aid in their prophylaxis. Some results suggest that Zn can directly interact with viral structural components, thereby influencing virus replication. It is also widely accepted that Zn affects immune responses at the cellular level as well as at the level of the recipient organism [108]. Interestingly, in cell culture studies, high Zn concentrations and the addition of compounds that stimulate cellular

import of Zn were found to inhibit the replication of various RNA viruses, including influenza virus [133].

In the present study we explored whether *E. faecium* affects the replication of swine influenza virus H1N1 and H3N2 in a macrophage (3D4/21) and epithelial cell line (MDBK). More importantly, we also explored systemic effects of *E. faecium* and high level Zn oxide feeding on SIV vaccination and infection in pigs.

## 2 MATERIALS AND METHODS

### 2.1 *In vitro* studies

#### 2. 1. 1 Virus

The Influenza A strains (A/Swine/Greven/IDT2889/2004(H1N1)) and (A/Swine/Bondelum/IDT5959/2007(H3N2)) were generous gifts from Dr. R. Dürrwald (Impfstoffwerk Dessau-Thornau, Germany).

#### 2. 1. 2 Cell lines

Madin-Darby bovine Kidney (MDBK) and Madin-Darby canine Kidney (MDCK) cells [170] used in this study were also generously provided from Dr. R. Dürrwald (Impfstoffwerk Dessau-Thornau, Germany). MDBK and MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM; PAN Biotech, Aidenbach, Germany) supplemented with 5% fetal calf serum (Hyclone, Utah, America), and 1% penicillin/streptomycin (Biochrom AG, Seromed, Berlin, Germany). The porcine continuous monomyeloid cell line 3D4/21 established from primary porcine alveolar macrophages [171] were kindly provided by Prof. A. Cencič (University of Maribor, Slovenia). 3D4/21 cells were maintained in Advanced Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Hyclone, Utah, America), and 1% penicillin/streptomycin (Biochrom AG, Seromed, Berlin, Germany).

#### 2. 1. 3 Probiotic *E. faecium*

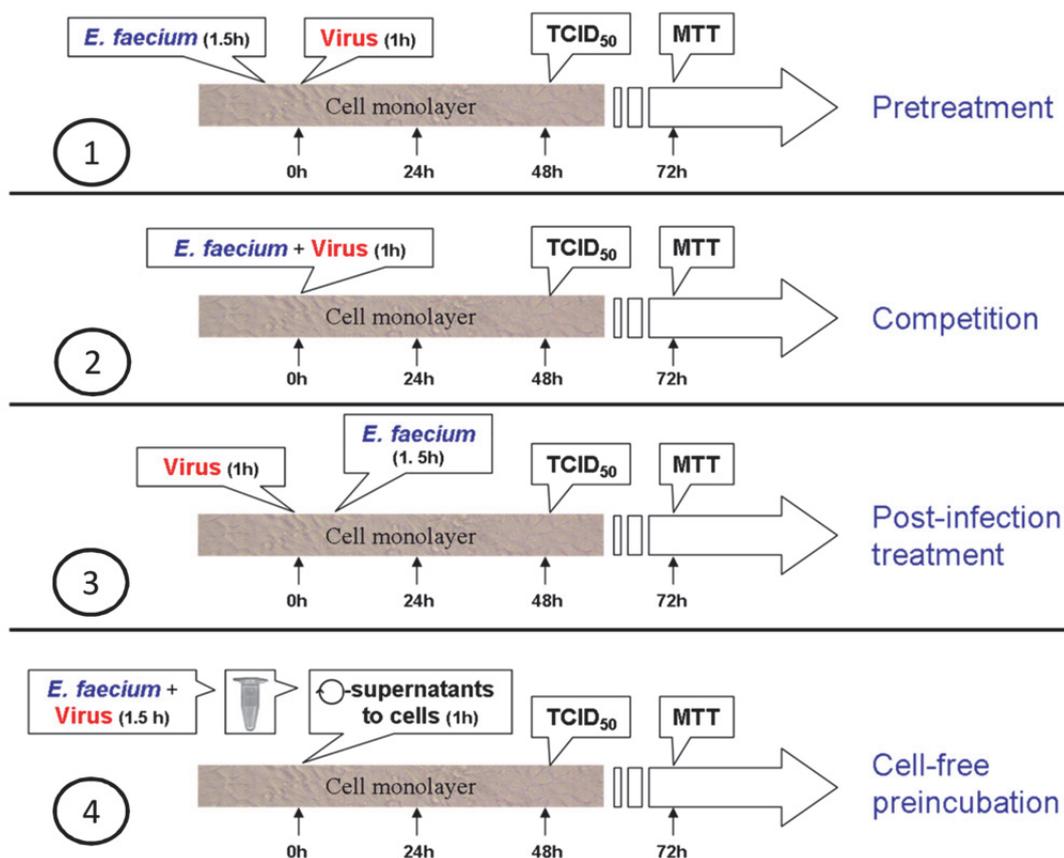
*E. faecium* NCIMB 10415 (Cylactin LBC ME10, DSM nutritional products Ltd, Kaiseraugst, Switzerland) was maintained in Todd-Hewitt broth (THB) medium (Roth, Kreuzlingen, Switzerland). One ml *E. faecium* was added to the 25 ml THB medium after overnight culture, OD value was monitored in comparison to blank control,  $6 \times 10^8$  bacteria was achieved when

OD value was 2.1 according to agar plate counting. *E. faecium* was transferred to DMEM before interference study.

### 2. 1. 4 Experimental design of interference experiments

To determine possible cytotoxic effects of *E. faecium*, different concentrations (1.00E+05, 1.00E+06, 1.00E+07 or 1.00E+08 colony forming units (CFU)/ml) were added to 3D4/21 and MDBK cell monolayers in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) for 72 h and cell viability was monitored by a methylthiazolyl-diphenyl-tetrazolium bromide (MTT) viability assay (see 2. 1. 7).

For interference studies, infection of cells with both strains of SIV was done at a multiplicity of infection (MOI) of 0.01. Briefly, *E. faecium* was applied before, together with or after virus at a concentration of  $10^6$  CFU/ml in DMEM medium without serum. In order to avoid any carry over effects, after the *E. faecium* treatment period, the *E. faecium* containing medium was aspirated. Then medium containing 1% penicillin/streptomycin was added to stop any propagation of *E. faecium*. The schematic in (figure 1) depicts the experimental setup for studying the interference between *E. faecium* with SIV-infection in the two cell culture systems. If *E. faecium* has any inhibiting potential, this allows us to define at what time during virus growth the addition of the probiotic is most effective. The preincubation setup shown in (panel 4, figure 1) should reveal whether the probiotic bacteria have a direct effect on the virus particles without any involvement of host cells. The MTT assay was used to measure the mitochondrial function of the cells subjected to the dual treatments, which serves as a viability index of metabolically active cells. After the experimental incubation period (Figure 1), the MTT assay was applied as described below. The percentage of metabolically active cells treated with probiotic bacteria and the percentage of protection from the virus induced cytopathic effect achieved was then calculated. MTT-values obtained from control cells without any virus and/or *E. faecium* treatment were set to 100% cell survival rate. The H1N1 strain was used on MDBK cells and the H3N2 strain was used on 3D4/21 macrophages in this study. All data represent the average values for a minimum of six wells of three independent experiments.



**Figure 1. Experimental design of dose response study of probiotic effect on SIV.**

(1) Pretreatment of cell monolayers with probiotics for 1.5 hours before SIV infection (Pretreatment). (2) Probiotics and virus are added together to the cells (Competition). (3) Treatment of cell monolayer with probiotics 1 hour after SIV infection (Post-infection). (4) After preincubation of SIV with probiotic bacteria, the mixed samples were centrifuged and the supernatants added to the cells (Preincubation). Virus titration for MDBK-cells was done at 48 h p.i. *E. faecium* was suspended in DMEM medium without serum before application to cells.

## 2. 1. 5 Cell culture

For passaging monolayers of MDBK or 3D4/21 cells, the cell culture medium was aspirated; cells were washed once with PBS (Biochrom AG, Seromed, Berlin, Germany) and cells were trypsinised with a mixture of trypsin/EDTA (Biochrom AG, Seromed, Berlin, Germany). Then new medium was added, the cells detached by gentle shaking and the cells suspension was transferred into a new cell culture flask (cell star, Greiner bio-one, Frickenhausen, Germany). MDBK and MDCK cells were split 1:5, 3D4/21 cells were split 1:3, depending on cell density,

twice a week. All cells were incubated at 37 °C and 5 % CO<sub>2</sub>. If an exact number of cells was needed, defined aliquots from the cell suspension were counted by cell counting chamber (Kisker, Steinfurt, Germany). The respective cell dilution was then seeded into cell plates/flasks (cell star, Greiner bio-one, Frickenhausen, Germany).

### **2. 1. 6 Virus propagation**

Stock virus of H1N1 and of H3N2 was propagated in Madin-Darby Bovine Kidney (MDBK) and in Madin-Darby canine Kidney (MDCK) cells, respectively. 80-90% confluent cells were infected with the respective virus in 10 ml medium of a moi of 0.001 and incubated for 1 h. Then cells were wash twice with DMEM medium and incubated for further three to five days until an overall cytopathic effect (CPE) could be observed. Virus containing cell culture supernatants were collected and frozen in aliquots from -20 to -80 °C. The titer of virus was determined by an established 50 % tissue culture infectious dose (TCID<sub>50</sub>) assay as described below.

### **2. 1. 7 MTT assay**

Cell monolayers were washed after the 72 h incubation period of experimental cells, 20 µl MTT (Sigma, Taufkirchen, Germany) in PBS (Biochrom AG, Seromed, Berlin, Germany) was then added to each well and the plates were further incubated at 37 °C in a CO<sub>2</sub> incubator (Thermo Scientific, Karlsruhe, Germany) for 1.5 h. Solubilisation of the formazan crystals formed during this period was achieved by the addition of dimethyl sulfoxide (DMSO). The absorbance (OD) at 570 nm was measured using a microplate reader (Tecan, Crailsheim, Germany). Cell survival rate was determined as bacteria average OD value / control average OD value.

### **2. 1. 8 TCID<sub>50</sub> assay**

This endpoint dilution assay quantifies the amount of virus required to kill 50% of infected hosts or to produce a cytopathic effect in 50% of inoculated tissue culture cells. For virus growth evaluation, 10-fold serial dilutions of culture supernatants from infected cells were

prepared and serial dilutions of the virus were added. After infection, indicator cells (MDBK or 3D4/21 cell as stated in result section) were stained by Giemsa (Sigma, Taufkirchen, Germany) and the CPE was recorded macroscopically and under the microscope. Each sample was assessed in quadruplicate as a minimum and input virus from non-treated cells was run as a control. The results of all TCID<sub>50</sub> assays were calculated according to the Reed and Muench method [172].

### **2. 1. 9 Assessment of nitric oxide (NO) release**

NO release was determined by measuring the amount of NO released into the culture medium by use of the Griess-Assay (Promega, Madison, USA) according to the manufacturer's instructions. 50 µl of each experimental sample was transferred into a 96 well plate in triplicate. Defined standard samples (0.1M sodium nitrite in water) were assessed in parallel to produce a standard curve. Then 50 µl of a sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) were added to each well at room temperature (protected from light) for 10 minutes, followed by 50 µl of NED (*N*-1-naphthylethylenediamine dihydrochloride) solution (0.1% *N*-1-naphthylethylenediamine dihydrochloride in water) were dispensed at room temperature (protected from light) for 10 minutes and absorbance (OD) at 570 nm was measured using a microplate reader (Tecan, Crailsheim, Germany) within 30 minutes. NO release in each sample was calculated by use of the nitrite standard curve generated in parallel.

### **2. 1. 10 RNA extraction**

After the treatment periods (compare panel 2, figure 1) by 2 h, 6 h and 24 h, 3D4/21 cells were collected from the wells. Total RNA was isolated from cell samples by use of the Gene MATRIX RNA Purification Kit (EURx, Gdnask, Poland) according to the manufacturer's instructions. 100 µl cell cultures were centrifuged and 400 µl lysate buffers were added to the cell pellet. Then the lysates were carefully bound to the homogenization spin-column and washed twice. RNA was eluted from the columns by adding 50µl RNase-free water directly onto the membrane. RNA then was ready for analysis/manipulations or could be stored at

-20°C for further analysis. The concentration and purity of samples were determined by use of a NanoDrop spectrophotometer (peQLab Biotechnologies, Erlangen, Germany).

### 2. 1. 11 cDNA synthesis

Reverse transcription (RT) was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. The polymerase chain reaction (PCR) components and cycling conditions are listed in (Table 1).

**Table 1. Reverse transcription protocol:**

Components	Cycling condition	
Template RNA (<1,000 ng)	11 µl	42°C 60 min
Oligo (dt)18 primer	1 µl	70°C 5 min
5*Reaction buffer	4 µl	
RiboLock RNase inhibitor	2 µl	
10mM dNTP Mix	1 µl	
RevertAid M MuLV Reverse Transcriptase	1 µl	
Total	20 µl	

### 2. 1. 12 Real-time PCR to assess the expression of immune mediators

Real-time PCR is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample. PCR reactions (Table 2) were performed in a total volume of 25 µl in an iCycler iQ detection system (Bio-Rad Laboratories, Munich, Germany). The expression of each gene was analyzed using the relative quantification method [173]. The PCR components and cycling conditions are listed in (Table 2). The designations of genes, the primer sequences, the annealing temperatures, and

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the sizes of the amplification are listed in (Table 3).

**Table 2. Real-time PCR protocol:**

Components		Cycling condition
2*SensiMIX SYBR	12.5 µl	95 °C 10 min
Forward primer (0.2 µM)	1 µl	94 °C 30 s
Reverse primer (0.2 µM)	1 µl	57 °C 30 s      40 repeats
Template (<1,000 ng)	2 µl	72 °C 45 s
Water	8.5 µl	4 °C 10 min
Total	25 µl	

**Table 3. PCR Primers**

Gene	Primer pairs (5'- 3')	Product (bp)	Source/Accession number in Genbank
β-Actin	Forward: CGGGACCTGACCGACTA	233	DQ845171.1
	Reverse: AAGGTCGGGAGGAAGGA		
IL-6	Forward: AACGCCTGGAAGAAGA	229	Ab194100
	Reverse: AACCCAGATTGGAAGC		
IL-10	Forward: GCATCCACTTCCCAACCA	446	EF433759
	Reverse: TCGGCATTACGTCTTCCAG		
IFN-α	Forward: GCT CCT GGC ACA AAT G	197	NM214393
	Reverse: GCTGCTGATCCAGTCC		
TNF-α	Forward: ACGCTCTTCTGCCTACTGC	388	NM214022
	Reverse: TGGGCGACGGGCTTATC		
TLR-3	Forward: AAC CAG CAA CAC GAC T	110	Ab111939
	Reverse: TTG GAA AGC CCA TAA A		

**2. 1. 13 Virus adsorption to *E. faecium* (preincubation assay)**

To investigate if virus could be trapped by probiotics, *E. faecium* (1.00E+06 CFU/ml) were mixed with 1.00E+04 SIV in a total of 1 ml DMEM for a 90 min co-incubation at 37 °C in a CO<sub>2</sub> incubator for 1.5 h. The mixture was then centrifuged at 3,500 rpm for 10 min (panel 4, figure 1). Sediments were prepared for quantification of virus by PCR and supernatants were used to determine unbound virus by infecting indicator cells (3d4/21 cells). Supernatants from virus only-samples were used as controls. Total RNA was isolated from sediments and M protein (101 bp) [174] of SIV was amplified and compared to virus only controls. The primers and probe, PCR components and cycling condition are listed in (Table 4).

**Table 4. Real-time PCR protocol:**

Components		Cycling condition
Template DNA (<1,000 ng)	5 µl	95 °C 10 min
5*Reaction buffer	5 µl	94 °C 15 s
H <sub>2</sub> O	10 µl	57 °C 30 s      45 repeats
dnTPs (200 µM)	0.5 µl	4 °C 10 min
MgCl <sub>2</sub> (1.5–2.0 mM)	1.5 µl	
Front primer (0.2 µM)	0.5 µl	AgATgAgTCTTCTAACCgAggTCg
Reverse primer 1 (0.2 µM)	0.5 µl	TgCAAAAACATCTTCAAgTCTCTg
Reverse primer 2 (0.2 µM)	1 µl	TgCAAARACACYTTCCAgtTCTCTg
Probe (100 nM)	0.75 µl	6FAM-TCAggCCCCCTCAAAGCCgA-TMR
Taq (250 nM)	0.25 µl	
Total	25 µl	

### **2. 1. 14 Electron microscopy**

Electron microscopy (EM) study was performed in collaboration with Juliane Rieger in the institute of veterinary anatomy, Freie Universität Berlin. In order to examine possible direct binding of virus to *E. faecium*, the cell-free preincubation assay was performed by mixing *E. faecium* with SIV at a bacteria-to-virus ratio of 10:1 for 1.5 h. After centrifugation for 10 min at 3500 rpm to sediment bacterial cells and after duplicate washing of sediments, the pellet was re-suspended in 1 ml Karnovsky's Fixative (5% glutaraldehyde + 4% formaldehyde in 0.1M cacodylate buffer + 50mg CaCl<sub>2</sub>/100ml). The samples were centrifuged again for 10 min at 2500 rpm and a drop (10 µl) was taken from the bottom of the tube and stained with 2 % phosphotungstic acid for 1 min. Finally, the samples were evaluated with a transmission electron microscope [69].

### **2. 1. 15 Statistical analysis**

Statistical analysis was performed in close collaboration with Sven Twardziok, Molekularbiologie und Bioinformatik, Charité Universitätsmedizin Berlin. All calculations were performed with IBM SPSS 19. Data analysis for virus titers and NO release were performed by two factorial ANOVA followed by a Posthoc Test (Scheffe). Data analysis for cytokine expression was performed by paired, two tailed t-test. P values of <0.05 were considered statistically significant. P values of <0.01 were considered statistically very significant. All data are given as the mean ± standard deviation.

### **2. 2 *in vivo* studies**

#### **2. 2. 1 Virus**

Influenza A virus (A/swine/Bissendorf/IDT1864/03 (H3N2)) was obtained from IDT-company (Dessau, Germany). Viral stocks were produced in Madin-Darby canine kidney (MDCK) cells.

#### **2. 2. 2 Vaccine**

The inactivated, trivalent vaccine Respiporc Flu3 (IDT, Dessau, Germany), which contains the three main swine influenza subtypes H1N1, H1N2 and H3N2 was used in our study:

A/sw/Haselunne/IDT2617/2003 (H1N1), A/sw/Bakum/IDT1769/2003 (H3N2),

A/sw/Bakum/1832/2000 (H1N2).

#### **2. 2. 3 Feed additives**

Concerning the diet, probiotic, *E. faecium* NCIMB 10415 was applied as a commercial probiotic feed additive (Cylactin LBC ME10, DSM nutritional products Ltd, Kaiseraugst, Switzerland) in a microencapsulated form and mixed to the diets of weaned piglets at a concentration of  $1 \times 10^9$  CFU / kg feed and ZnO was added at three different concentrations (Zn<sup>low</sup>: 80 mg/kg diet (natural Zn content); Zn<sup>med</sup>: 150 mg/kg diet (max. allowed EU level); Zn<sup>high</sup>: 2500 mg/kg diets (pharmacological level)). The Zn<sup>low</sup> diet comprises the regular feed of the animals and therefore animals fed this diet are considered the control groups throughout the study.

#### **2. 2. 4 Animals and experimental setup**

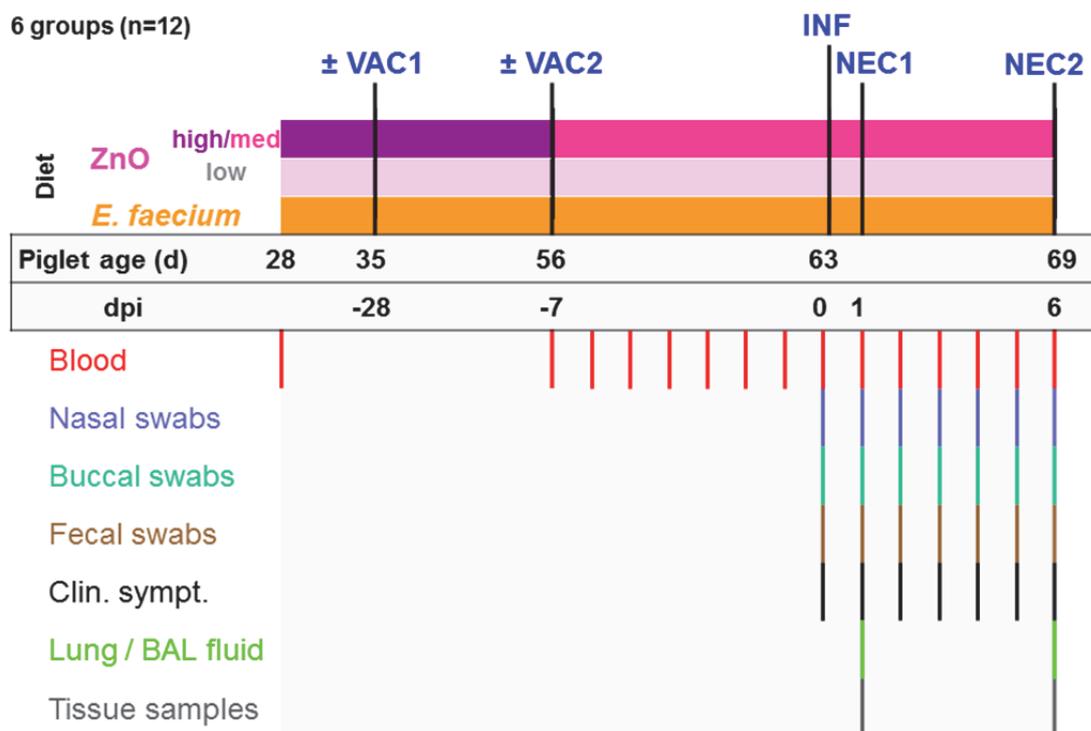
All pigs involved in this study were approved by the local animal welfare authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Rostock, Mecklenburg-Vorpommern, Germany) under the registration number 44/12. Piglets (n = 72) were raised at the Institute of Animal Nutrition, Freie Universität Berlin and weaned at the age of 28 days of age. Pigs were then randomly assigned to three different diets (control, Zn or *E. faecium*) and kept in groups

## Materials & Methods

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of 6 (2 pens per diet). As shown in (Figure 2), high Zn levels were fed only until the age of 56 days in order to avoid toxic effects. At this point, the diet was switched to the medium concentration Zn<sup>med</sup> (150 ppm) one week before SIV infection. Half of the piglets (one pen) were vaccinated intramuscularly with above mentioned anti-flu vaccine (VAC) twice on day 35 and 56. In total, there were 6 treatment groups containing 12 piglets each. The challenge trial was performed in collaboration with the Friedrich-Loeffler-Institute (FLI) on the Island Riems, Germany. Five days before virus infection, all piglets were transported from Berlin to the BSL (Bio-safety level) 3\* facility at FLI, where they housed in HEPA-filtered isolation units at a constant 27 °C. Experimental procedures were performed by Dr. Elke Lange and technicians, Abteilung für experimentelle Tierhaltung und Biosicherheit, Friedrich-Loeffler-Institut supported by Dr. Michael Burwinkel and Weidong Chai, Institut für Virologie, Freie Universität Berlin. All pigs were tested negative for the presence of SIV antibodies by ELISA (ID Screen Influenza A competition, ID.vet, Grabels, France) prior to infection. At 63 days of age, all piglets were inoculated by the intranasal route with 2 x 1 ml of SIV H3N2 with a titer of 10<sup>6.3</sup> TCID<sub>50</sub>/ml using a LMA MA intranasal mucosal atomization device (Teleflex Medical GmbH, Kernen, Germany). Half of the piglets from each group were killed on 1 and 6 dpi by i.v. injection of T61 after intramuscular induction of anesthesia with 20-30 mg ketamine /kg body weight (Ursotamin, Serumwerk, Bernburg AG, Bernburg, Germany) and 1-2 mg azaperon /kg BW (Stresnil, Janssen-Cilag GmbH, Neuss, Germany). Histopathological analyses were carried out in collaboration with Juliane Rieger and Karin Briest-Forch in the Institute of Veterinary Anatomy, Freie Universität Berlin.

## Experimental design – SIV trial



**Figure 2. Schematic of set-up of SIV challenge trial.**

ZnO: Zinc oxide; Zn<sup>low</sup>: 80 mg/kg diet (natural Zn content of control feeds); Zn<sup>med</sup>: 150 mg/kg diet (max. allowed EU level); Zn<sup>high</sup>: 2500 mg/kg diets (pharmacological level); VAC1: first vaccination; VAC2: second vaccination; INF: infection with a H3N2 swine influenza virus; NEC1: necropsy at 1 dpi; NEC2: necropsy at 6 dpi.

### 2. 2. 5 Clinical follow-up and sampling

During the experiment, animals were clinically monitored daily for the development of clinical signs including fever, fatigue, anorexia, dyspnea and cough. Body weights were recorded weekly after weaning before infection and at necropsy on 1 and 6 dpi after exsanguination. Blood samples were taken daily after the second vaccination for serological analyses (Figure 2). Nasal, buccal and fecal swabs were collected daily for the analysis of virus shedding. At the day of necropsy, samples were taken from the nasal turbinates and lungs (apical, middle and accessory lobes). Samples of all organs were prepared for histological analysis.

Bronchoalveolar lavage fluid (BALF) was also obtained via the trachea after the lungs were removed without serum.

### **2. 2. 6 Pathology**

Pathological examinations were done by Dr. Angele Breithaupt, Institut für Veterinärpathologie, Freie Universität Berlin, supported by Dr. Michael Burwinkel, Institut für Virologie, Freie Universität Berlin. At necropsy, the lungs were immediately examined macroscopically and, additionally, photographs were taken for further analysis. Lung sections from the portion most consistently affected by gross lesions (tissue consolidation) were stained using a hematoxylin/eosin (HE) standard staining protocol [175] and examined microscopically. Small sections of all the above-mentioned organs were fixed in 10% buffered formalin. Fixed tissues were dehydrated, embedded in paraffin and 5 µm sections were cut for histological staining. Examination of tissue sections from this study was carried out blindly by a veterinary pathologist. Lesion severity was scored by the distribution of lesions within the sections examined as follows: 0 - no visible changes; 1 - mild changes, minimally different from the normal; 2 - moderate changes; 3 - severe and diffusely distributed changes.

### **2. 2. 7 Antibody ELISA**

The development of an influenza virus-specific immune response was analyzed by a commercially available ELISA kit targeting the viral nucleoprotein (ID Screen Influenza A Antibody Competition ELISA (ID.vet, Grabels, France)) according to the manufacturer's instructions. Capture antibodies were pre-coated on the bottom of the 96-well plate. The standard and samples were added to the wells and incubated to allow target proteins to bind for 1 h. The wells were washed to remove unbound material and then second antibodies (HRP conjugate) were added. Next, the chromogenic substrate for HRP was added and the subsequent enzymatic reactions turn the solution to blue. Finally, the reaction was stopped by stop solution, turning the solution yellow in proportion to the amount of target protein in the sample. The optical density of the reaction was measured at 450 nm ( $OD_{450nm}$ ) with a microplate reader (Tecan, Crailsheim, Germany). Results were reported as the ratio (S/N) of

OD<sub>450nm</sub> between the result of a sample and negative control included in the kit (positive cut-off: S/N=0.55).

### **2. 2. 8 Hemagglutination inhibition (HI) assay**

The hemagglutination inhibition (HI) assay was performed using 0.5 % chicken erythrocytes for hemagglutination and 8 hemagglutinating units of A/swine/Bissendorf/IDT1864. Sera were pretreated with receptor destroying enzyme (Cholera filtrate; Sigma-Aldrich, St. Louis, USA) to remove nonspecific inhibitors and adsorbed onto chicken erythrocytes to remove agglutination factors. The tests were then performed according to standard procedures [176] in twofold dilutions starting at 1:20.

### **2. 2. 9 Viral RNA quantification from swabs**

Nasal, buccal and fecal swabs were taken, placed in vials containing serum-free cell-culture media, and stored at -80 °C until further analysis. Virus quantification was done by Dr. Bernd Hoffmann and technicians, Institut für Virusdiagnostik, Friedrich-Loeffler-Institut. Viral RNA was extracted from nasal and buccal swabs taken at 0, 2, 4, 6 dpi and from fecal swabs at 3 dpi using the MagAttract DNA Mini M48 Kit (Qiagen, Hilden, Germany) on the KingFisher Flex Magnetic Particle Processors (Thermo Fisher Scientific, Waltham, USA). Swabs were eluted with 1000 µl serum-free cell-culture medium. Then 100 µl of the medium were used for extraction and the RNA was eluted with 100 µl AVE elution buffer. Real-time qPCR for quantification of SIV gene copy numbers was performed using a pan-Influenza A-M1.2 assay [177] and an appropriate in-vitro transcribed RNA standard.

### **2. 2. 10 Differential cell count**

To evaluate changes of cellular composition in the peripheral blood after SIV infection, 150 µl of whole blood were analyzed using an automated XT-2000iV hematology analyser (Sysmex Corporation, Hyogo, Japan) and the number of neutrophils, lymphocytes, and monocytes was determined.

### 2. 2. 11 Flow cytometry

Peripheral blood mononuclear cells (PBMC) were subjected to multicolor immunostaining with porcine cell surface markers for flow cytometry analysis using a BD FACSCanto (BD Biosciences, Heidelberg, Germany). Each heparinized blood sample (50  $\mu$ l) was initially stained with a defined antibody mix 1 (Table 5). Respective isotypes were also included in the assay. After incubation for 15 min in the dark at 4 °C, cells were washed with fluorescence-activated cell sorting (FACS) buffer (0.1% BSA, 0.035% sodium bicarbonate and 0.02% sodium azide in HBSS) and centrifuged for 5 min at 700 x g. Then, antibody mix 2, mix 3 or mix 4 (Table 5) were successively added and cells were washed and centrifuged. After the last wash step, contaminating erythrocytes were lysed by adding 100 $\mu$ l of lysis buffer (10-fold concentrated stock: 8.3 g  $\text{NH}_4\text{Cl}$ , 1.0 g  $\text{KHCO}_3$ , 0.37 g  $\text{Na}_4\text{EDTA}$  and 100 ml  $\text{H}_2\text{O}$  adjust to pH 7.4 with NaOH and sterilized with 0.22 $\mu$ m filter) and samples were analyzed. Lung mononuclear cells were isolated from freshly euthanized piglets after removal of lungs with trachea and bronchus. The left lungs were lavaged with 50 ml of PBS (pH 7.4) using a flexible tube and the collected bronchoalveolar lavage (BAL) samples were centrifuged at 300 x g for 10 min at 4 °C. The pellet was re-suspended in FACS buffer and stained as described above. Flow cytometry measurements were performed by Ulrike Blohm, Institut für Immunologie, Friedrich-Loeffler-Institut.

Results from flow cytometry were analyzed using the FlowJo software (Treestar, Ashland, USA). Based on  $\gamma\delta$ -T cell receptor (gdTCR), CD3, CD4, CD8, CD2 and CD21 staining characteristics, each subpopulation was then further grouped as follows:  $\gamma\delta$ -T cells (gdTCR<sup>+</sup>CD3<sup>+</sup>CD2<sup>+</sup>CD8<sup>+</sup>); T-helper (Th) cells (gdTCR<sup>-</sup>CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>); activated T helper cells (gdTCR<sup>-</sup>CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>high</sup>); cytotoxic T lymphocytes (CTLs) (gdTCR<sup>-</sup>CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup>); Th/memory cells (gdTCR<sup>-</sup>CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>); natural killer (NK) cells (gdTCR<sup>-</sup>CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>high</sup>) and antibody-forming and/or memory B cells (gdTCR<sup>-</sup>CD3<sup>-</sup>CD2<sup>+</sup>CD21<sup>-</sup>).

**Table 5. Primary and secondary antibodies used for flow cytometry staining**

Mix	Antibody	Isotype	Labeling	Clone	Source
1	Mouse anti-pig CD2	IgG2a	None	MSA 4	Hybridoma supernatant
	Mouse anti-pig CD25	IgG1	None	K231.3B2	AbD Serotec
	Mouse anti-pig TCR $\gamma\delta$	IgG2b	None	PPT 16	Hybridoma supernatant
2	Goat anti-mouse IgG1	IgG1	DyLight® 405	polyclonal	Dianova
	Goat anti-mouse IgG2a	IgG2a	APC –Cy™7		Southern Biotech
	Goat anti-mouse IgG2b	IgG2b	PE	polyclonal	Dianova
3	Mouse anti-pig CD3	IgG2a	Alexa Flour®648	BB23-8E6-8C8	BD Pharmingen
	Mouse anti-pig CD4	IgG2b	PerCP-Cy™5.5	74-12-4	BD Pharmingen
	Mouse anti-pig CD8 $\alpha$	IgG2a	FITC	76-2-11	Southern Biotech
	Mouse anti-pig CD21	IgG1	Biotin	BB6-11C9.6	Southern Biotech
4	Streptavidin		PE/Cy™7		Southern Biotech

## 2. 2. 12 Statistical analysis

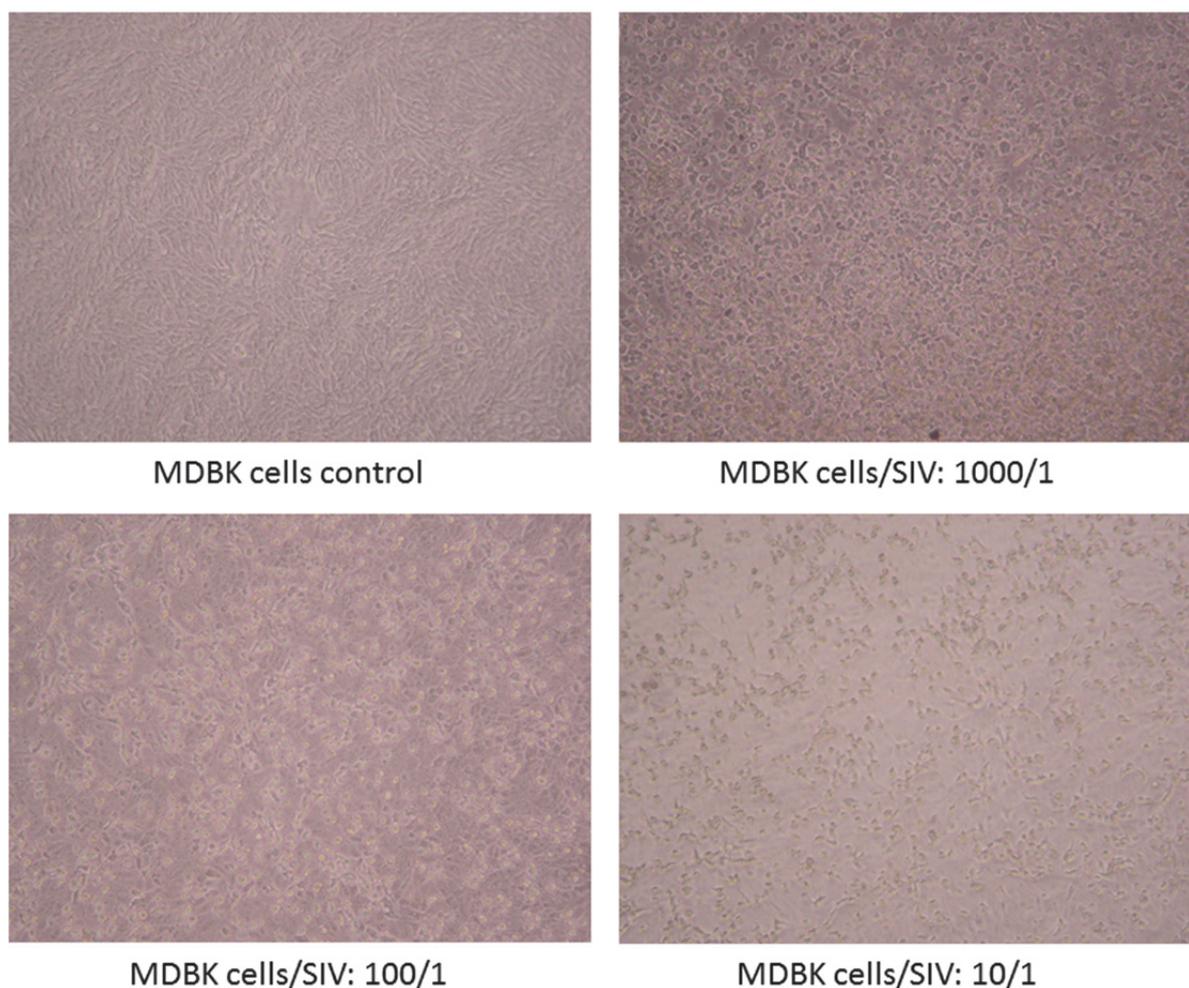
Statistical analysis was performed in close collaboration with Sven Twardziok in the Molekularbiologie und Bioinformatik, Charité Universitätsmedizin Berlin. Results were analyzed by a mixed model with fixed effects (time, diet, time\*diet (ELISA and HI assay data); diet, vaccination, diet\*vaccination (lesion score data); time, diet, vaccination, time\*diet, time\*vaccination, diet\*vaccination, time\*diet\*vaccination (qRT-PCR, blood count, flow cytometry data)) and one random effect (animal). Post-hoc tests (LSD) were applied in case of significant effects. Calculations were performed with SPSS® Version 21 (IBM, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).



## Results

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obtained on 3D4/21 cells which also showed a dose-dependence of the cytopathic effect induced by SIV (not shown). The highest titer of SIV on MDBK cells was  $1 \times 10^7$  TCID<sub>50</sub>/ml, the highest titer of SIV on 3D4/21 cells was  $6 \times 10^6$  TCID<sub>50</sub>/ml.



**Figure 4. SIV H1N1 propagation in MDBK cells at 48 h.**

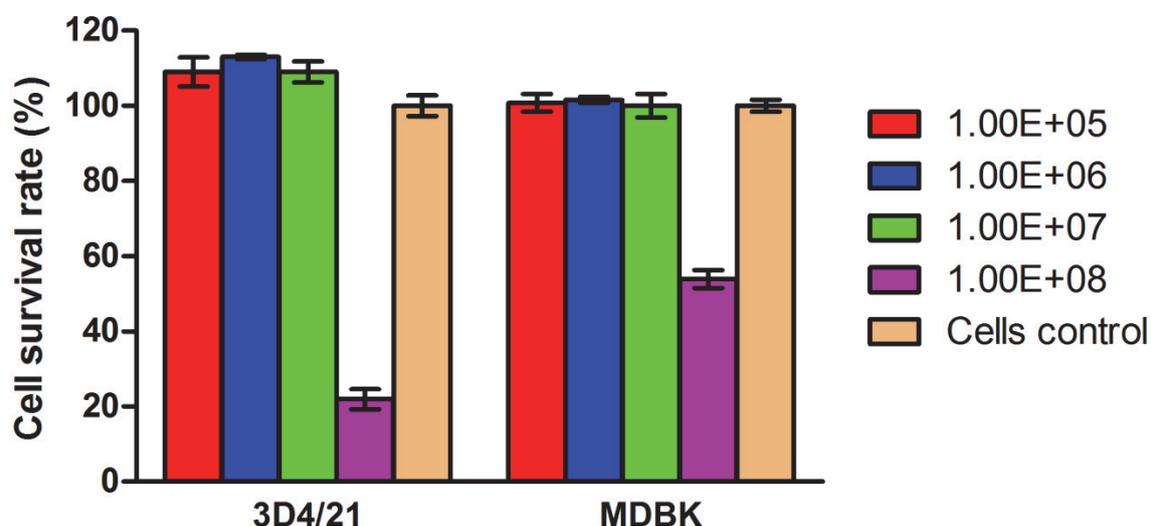
Different concentrations of SIV H1N1 virus were added to the 80–90 % confluent MDBK cell monolayer, photographs were taken under the microscope at 48 h.

### **3. 1. 3 Cytotoxicity effect of *E. faecium* on 3D4/21 and MDBK cells**

Before the inhibiting potential of a probiotic on virus multiplication can be assessed *in vitro*, care has to be taken that *E. faecium* has a cytotoxic effect on the cells by itself. Cells cultures were therefore subjected to increasing concentration of *E. faecium* and their viability monitored. Cells cultures were therefore subjected to increasing concentration of *E. faecium*.

## Results

The results of such cytotoxicity assay with *E. faecium* in both 3D4/21 and MDBK cells are shown in (Figure 5). Compared to control cells (100% cell survival rate), the application of *E. faecium* on the cell lines examined did not lead to any detrimental effects on cell integrity or metabolism unless the concentration exceeded of  $1 \times 10^7$  CFU/ml. As seen from the results compiled in (Figure 5), only *E. faecium* at the highest concentration ( $1 \times 10^8$  CFU/ml) had a cytotoxic effect, especially for the macrophage cell line 3D4/21. Under the same conditions a proportion of about 60% of the MDBK-cells still survived in the presence of this probiotic. Based on these results,  $1 \times 10^6$  CFU/ml of *E. faecium* was applied for the interference studies described below. Growth of the probiotic was monitored by assessing aliquots on THB agar plate with or without antibiotic. During the full time incubation period of 48 hours, a 10 fold increase of the number of probiotic bacteria was recorded when no antibiotic was present in the medium. However, no growth of probiotic bacteria at all was observed with the presence of antibiotic in the growth medium.

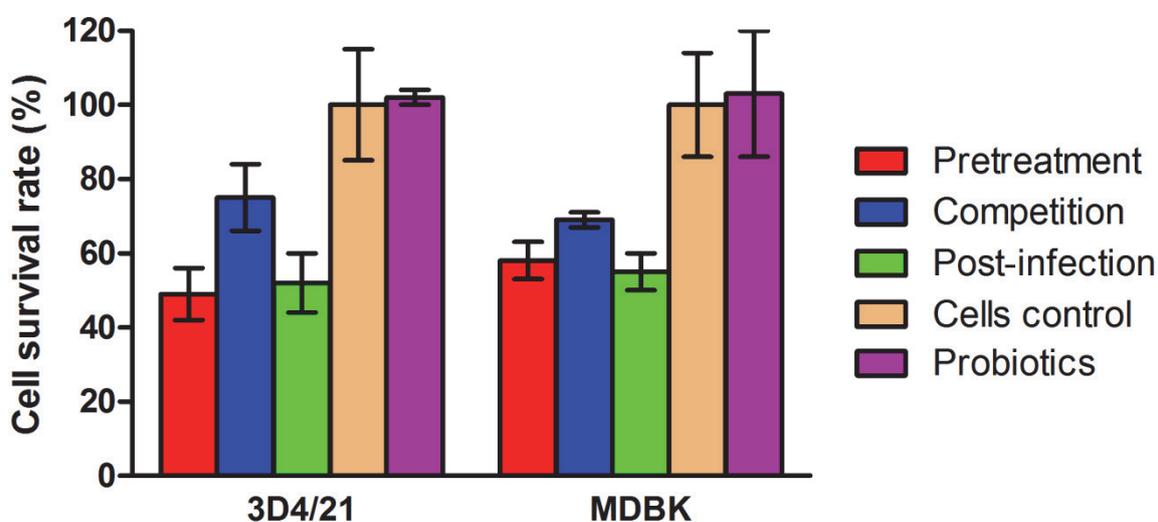


**Figure 5. Cytotoxicity of *E. faecium* on 3D4/21 and MDBK cells.**

Different concentrations of *E. faecium* ( $1.00 \times 10^5$ ,  $1.00 \times 10^6$ ,  $1.00 \times 10^7$ ,  $1.00 \times 10^8$  CFU/ml) were added to 3D4/21 and MDBK cell monolayers at sub-confluency and cell viability assessed by an MTT assay after a 72 h exposure. Cell survival rates are given as bars taking non-treated cells as 100%. The means  $\pm$  standard deviations from three independent experiments are shown.

### 3. 1. 4 Effect of *E. faecium* on SIV infected cells as detected by MTT assay

As expected from the above cytotoxicity study,  $1 \times 10^6$  CFU/ml of *E. faecium* did not affect the viability of uninfected 3D4/21- and MDBK-cells (last bar for each of the cell types shown in Figure 6). While SIV already at 48 h p.i had destroyed the cell monolayers completely (defined as 0 % survival, compare figure 4), each of the treatment modalities with the above concentration of *E. faecium* resulted in a rescue of the cells from SIV infection. Among three setup conditions compared, the rescue of cells was most pronounced, when the probiotic bacteria and SIV-inoculum are added to the monolayers together for 60 min (competition). This resulted in an 80 % protective effect for 3D4/21 and in a 70 % protective effect on MDBK cells. But even a pretreatment of the cells with *E. faecium* and the addition of the probiotic after completion of SIV-infection both resulted in a significant rescue of the cells from “death” through SIV-infection in (Figure 6).



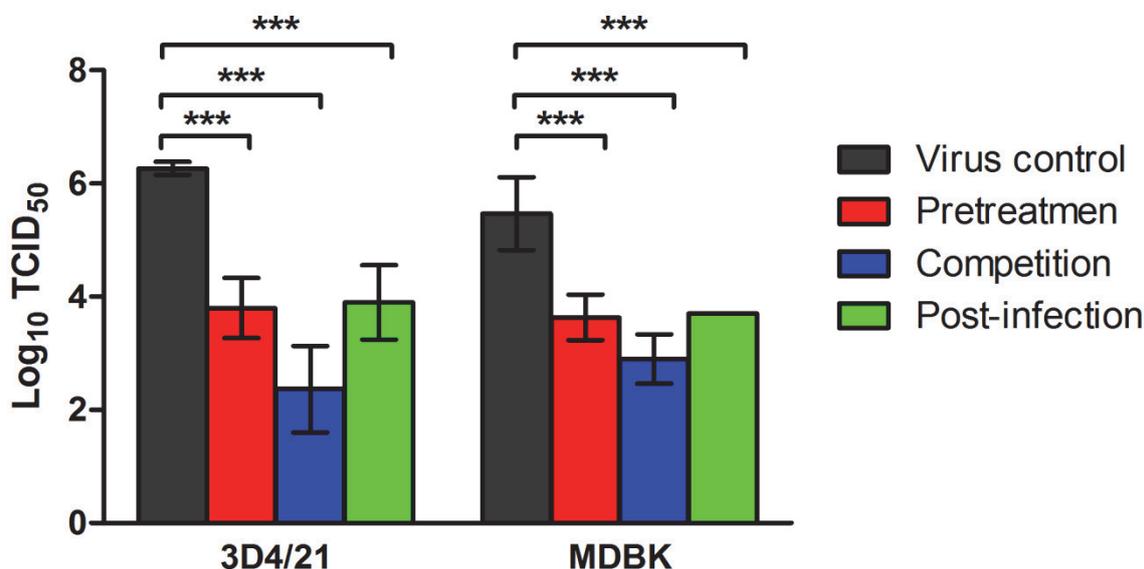
**Figure 6. Cell viability of 3D4/21- and MDBK-cells after treatment with *E. faecium*.**

Pretreatment: *E. faecium* was present on the cells for 90 min prior to SIV infection; Competition: *E. faecium* and SIV were added simultaneously for 1 h; Post-infection: *E. faecium* was added for 90 min after the 1h SIV infection period. Results are expressed as percent cell survival rates where non-treated and non-infected cells served as controls (set at 100 % survival rate) and SIV-infected cells without *E. faecium* treatment as the complete damage marker (set at 0 % survival rate). Results represent means  $\pm$  standard deviations

from three independent experiments.

### 3. 1. 5 Virus titer reductions in cells treated with *E. faecium*

The effect of *E. faecium* treatment on virus multiplication was validated by the TCID<sub>50</sub> assay. As shown in (Figure 7), the virus titer was decreased significantly after treatment of both types of host cells with *E. faecium*, but the degree of inhibition differed depending on whether the probiotic was present before, during or after infection with SIV. A up to 4 Log<sub>10</sub> TCID<sub>50</sub> reduction was obtained when *E. faecium* and SIV were present on the monolayers simultaneously indicating that direct competition between SIV and the probiotic for presently unknown entities results in the most effective inhibition of virus production (see below). These results are in line with those from the cell viability assay of SIV-infected cells treated with the probiotic shown in (Figure 6). Probiotic *E. faecium* induced inhibition of SIV with both types of host cells, but it appears to be somewhat more effective in the macrophage line 3D4/21. However, this could also be due to the lower SIV-titers reached in the non-treated MDBK-cells which was about one log-unit less than with non-treated 3D4/21-cells.



**Figure 7. Influence of *E. faecium* on virus production in SIV infected cells.**

10<sup>6</sup> CFU/ml *E. faecium* were added for 60 or 90 min to cells in 96-well plates according to the experimental design described in (Figure 1). Infection throughout was with SIV at 0.01 MOI. At

48 h p.i. (3d4/21 cells) or 96 h p.i. (MDBK cells), the supernatants were collected and virus titers determined by TCID<sub>50</sub>. Results are means ± standard deviations from three independent experiments. \*\*\*P < 0.001.

### 3. 1. 6 *E. faecium* increases the production of NO

It is known from the literature [178] that, beside multiple other functions, nitric oxide (NO) is also an important physiological messenger and effector molecule for antiviral effects. Assessment of the secretion of NO under the influence of *E. faecium* revealed a most significant stimulating effect for 3D4/21 cells. As shown in (Figure 8), *E. faecium* increased the production of NO in both non-infected (bar on right side) and SIV-infected cells. As with the results shown above, the strongest stimulation was reached by *E. faecium* added to the host cells simultaneously with the virus (“competition”, blue bar). In MDBK-cells, the stimulation of NO release through *E. faecium* treatment was much less pronounced. However the results shown in (Figure 8) indicate the same tendency as for 3D4/21-cells and are significant for the probiotic induced stimulation of NO in non-infected cells [72].

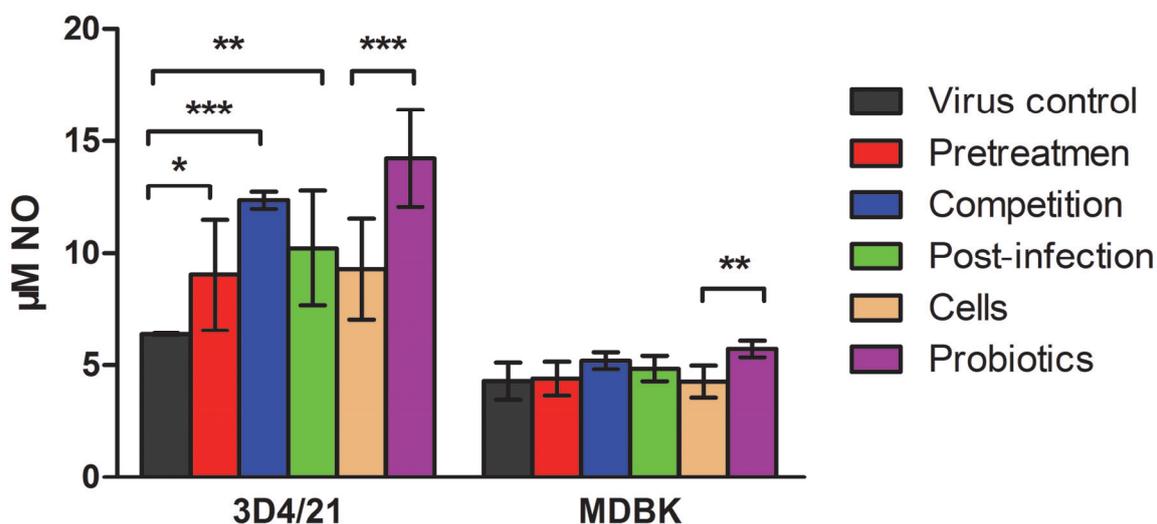
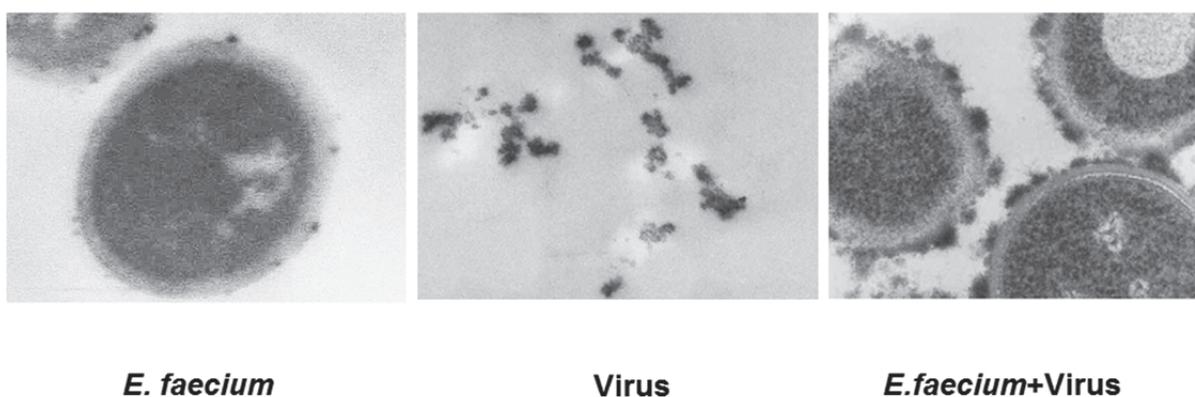


Figure 8. Effect of *E. faecium* on the nitric oxide (NO) release from 3D4/21 and MDBK cells.

Released NO in the supernatant was measured by Griess assay according to the modalities described in (Figure 1) on 3D4/21 and MDBK cells. Cells only and cells treated with *E. faecium* are shown in the last two columns of each group. Results are means  $\pm$  standard deviations from three independent experiments. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

### 3. 1. 7 Virus adsorption by *E. faecium*

It is possible that influenza virus particles could be engaged in direct physical interaction with the probiotic bacteria which may lead to a loss of infectivity. To address this question we included an experiment where virus particles were mixed with probiotic bacteria in a test tube and incubated for 1.5 h at room temperature (panel 4 in Figure 1 termed “preincubation”). After low speed centrifugation of the mixture to sediment *E. faecium*, samples were subjected to electron microscopical analysis. The micrographs shown indicate that virus particles seemed to be attached to the *E. faecium* surface (Figure 9). If SIV particles are trapped by the bacterial cells of *E. faecium*, virus titers should be reduced in the supernatants of the preincubation mixtures when compared to the controls where preincubation occurred with virus only. As seen from the data in (Table 6), virus titers were reduced by about two log-units in both types of host cells.



**Figure 9. Attachment of SIV particles to *E. faecium*.**

Samples of the resuspended pellets of virus and bacteria mixture from the preincubation assay were stained and examined by electron microscopy.

**Table 6. Loss of infectivity by direct physical interaction of SIV and *E. faecium***

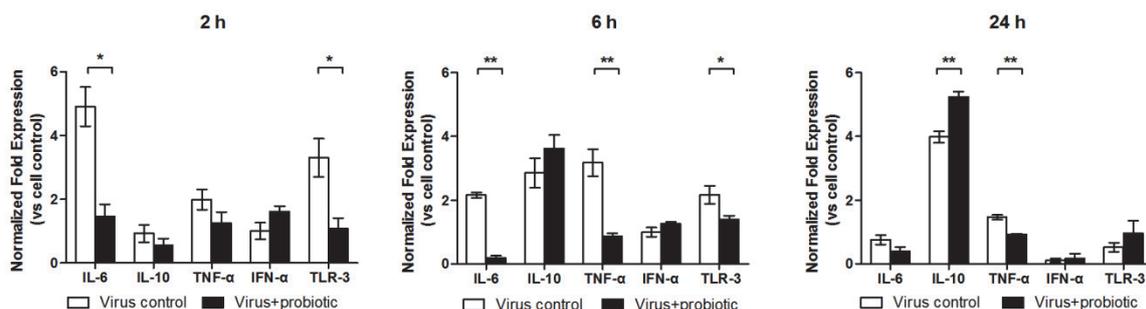
	3D4/21		MDBK	
	Virus Control	Virus+ <i>E. faecium</i>	Virus Control	Virus+ <i>E. faecium</i>
TCID <sub>50</sub>	6.27±0.12	3.63±0.15**	5.47±0.64	3.60±0.53**

After preincubation of SIV and *E. faecium* for 1.5 h, the mixture was centrifuged and supernatants were transferred onto monolayers of 3D4/21- and MDBK-cells to determine the virus titers by TCID<sub>50</sub>. As a control, SIV was preincubated without adding any *E. faecium* and the samples processed in parallel to the ones with the probiotic. Results are means ± standard deviations from three independent experiments. \*\*P< 0.01.

### 3. 1. 8 Cytokine expression under the influence of *E. faecium*

The observed inhibition of virus multiplication and stimulation of NO in the experiments where the probiotic was added to the cell (Figure 6, Figure 7) indicate, that *E. faecium* may influence cellular factors of innate defense which affect virus growth. We therefore analyzed the expression of cytokines and TLRs in 3D4/21-cells which are more likely to potentially modulate virus production than MDBK cells. The results from quantitative RT-PCR shown in (Figure 10) reveal a decreased expression of IL-6, TLR-3 and TNF-α in samples from *E. faecium* treated cells when compared to the non-treated samples (SIV-infected 3D4/21-cells only). On the other hand, the regulatory and potentially immunosuppressive cytokine IL-10 showed a low expression at 2 h, but increased strongly at 6 h and 24 h in the probiotic-treated cultures (Figure 10). Furthermore, *E. faecium* promoted an increased expression of IFN-α, at 2 h, 6 h and 24 h post SIV infection. However, due to the lacking significance of the values for the virus group and the *E. faecium* treated group, this effect can only be regarded as a tendency at most.

## Results



**Figure 10. Expression of cellular mediators of defense at 2 h, 6 h and 24 h.**

Cellular immune response of 3D4/21 cells to SIV infection after a 1h treatment of cells with or without the presence of  $10^6$  CFU/ml *E. faecium* during the infection period (“competition”, compare Figure 1). Selected cytokines (IL-6, IL-10, TNF- $\alpha$ , IFN- $\alpha$  and TLR-3) were measured by qRT-PCR at 2 h, 6 h and 24 h after infection. Results are means  $\pm$  standard deviations from three independent experiments. \*P < 0.05, \*\*P < 0.01.

### 3. 2 *in vivo* studies

Seventy-two weaned piglets were fed three different diets containing either  $1 \times 10^9$  colony forming units (CFU)/kg *E. faecium* or high (2,500 ppm) or normal levels of Zn oxide (80 ppm, control). Half of the piglets were vaccinated intramuscularly (VAC) twice with an inactivated trivalent SIV vaccine. All piglets were then infected intranasally with H3N2 SIV

#### 3. 2. 1 Clinical symptoms and weight gains

Clinically, SIV infection caused only mild symptoms (Figure 11) with fever ( $\geq 40^\circ\text{C}$ ) occurring only sporadically. Average body temperatures were lowest in the *E. faecium*+VAC group throughout the observation period after SIV infection. On 2 days post-infection (dpi) the piglets from the SIV infected control groups showed significantly elevated body temperatures compared to the 2 other treatment groups which had received feed supplementation with Zn or *E. faecium*.

Concerning body weights, significantly higher weekly weight gains were observed before infection in the *E. faecium* groups during the period from 39 to 46 and from 46 to 53 days of age regardless of vaccination (Figure 12A,). Comparing the dead body weights after exsanguination it was apparent that mean body weights in all Zn and *E. faecium* fed groups had increased after SIV infection from 1 dpi to 6 dpi, while it had declined in the control groups (Figure 12 B).

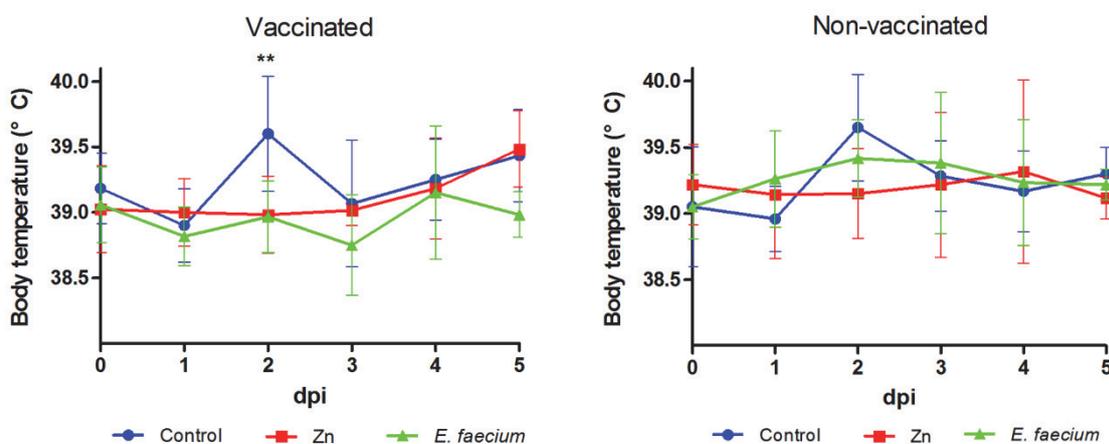
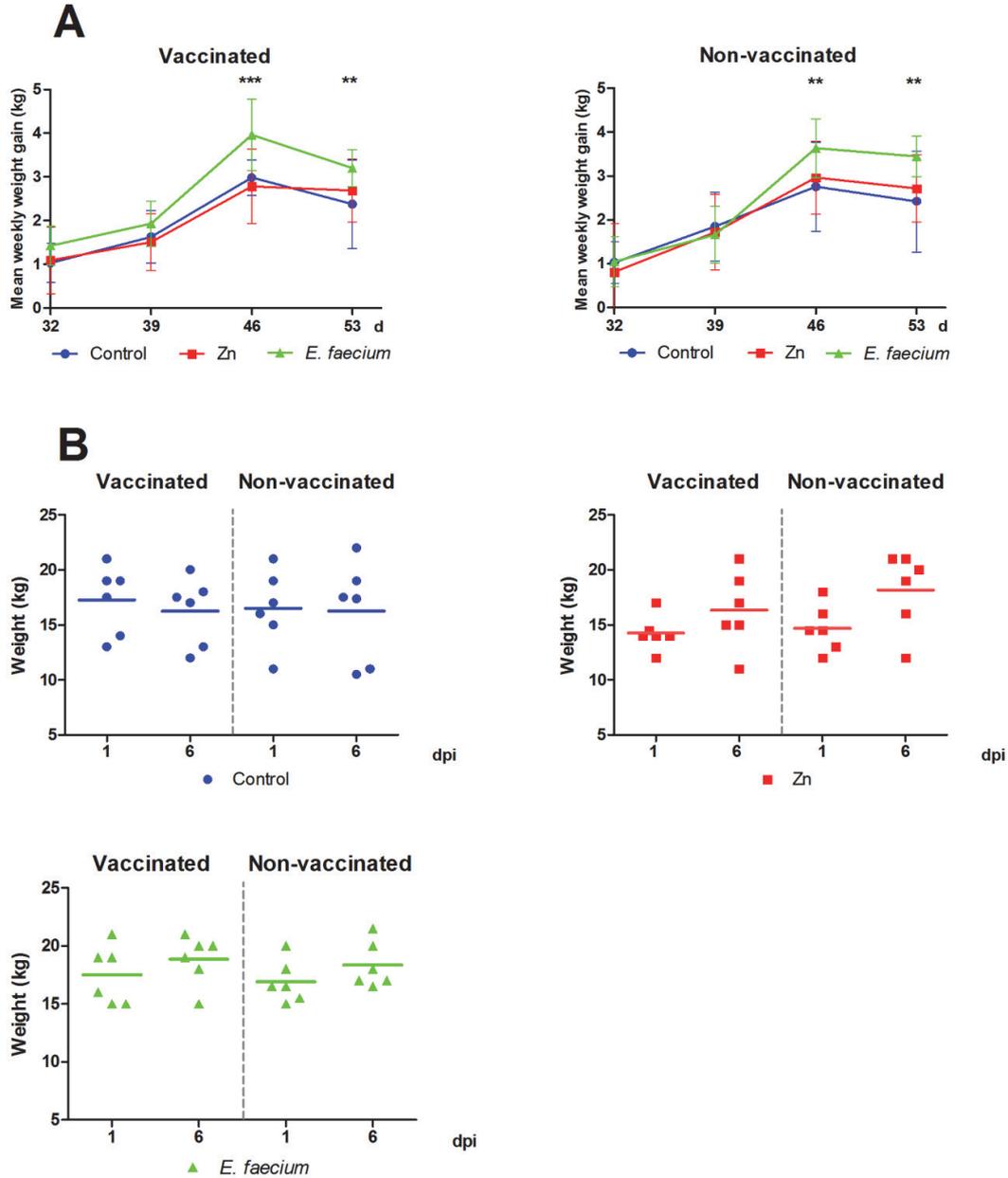


Figure 11. Body temperatures.

## Results

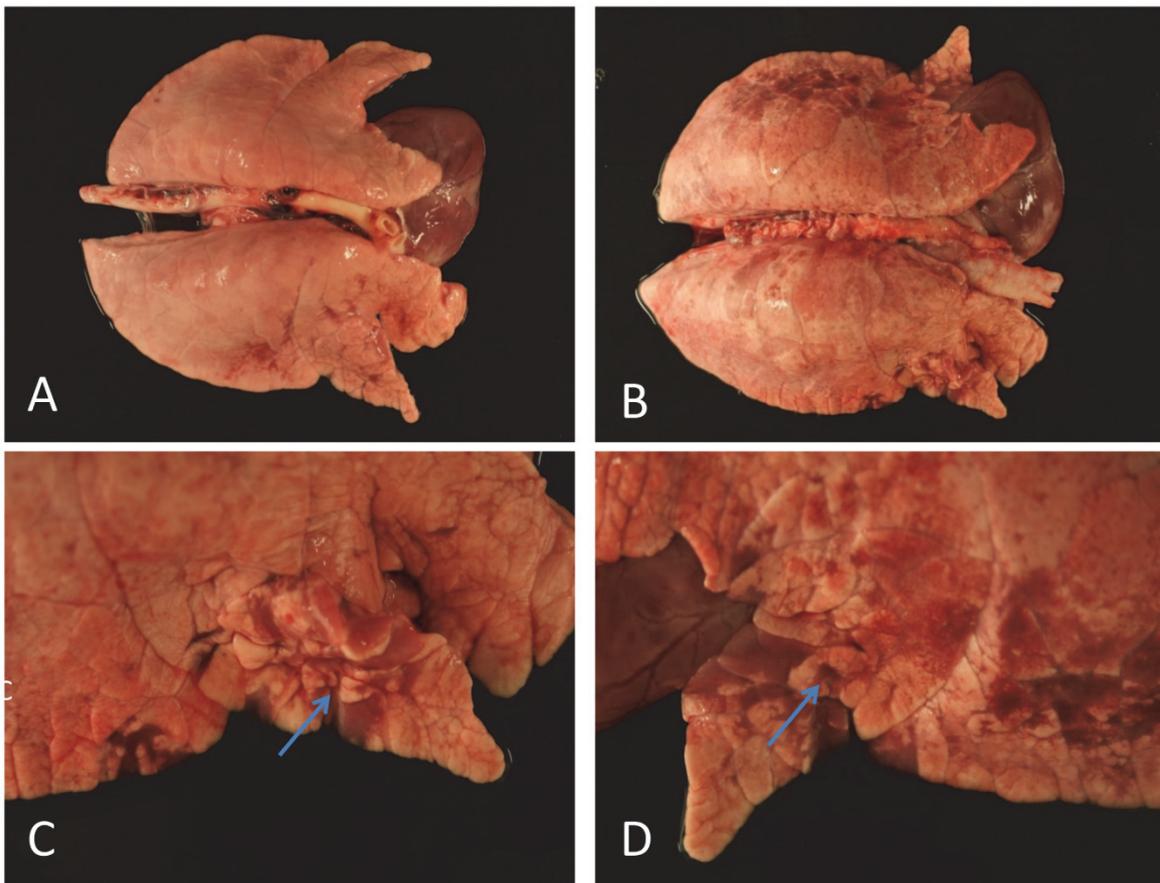
Individual body temperatures were measured rectally daily after infection. Each bar represents the mean value  $\pm$  standard deviation from 6 pigs. A significant difference is shown for the *E. faecium*+VAC compared to Zn<sup>low</sup>+VAC group (\*\*: P<0.01).



**Figure 12. Animal weight analyses. (A)** Mean weekly weight gain before virus infection. Each bar represents the mean value  $\pm$  standard deviation from 12 pigs (\*\*: P<0.01. \*\*\*: P<0.001). **(B)** Mean body weights on the indicated day after virus infection. Weights were measured after exsanguination.

### 3. 2. 2 Gross Pathology

Vaccination reduced the appearance frequency of parenchymal consolidation in the lungs of piglets in all diet groups at 6 dpi (Figure 13 A). Non-vaccinated animals showed more lesions macroscopically at 6 dpi (Figure 13 B, C, D). The right middle lung lobes exhibited the highest frequency and extent of lesions macroscopically; therefore, sections from this lobe were further analyzed and scored after histopathology examination.

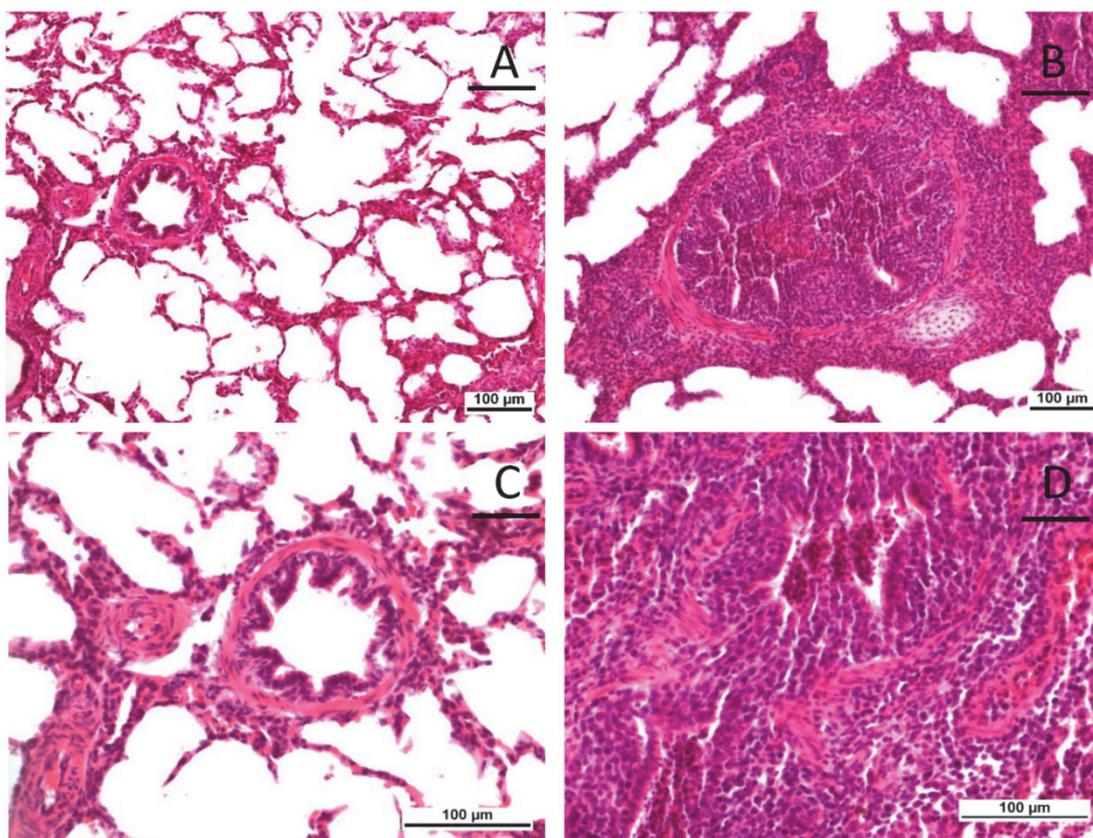


**Figure 13. Exemplary gross lesions in lungs after SIV infection.**

**(A)** Lung from a vaccinated piglet at 6 dpi. **(B)** Lung from a non-vaccinated piglet at 6 dpi. **(C and D)** Detailed pictures of lung B showing focal areas of tissue consolidation (arrows).

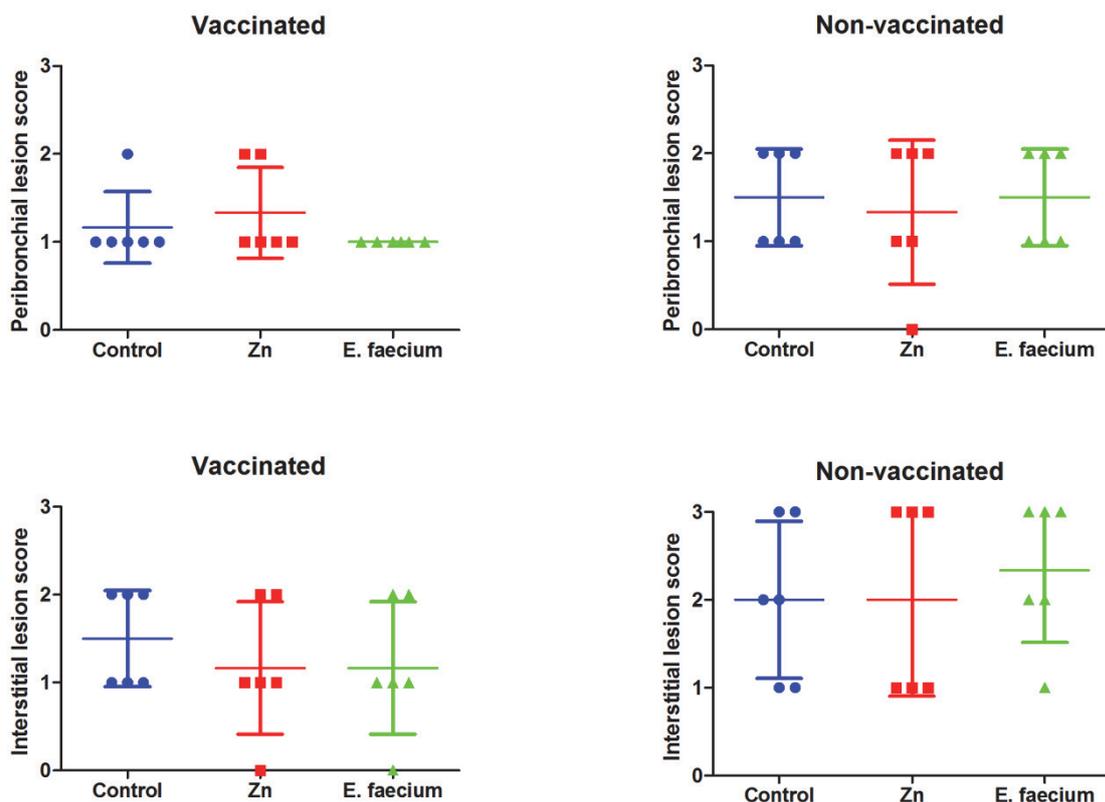
### 3. 3. 3 Histopathology and lesion score by HE staining

Success of vaccination was also seen microscopically by HE staining (Figure 14). Affected pigs revealed a mild (score 1) to severe (score 3) bronchointerstitial, lymphocytic dominated pneumonia (Figure 15). Sporadically bronchioles and alveoli contained cellular debris with lymphocytes, fewer histiocytes and scattered neutrophils accompanied by bronchiolar epithelial degeneration and necrosis. In the vaccinated groups a reduced frequency of moderate (score 2) peribronchial lesions and a prevention of severe (score 3) interstitial lungs lesion was observed, but no significant differences between different diets were apparent in vaccinated and non-vaccinated animals.



**Figure 14. Microscopical examination of lung sections.**

**(A and C)** HE stained lung of a vaccinated piglet with normal bronchial epithelial lining and absence of infiltrates of inflammatory cells. **(B and D)** HE stained lung of a non-vaccinated piglet with extensive infiltration predominantly of lymphocytes in the interstitium and around bronchi and bronchioli.

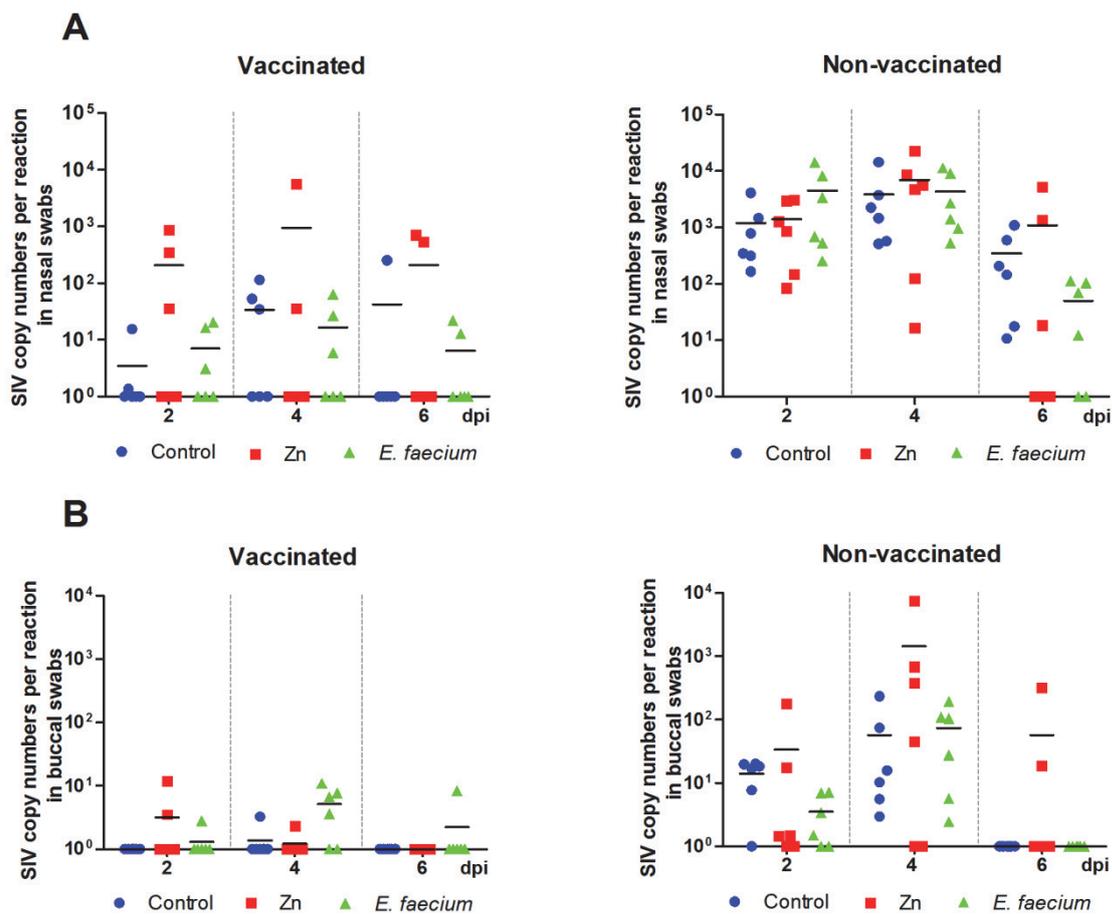


**Figure 15. Pathohistological lesion scoring.**

Scores of lung lesions in the right middle lobes (0 - no visible changes; 1 - mild changes, minimally different from the normal; 2 - moderate changes; 3 - severe and diffusely distributed changes).

### 3. 2. 4 Virological analysis

Virus shedding after challenge infection was analyzed by quantitate real-time RT-PCR (qRT-PCR) in nasal and buccal swabs before infection and at 2, 4 and 6 dpi (Figure 16) and in fecal swabs from 3 dpi. No virus genomes were detectable in samples before infection and in the fecal swabs (not shown). Generally, the vaccinated groups had lower viral loads when compared to the non-vaccinated groups in both nasal and buccal swabs (Figure 16). There were no significant differences, however, between the dietary treatment groups.



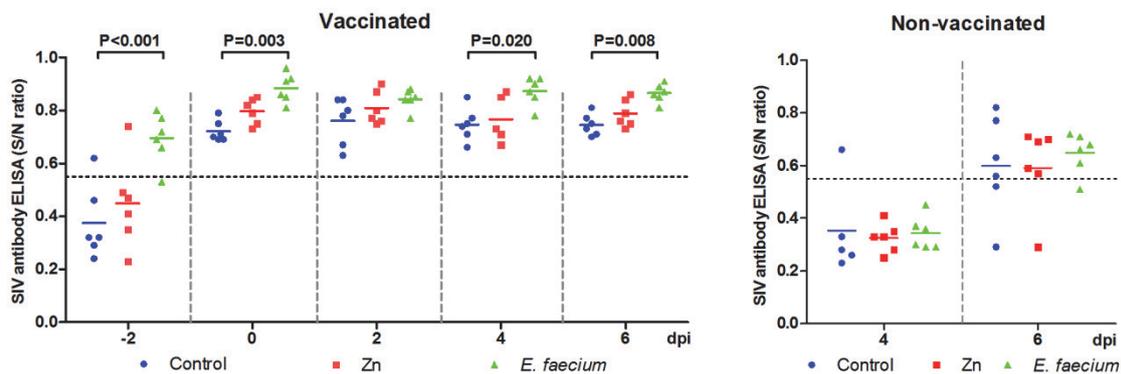
**Figure 16. Virus shedding in swabs determined by qRT-PCR.**

Virus shedding in nasal (**A**) and buccal swabs (**B**). SIV genome copy numbers were detected in swab eluates. All swabs taken at the day of infection (0 dpi) were negative.

### 3. 2. 5 Antibody ELISA

At the day of infection, 7 days after the second vaccination, all piglets had developed antibodies as detected with the NP protein ELISA (Figure 17). Significantly higher H3N2-specific antibodies were detected in the *E. faecium*+VAC group 2 days before ( $P=0.027$ ) and on the day of challenge infection ( $P=0.003$ ) as well as on 4 ( $P=0.020$ ) and 6 dpi ( $P=0.008$ ). For the non-vaccinated piglets, positive antibodies could barely be detected at 6 dpi.

## Results

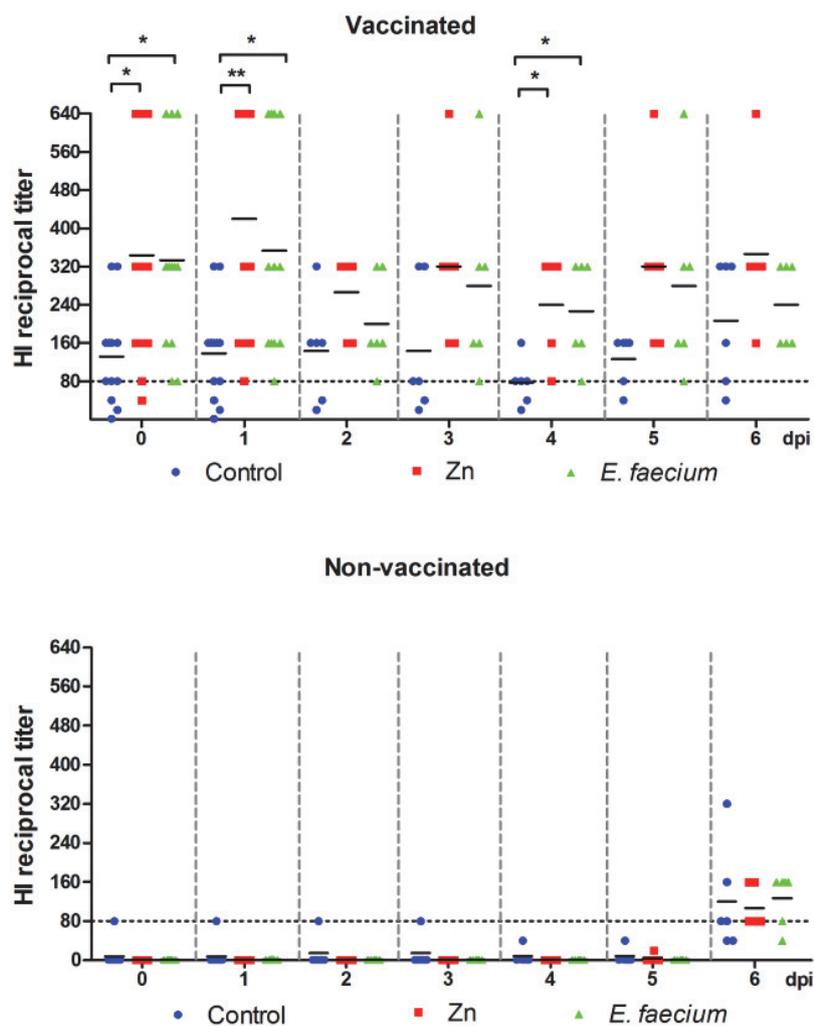


**Figure 17. SIV antibody ELISA.**

SIV-specific antibodies were detected in swine sera by competition ELISA targeting NP from -2 dpi to 6 dpi. The dotted line indicates the threshold above which values are considered positive.

### 3. 2. 6 HI assay

The ELISA results were confirmed by an HI assay which covers receptor blocking antibodies (Figure 18). Significantly higher antibody titers were detected in the *E. faecium*+VAC group on the day of SIV infection (0 dpi, P<0.05), 1 dpi (P<0.05) and 4 dpi (P<0.05). Significantly higher antibodies were also detected in the Zn+VAC groups on the day of SIV infection (P<0.05), 1 dpi (P<0.01) and 4 dpi (P<0.05). For the non-vaccinated piglets, antibodies could barely be detected at 6 dpi.



**Figure 18. Hemagglutination inhibition (HI) antibody titers.**

Two-fold serum dilutions starting at 1:20 were examined. Values  $\geq 80$  (dotted line) are considered positive.

### 3. 2. 7 Differential cell count

Hematological parameters in peripheral blood were examined after SIV infection using an automated analyzer. As shown in (Table 7), the numbers of monocytes and lymphocytes showed no differences between the groups, whereas reduced numbers of neutrophils were observed in the Zn groups.

## Results

**Table 7. Blood count.** Cellular composition of peripheral blood (mean numbers (100/ $\mu$ l)  $\pm$ SD) from 6 piglets/group infected with influenza virus.

Cell type	dpi	Control	Zn	<i>E. f.</i>	Control+VAC	Zn+VAC	<i>E. f.</i> +VAC
Neutrophils (20-70)	0	87.6 $\pm$ 23.5	53.3 $\pm$ 12.2	83.9 $\pm$ 33.8	80.2 $\pm$ 14.8	75.6 $\pm$ 15.5	76.0 $\pm$ 11.6
	3	92.8 $\pm$ 12.9	77.9 $\pm$ 31.2	94.9 $\pm$ 21.9	100.3 $\pm$ 28.22	88.2 $\pm$ 22.1	109.4 $\pm$ 43.9
	6	94.9 $\pm$ 6.9	84.9 $\pm$ 40.3	102.3 $\pm$ 15.5	70.1 $\pm$ 19.1	86.1 $\pm$ 19.4	91.6 $\pm$ 26.6
	0	87.8 $\pm$ 15.4	103.2 $\pm$ 27.2	97.0 $\pm$ 35.1	89.3 $\pm$ 10.1	85.8 $\pm$ 16.8	94.7 $\pm$ 9.5
	3	92.0 $\pm$ 13.1	107.4 $\pm$ 16.7	94.0 $\pm$ 13.3	89.1 $\pm$ 23.8	103.9 $\pm$ 20.1	111.7 $\pm$ 15.6
	6	93.1 $\pm$ 24.3	86.4 $\pm$ 21.4	81.1 $\pm$ 5.9	69.8 $\pm$ 14.0	89.7 $\pm$ 12.1	79.7 $\pm$ 9.6
Lymphocytes (60-340)	0	15.2 $\pm$ 7.0	14.3 $\pm$ 4.5	13.4 $\pm$ 2.5	13.2 $\pm$ 3.0	14.6 $\pm$ 2.3	13.1 $\pm$ 2.6
	3	13.4 $\pm$ 3.7	17.6 $\pm$ 4.3	19.1 $\pm$ 7.0	15.5 $\pm$ 5.3	17.2 $\pm$ 3.3	15.2 $\pm$ 3.6
	6	17.6 $\pm$ 4.4	17.1 $\pm$ 4.2	13.3 $\pm$ 2.8	12.1 $\pm$ 2.9	16.8 $\pm$ 6.0	9.0 $\pm$ 2.0
	0	15.2 $\pm$ 7.0	14.3 $\pm$ 4.5	13.4 $\pm$ 2.5	13.2 $\pm$ 3.0	14.6 $\pm$ 2.3	13.1 $\pm$ 2.6
	3	13.4 $\pm$ 3.7	17.6 $\pm$ 4.3	19.1 $\pm$ 7.0	15.5 $\pm$ 5.3	17.2 $\pm$ 3.3	15.2 $\pm$ 3.6
	6	17.6 $\pm$ 4.4	17.1 $\pm$ 4.2	13.3 $\pm$ 2.8	12.1 $\pm$ 2.9	16.8 $\pm$ 6.0	9.0 $\pm$ 2.0
Monocytes (0-9)	0	15.2 $\pm$ 7.0	14.3 $\pm$ 4.5	13.4 $\pm$ 2.5	13.2 $\pm$ 3.0	14.6 $\pm$ 2.3	13.1 $\pm$ 2.6
	3	13.4 $\pm$ 3.7	17.6 $\pm$ 4.3	19.1 $\pm$ 7.0	15.5 $\pm$ 5.3	17.2 $\pm$ 3.3	15.2 $\pm$ 3.6
	6	17.6 $\pm$ 4.4	17.1 $\pm$ 4.2	13.3 $\pm$ 2.8	12.1 $\pm$ 2.9	16.8 $\pm$ 6.0	9.0 $\pm$ 2.0
	0	15.2 $\pm$ 7.0	14.3 $\pm$ 4.5	13.4 $\pm$ 2.5	13.2 $\pm$ 3.0	14.6 $\pm$ 2.3	13.1 $\pm$ 2.6
	3	13.4 $\pm$ 3.7	17.6 $\pm$ 4.3	19.1 $\pm$ 7.0	15.5 $\pm$ 5.3	17.2 $\pm$ 3.3	15.2 $\pm$ 3.6
	6	17.6 $\pm$ 4.4	17.1 $\pm$ 4.2	13.3 $\pm$ 2.8	12.1 $\pm$ 2.9	16.8 $\pm$ 6.0	9.0 $\pm$ 2.0

### 3. 2. 8 Cellular immune responses

Flow cytometry of immune cell phenotypes of PBMC subpopulations was performed from 0 dpi to 6 dpi (Figure 19). Virus infection led to a slight decrease in the frequency of Th cells until 6 dpi. In contrast, increased percentages of CTLs, Th/memory cells, antibody-forming and/or memory B cells, and NK cells were observed until 6 dpi in both vaccinated and non-vaccinated animals.

Regarding the dietary effect of supplementation, no significant differences between treatment groups were observed for any subpopulation before infection (Figure 19, 0 dpi). After challenge infection, significant differences were found only at single time points. For instance, higher CTL percentages ( $P < 0.05$ ) were found in the Zn+VAC group compared to the control group at 5 dpi. In the non-vaccinated groups, higher CD4<sup>+</sup>CD8<sup>+</sup> T cell percentages ( $P < 0.05$ )

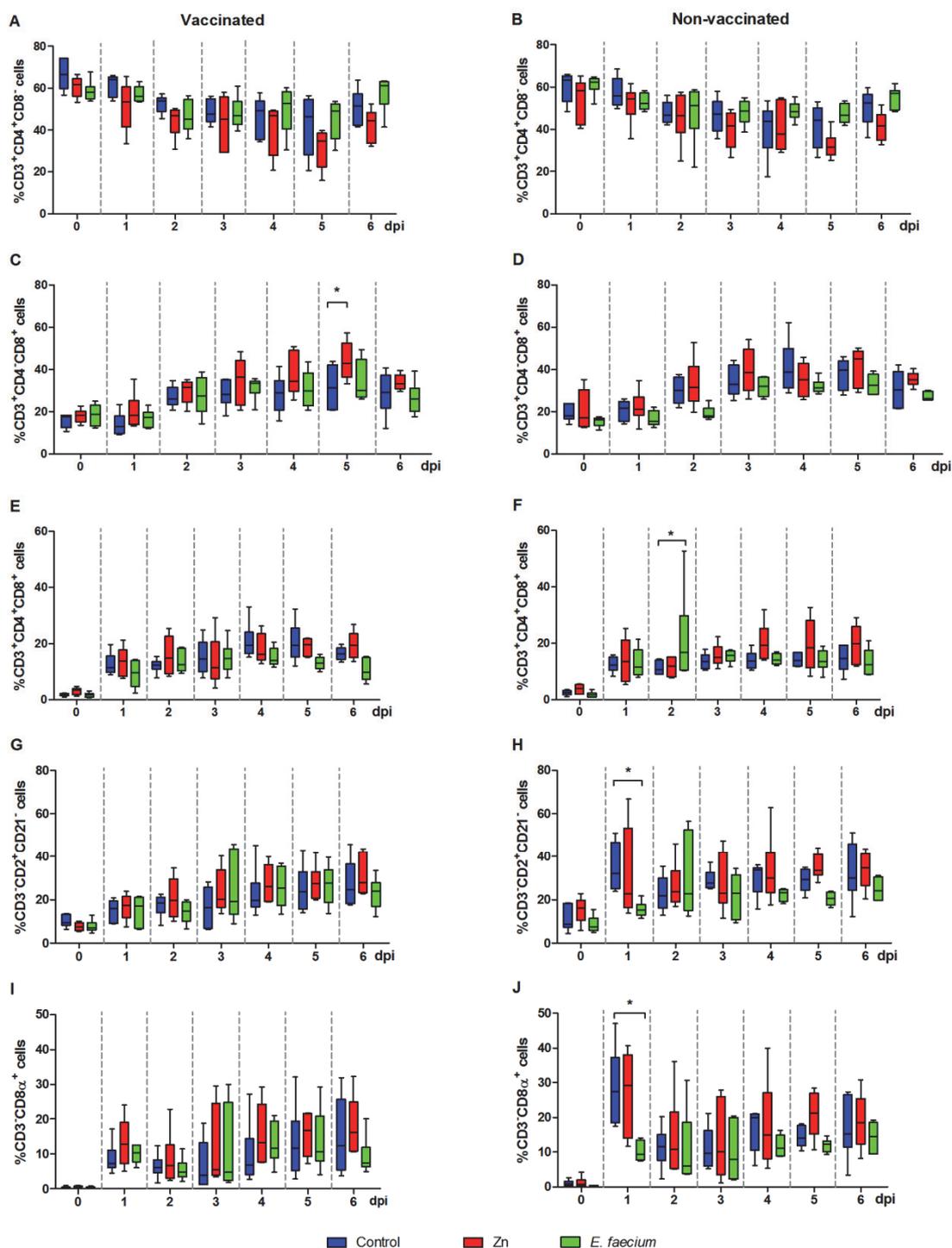
## Results

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were found in the *E. faecium* group at 2 dpi. Finally, significantly lower antibody-producing and/or memory B cell numbers were observed in the *E. faecium* group compared to the control group ( $P < 0.05$ ) at 1 dpi in non-vaccinated pigs.

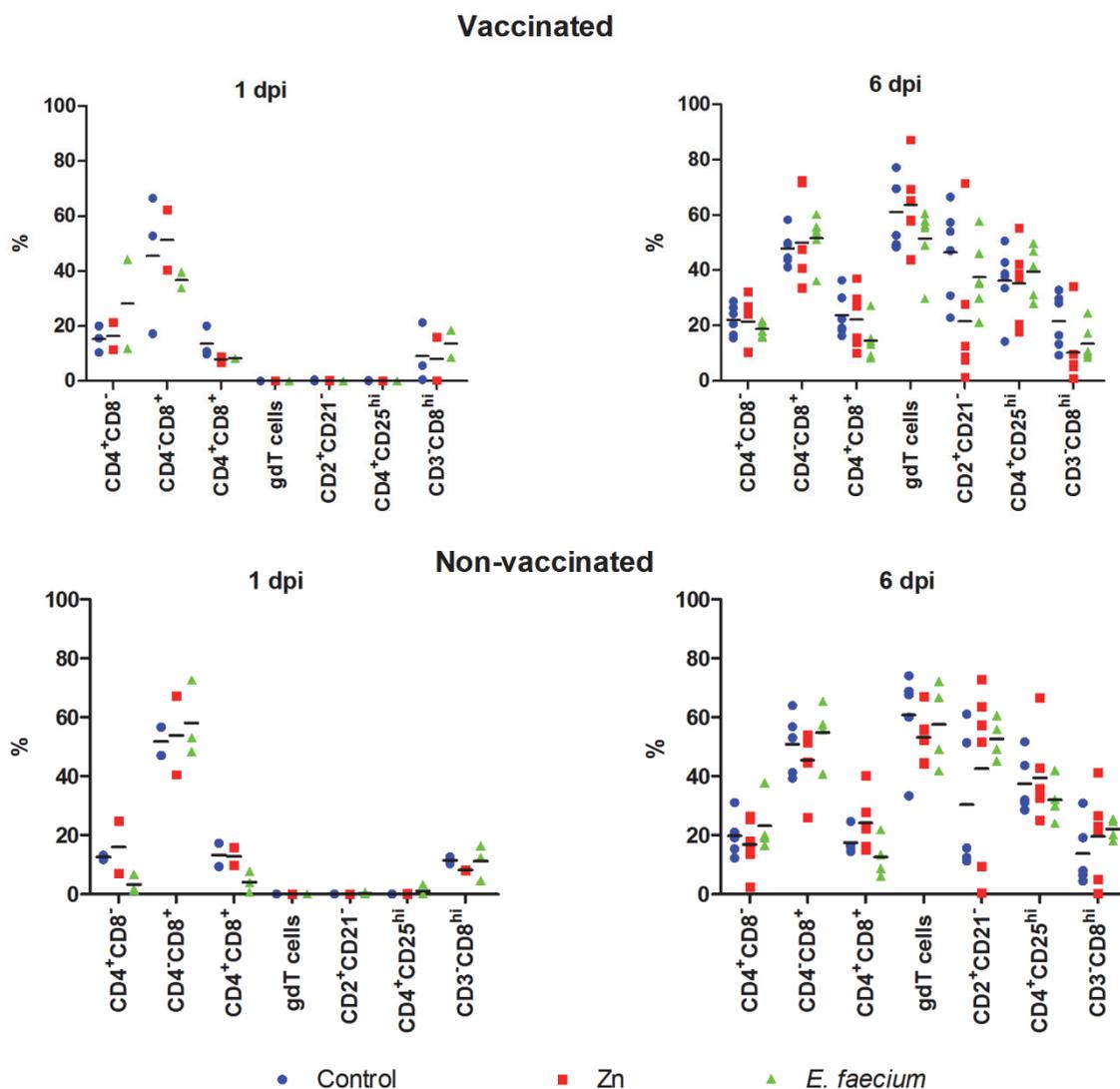
Immune cell phenotypes of BAL cells were examined after necropsy on 1 dpi and 6 dpi. We observed an increase of  $\gamma\delta$  T-cell, activated B-cell and activated T-cell percentages at 6 dpi after infection compared to 1 dpi, but no influence of vaccination and dietary treatment could be recorded (Figure 20).

## Results



**Figure 19. Comparison of immune cell subsets of PBMC.**

Percentages of Th cells (A, B), CTLs (C, D), Th/memory cells (E, F), antibody-forming/memory B cells (G, H) and NK cells (I, J) in PBMCs from 0 to 6 dpi. Each bar represents the mean value  $\pm$  standard deviation from 6 piglets (\*: P < 0.05).



**Figure 20. Comparison of immune BAL cell subsets.** Percentages of Th cells (CD4<sup>+</sup>CD8<sup>-</sup>); CTLs (CD4<sup>+</sup>CD8<sup>+</sup>); Th/memory cells (CD4<sup>+</sup>CD8<sup>+</sup>);  $\gamma\delta$  T cells (CD2<sup>+</sup>CD8<sup>+</sup>); antibody-producing and/or memory B cells (CD2<sup>+</sup>CD21<sup>-</sup>); activated Th cells (CD8<sup>-</sup>CD25<sup>high</sup>), and NK cells (CD3<sup>-</sup>CD8<sup>high</sup>) at 1dpi and 6 dpi in vaccinated (upper panel) and non-vaccinated (lower panel) animals.



## 4 DISCUSSION

### 4.1 *In vitro* study

Probiotics have been mainly studied in the context of bacterial infections of the gastrointestinal tract which is the natural target tissue of probiotics. However, there are a few reports which indicate that upon oral intake, probiotics can also affect infections of the respiratory tract [61,99,100]. The underlying mechanism for such effect is associated with the impact that probiotics have on different forms of the immune system [89,90]. There are also reports in the literature [63,75,76,79] where probiotics induce antiviral activity *in vitro* and are even applied as a medical treatment against persistent virus infections in humans and animals.

In this study, zoonotic swine influenza viruses served as a novel object to test for the antiviral potential of the probiotic *E. faecium* and to shed light on its mechanisms of action. Two different SIV strains were chosen which are currently circulating in the pig population, H1N1 and H3N2. As an established model for the present *in vitro* study an epithelial- (MDBK-cells) and a porcine alveolar macrophage cell line (3D4/21-cells) were utilized. We present the results from *in vitro* experiments using porcine H1N1- and H3N2-influenza virus in MDBK- and 3D4/21 cells, respectively, which demonstrate that at the cellular level, the probiotic *E. faecium* effectively protects host cells from swine influenza virus infection and the data presented on the influenza system are in support of the above mentioned hypotheses published on other host-virus system [61,99,100], that probiotics are not only useful to inhibit enteric viruses, but may also have potential for the control of respiratory viruses.

It can be argued that the concentration of the probiotic utilized here may not reflect the situation in the target tissue *in vivo*. However, the concentration chosen for treatment of the cell cultures ( $10^6$  CFU/ml) reflects the same concentration which was determined in the gut of piglets fed *E. faecium* as a supplement during previous feeding trials in our research consortium [66]. In order to find out during which period of the SIV replication cycle the

probiotic has the most stringent effect, *E. faecium* was added for maximally 90 min to the host cells either before, during or after virus infection (Figure 1). The results indicate that the simultaneous addition of virus and *E. faecium* to the host cell monolayer apparently allowed for the most effective interference of the probiotic with SIV and/or the host cell leading to a maximal cell rescue and to a pronounced inhibition of virus multiplication. As seen from (Figure 6, Figure 7), this experimental setup (termed “competition”) resulted in a 4 log-unit reduction of virus titer and in a concomitant rescue of cell viability. Since both a 1 h exposure of the monolayers to *E. faecium* before SIV-infection and a 1 h treatment after completion of virus infection led to a 2-3 log-unit loss of virus titer, it is reasonable to hypothesize that the probiotic alters host cell factors which apparently leads to an inhibition of influenza virus multiplication. Most likely candidates for such factors are mediators of cellular defense processes.

The expression of NO and its subsequent increased activity has previously been reported to play a role in the host response to multiple viral families, and in various host species [178,179]. There are also reports that pretreatment with NO donor compounds significantly suppressed replication of astrovirus [180]. In addition to its antiviral properties, NO has been described to modulate intestinal barrier function, gut motility, iron transport, and has been implicated in numerous infections and non-infectious diseases [180]. We found that *E. faecium* increased the expression of NO in both 3D4/21 and MDBK cells (Figure 8). All the samples collected after treatment with *E. faecium* showed significantly increased NO-values when compared to the non-treated counterparts, especially in the 3D4/21 cell line. This is consistent with the hypothesis that high NO levels are correlated with decreased SIV production [178,180].

Since *E. faecium* acts most inhibitory when it is added together with the virus particles during the 1 h experimental incubation period, we assessed whether SIV might be physically trapped or inactivated by the probiotic bacteria. This simultaneous addition of virus and probiotic was mimicked in a mixed incubation designated “preincubation assay” in the experimental setup shown (panel 4, Figure 1). As illustrated by electron microscopy (Figure 9), in such incubation mixtures virus particles are indeed bound to the surface of *E. faecium*. The results of virus titrations of samples taken from the supernatant after centrifugation of such suspensions

summarized in (table 6) show that a substantial portion of the input virus particles has indeed been trapped by the bacteria concentrated in the sediment. Presently it is not possible to make any conclusions with regard to the quantitative trapping capacity or to the distinction between receptor involvement and/or electrostatic effects. However, the trapping of SIV by a probiotic bacterium observed here, should certainly be followed up to identify the structures involved and also to determine the specificity of binding. With the direct trapping of virus particles through *E. faecium* bacterial cells and the induction of NO- expression, two antiviral functions of the probiotic may operate synergistically and add up to produce a more severe inhibition of SIV under the experimental conditions at the cellular level. However the matters may even be more complicated.

There is now growing evidence that even at the cellular level, probiotics could also affect the expression of cytokines and other immune mediators relevant for the innate immune response to viral infections [88,181]. We determined the expression of selected mediators known to be involved in cellular defense processes: IFN- $\alpha$ , IL-6, TNF- $\alpha$ , IL-10, and TLR-3 (Figure 10) in SIV-infected host cells (3D4/21 cells) under the influence of *E. faecium*.

The immune effects of IFN- $\alpha$  have previously been exploited to treat several diseases [182]. IFN- $\alpha$  is extensively used to prevent and treat viral respiratory diseases such as flu [183-185]. However, there is also a report that influenza viruses can escape the antiviral activities of interferon by mutation [49]. As seen from (Figure 10), *E. faecium* promoted an increased expression of IFN- $\alpha$  in 3D4/21 cells. However, the difference between the values generally rated as non-significant, thus IFN- $\alpha$  can be ruled out as the main immunoregulatory cytokine which could lead to an *E. faecium* induced inhibition of SIV-infection in cell culture.

IL-10 has been known as a regulatory cytokine in the activation and effector function of T lymphocytes, monocytes, and macrophages [186]. Another published study suggests that IL-10 is associated with the immune responses to pathogens [187]. In the present study, IL-10 was found to be stimulated by the probiotic treatment which was repressed early after virus infection but then expressed at higher levels later in infection to control the strong initial inflammatory response to SIV infection. Interestingly, this cytokine is particularly enhanced in the macrophage cell line upon *E. faecium* treatment and thus is regarded as a candidate

cytokine to support cellular control of SIV infection.

In this study, two pro-inflammatory cytokines were found to be significantly reduced in SIV-infected 3D4/21-cells upon treatment with the probiotic, IL-6 and TNF- $\alpha$  (compare Figure 10). IL-6 and TNF- $\alpha$  are known to participate in non-specific and specific antiviral immune response to influenza virus infection [181]. Secretion of IL-6 by macrophages is known to play an indirect immunoregulatory role in the immune responses to viral infection [188], and TNF- $\alpha$  acts as an inflammatory cytokine by triggering a cascade of cytokine production [50,189]. Since both IL-6 and TNF- $\alpha$  are downregulated in the presence of *E. faecium* in SIV-infected 3D4/21 cells, the reduced inflammatory response caused by some cytokines at the cellular level may contribute to the antiviral effect of the probiotic.

The expression of Toll-like receptors has been described as being fundamental in the host defense against pathogenic challenges since they trigger innate immune responses [190]. Among those, Toll-like receptor 3 (TLR-3) was the first identified antiviral TLR to have a central role in the host response to viruses [191]. Our experimental data show that the treatment of SIV-infected 3D4/21-cells with *E. faecium* led to a decreased expression of TLR-3 at 2 h and 6 h post infections when compared to virus infected cells without *E. faecium*. This suggests that the probiotic-induced modulation of this receptor may have a role in its antiviral function. Another explanation could be that - in line with our observation that SIV particles could be trapped by the probiotic, fewer virus particles may interact with the host cells and thus, fewer TLR-3 proteins are induced. At any rate, the function of TLR-3 in the antiviral defense need to be further investigated.

The results presented altogether show that the probiotic *E. faecium* quite effectively inhibits the multiplication of swine influenza viruses in relevant cell culture systems. The antiviral mechanism of this probiotic is probably manifold since it was found to act on both the virus particles and the host cells. However, at least a few inhibitory parameters could be identified: *E. faecium* bacteria are able to adsorb SIV-particles and to alert the cells by mediating a rapid antiviral response through modulating the expression of defense relevant mediators. Amongst these IL-6, TNF- $\alpha$ , IL-10, IFN- $\alpha$  and TLR-3 were identified as entities modulated by the probiotic treatment. It is realized that *E. faecium* can induce many more complex reactions in

## Discussion

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a treated tissue and that the results presented are quite limited, because only a few mediators were assessed in this study. However, one common denominator of probiotic action could be NO which is a mediator affected by many cellular signaling cascades. In line with publications for other virus-host systems, our results also point to a central role of NO which is stimulated upon the treatment with the probiotic and which may mount an improved cellular defense response against SIV-infection in tissues which were stimulated with a probiotic.

On the basis of the available evidence shown here for swine influenza virus, it appears that already at the cellular level *E. faecium* as a probiotic feed (or food) additive has the potential of reducing influenza virus infections in farm animals.

### 4. 2 *in vivo* study

Concerning the *in vivo* study, we investigated the effects of feed supplementation with *E. faecium* or higher dietary ZnO levels on vaccination against and challenge with swine influenza A virus in piglets. Clinical follow-up, virological outcome, as well as humoral immune and cellular immune responses were recorded. To our knowledge, such information on the impact of probiotics and Zn in pigs, or any livestock is the first to be collected and described. The data presented provides an important contribution with respect to the assessment of the usefulness of feed supplementation on an important viral disease.

Challenge infection with H3N2 SIV caused mild symptoms, which is in line with observations from other studies [192] and confirms the importance of good sanitary status, as provided during the experimentation here, in the prevention of secondary infections. The observation of significantly higher body weight gains in the *E. faecium* treatment groups after weaning and before infection was also made in other studies [67,124], whereas the growth-promoting effect of the Zn diet observed by others [114,123,124] could not be confirmed. Comparing body weights after challenge infection, it appeared that mean body weights of piglets in all Zn and *E. faecium* increased from 1 dpi to 6 dpi, while it decreased in the control groups (Figure 12B). These results might indicate a better and faster recovery from infection and anorexia of reduced duration in the probiotic and Zn groups.

In this study, lungs from non-vaccinated animals showed more extensive macroscopical lesions (Figures 13 and 14) than those from vaccinated animals. Microscopical evaluation also revealed that vaccination reduced the severity of microscopic lesions (Figure 15). However, a dietary influence of probiotic and Zn on these features was not apparent.

The most prominent finding obtained in this study was the development of higher SIV-specific ELISA- and HI- antibody levels in the Zn and particularly *E. faecium* treated vaccine groups two days before as well as on the day of virus infection (Figures 7 and 8). The increased antibody response to vaccination in the group receiving the higher Zn level diet compared to the normal diet group might indicate that a suboptimal Zn supply in the control group was restored, since it has been shown that a Zn deficiency impairs B-cell function [193]. It is also

possible that a normal antibody response in the control group was improved by the additional Zn supply, although this has not been shown elsewhere yet. The data also demonstrate that dietary supplementation with *E. faecium* was able to boost antibody levels. Similar observations were made in a previous study using *Bacillus cereus* in non-infected piglets [194]. However, we and others can only speculate about the possible mechanisms of how antibody titers to a vaccine applied parenterally might be enhanced by oral probiotics. We applied the influenza vaccine intramuscularly and we assume that immune responses were mainly generated in the tributary (axillary) lymph nodes. Some communication must, therefore, exist between probiotic bacteria in the gut and the cells initiating immune responses at a distant site to explain the observed effect. It was previously argued that (subcellular) fragments of probiotics may enter the bloodstream and as such have a very direct albeit weak adjuvant effect at a distant lymph node [64]. Another possible explanation could be that during feed intake some probiotic fragments might be inhaled and/or directly get in contact with epithelial cells in the nasopharynx and induce cytokines or other signaling molecules with an adjuvant effect. Interestingly the *E. faecium* group diet was based on the control ( $Zn^{low}$ ) diet, thus, not only could a possible lack of Zn be compensated by the probiotic supplement but also there could be a possible synergistic effect between *E. faecium* and optimal or elevated Zn for the induction of even higher antibody levels.

Our data also shows that vaccination did not result in sterile immunity but reduced the number of animals shedding virus as well as the amount of virus shed from the nose and buccal sites (Figure 16). Fecal shedding was also tested but, in agreement with the literature [195], no virus could be detected. Despite higher antibody levels, a stronger reduction of virus shedding was not achieved by *E. faecium* or Zn supplementation in vaccinated animals. As reported by others [196,197], an increase in antibody levels does not necessarily mean that these antibodies exhibit high specificity or affinity. This is especially true for antibodies induced by inactivated vaccines where, unlike following live vaccine administration or natural infection, virus is not delivered to secondary lymphatic organs and presented by dendritic cells to elicit optimal virus-neutralizing antibody responses.

## Discussion

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Hematology revealed transiently reduced neutrophil numbers in both vaccinated and non-vaccinated animals receiving the Zn diet. Zn-induced neutropenia has been described in the literature [198]. Obviously, in this study, neutrophil numbers were still sufficient to avoid negative effects on the course of SIV infection. It needs to be emphasized that the Zn diet (2500 ppm) was reduced to a Zn<sup>med</sup> diet (250 ppm) before infection to reduce the possibility of toxic effects. We, therefore, cannot rule out that, if continued, the high Zn doses might have had negative effects on health of the individuals.

According to the literature on cellular immune responses, CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as antibody-producing B cells make an important contribution to the control of influenza virus replication and virus clearance during infection [199,200]. Th cells primarily stimulate antibody and cytokine production as well as proliferation of CTLs. The CTL response is mainly directed against the more conserved influenza virus proteins, M and NP. Consequently, a robust CTL response can also confer protection against heterologous influenza A virus challenge [53,199]. Inactivated vaccines are poor inducers of cellular immune responses [201]. Accordingly, we observed no significant effect of vaccination on cellular immune responses. Only a slight decrease in Th cells and a concomitant equally slight increase of CTL and antibody-producing B cell percentages was recorded from 1 to 6 dpi in PBMCs. Regarding dietary effects, we found significant differences between the *E. faecium* and Zn groups and the control group only at single time points. However, no prolonged effects were detected. We also compared the percentages of immune cell phenotypes in cells of the BAL fluid after necropsy, since the proliferation responses in peripheral blood does not fully reflect those at the site of infection [202]. We found increased percentages of  $\gamma\delta$  T-cells, activated B-cells and activated T-cells at 6 dpi compared to those on 1 dpi in vaccinated and non-vaccinated animals, but no influence of dietary treatment. Thus it seems that *E. faecium* and Zn supplementation neither systemically nor locally changed the cellular immune response to SIV infection substantially.

In summary, the results presented here suggest that high doses of ZnO and particularly *E. faecium* fed as supplements to piglets can increase humoral immune responses following SIV vaccination and support recovery from clinical illness caused by SIV infection. However, the increased antibody response does not significantly affect virus shedding or prevent the

## Discussion

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development of lung lesions after challenge infection. Future studies are needed to reveal if used in combination with an appropriate vaccine, feed supplementation with ZnO and/or *E. faecium* might potentiate an antibody response to allow for a reduction of virus shedding.



## 5 SUMMARY

### **Effect of dietary *Enterococcus faecium* NCIMB 10415 and zinc oxide on porcine influenza virus infection *in vitro* and *in vivo***

Swine influenza virus (SIV) causes severe suffering at the animal level and significant economic losses in the swine industry worldwide. Since epithelial cells in pig trachea contain both human and avian type receptors ( $\alpha$  2, 6- and  $\alpha$  2, 3-linked sialic acid, respectively), pigs are supposed to be the “mixing vessels” for a wide range of influenza A viruses and as the potential source for new human-avian influenza A virus reassortants. Therefore the control of swine influenza viruses plays an important role both from the animal health and from the public health point of view. To address this question, two studies were performed in porcine model - *In vitro* and *in vivo*. Concerning the *in vitro* study, we assessed the inhibitory potential of the probiotic *Enterococcus faecium* NCIMB10415 on the replication of two porcine strains of influenza virus (H1N1 and H3N2 strain) in a continuous porcine macrophage cell line (3D4/21) derived from lung macrophages and in the continuous epithelial cell line, MDBK cells. Cell cultures were treated with *E. faecium* at the non-toxic concentration of  $1 \times 10^6$  CFU/ml in growth medium for up to 90 min before, during and after SIV infection. After further incubation of cultures in probiotic-free growth medium, cell viability and virus propagation were determined at 48 h or 96 h post infection in 3d4/21 and MDBK cells, respectively. The results obtained reveal an almost complete recovery of viability of SIV infected cells and an inhibition of virus multiplication by up to four log units in the *E. faecium* treated cells. In both 3D4- and MDBK-cells a 60 min treatment with *E. faecium* stimulated NO release which is in line with published evidence for an antiviral function of NO. Furthermore, *E. faecium* caused a modified cellular expression of selected mediators of defense in 3D4-cells: while the expression of TNF- $\alpha$ , TLR-3 and IL-6 were decreased in the SIV-infected and probiotic treated cells, IL-10 was found to be increased. Since we obtained experimental evidence for the direct adsorptive trapping of SIV through *E. faecium*, at the cellular level, this probiotic microorganism inhibits influenza viruses by at least two mechanisms, direct physical interaction and strengthening of

innate defense.

Concerning the *in vivo* study, we tested if probiotic *Enterococcus faecium* NCIMB 10415 or zinc (Zn) oxide as feed supplements could provide beneficial effects on SIV vaccination and infection in piglets. Seventy-two weaned piglets were fed three different diets containing either *E. faecium* or high (2,500 ppm) or normal levels of Zn oxide (natural Zinc content: 50-80 ppm, control). Half of the piglets were vaccinated intramuscularly (VAC) twice with an inactivated trivalent SIV vaccine. All piglets were then infected intranasally with H3N2 SIV. Clinically, significantly higher weekly weight gains were observed in the *E. faecium* group before virus infection, and piglets in Zn and *E. faecium* groups gained weight, while those in the control group lost weight. Using ELISA, we found significantly higher H3N2-specific antibody levels in the *E. faecium*+VAC group 2 days before and at the day of challenge infection as well as at 4 and 6 days after challenge infection. Higher hemagglutination inhibition (HI) titers were also observed in the Zn+VAC and *E. faecium*+VAC groups at 0, 1 and 4 days after infection. However, there were no significant differences in virus shedding and lung lesions between the dietary groups. Compared to the control group, significantly higher CD4<sup>+</sup>CD8<sup>+</sup> and CD8<sup>+</sup> (cytotoxic T lymphocytes, CTL) were detected in the Zn and *E. faecium* groups at various time points after infection as determined by flow cytometry. Our results suggest that feeding high doses of zinc oxide and particularly *E. faecium* could beneficially influence humoral immune responses after vaccination and recovery from SIV infection, but not affect virus shedding and lung pathology.

## 6 ZUSAMMENFASSUNG

### Wirkung von Nahrungs *Enterococcus faecium* NCIMB 10415 und Zink oxid auf Schweine influenza virus Infektion *in vitro* und *in vivo*

Schweineinflenzaviren (SIV) verursachen bei Tieren erhebliches Leiden und gravierende Verluste in der weltweiten Schweineproduktion. Weil die Epithelzellen in der Trachea des Schweins sowohl den Humantyp als auch den aviären Typ des Rezeptors ( $\alpha$  2, 6- und  $\alpha$  2, 3-verknüpfte Neuraminsäure) enthalten, werden Schweine als die Mischbatterien für ein weites Spektrum unterschiedlicher Influenza A Viren betrachtet und damit als die potenzielle Quelle neuer human- und vogelgängiger neuer Reassortanten. Deshalb spielt die Kontrolle der Schweineinflenzaviren im Hinblick auf die Tiergesundheit und ebenso auf das öffentliche Gesundheitswesen eine wichtige Rolle. Um einen Beitrag zu liefern, der beiden Gesichtspunkten Rechnung trägt, wurden unter Verwendung eines porcinen Modellsystems zwei Studien zur Kontrolle von Schweineinflenzaviren unternommen – *in vitro* und *in vivo*. Das *in vitro*-Experiment betreffend wurde untersucht, ob das Probiotikum *Enterococcus faecium* NCIMB10415 Potenzial zur Vermehrungshemmung von zwei porcinen Influenza virus Stämmen (H1N1 and H3N2) besitzt. Als Wirtssysteme wurden eine aus Lungenmakrophagen abgeleitete, kontinuierliche porcine Makrophagenzelllinie (3D4/21) sowie eine kontinuierliche Epithelzelllinie, MDBK-Zellen, verwendet. Die Zellkulturen wurden für bis zu 90 Minuten vor, während oder nach der Infektion mit SIV einer nicht-toxischen Konzentration von  $1 \times 10^6$  CFU/ml *E. faecium* im Wachstumsmedium ausgesetzt. Nach einer weiteren Inkubation der Zellen in Wachstumsmedium ohne Probiotikum wurden nach 48 (3d421/) bzw. 96 Stunden (MDBK-Zellen) die Viabilität der Zellen wie auch die bis dahin erfolgte Virusvermehrung bestimmt. Die erhaltenen Ergebnisse offenbarten eine annähernd normale Viabilität der mit *E. faecium* behandelten SIV-infizierten Zellen und damit einen wirksamen Schutz der Zellen vor der SIV-Infektion. Die Virustitrationen ergaben, dass die Virusvermehrung in den mit *E. faecium* behandelten Zellen um bis zu vier Log-Stufen reduziert war. Eine 60-minütige Behandlung mit *E. faecium* führte sowohl in 3D4- als auch in

den MDBK-Zellen zur Stimulation der NO-Sekretion, was mit Literaturdaten in Einklang steht. Zudem verursachte *E. faecium* insbesondere in 3d4-Zellen eine Veränderung der zellulären Expression ausgewählter Mediatoren der zellulären Abwehr. Während die Expression von TNF- $\alpha$ , TLR-3 und IL-6 in SIV-infizierten und Probiotika-behandelten Zellen reduziert waren, konnte für IL-10 eine gesteigerte Expression beobachtet werden. Die Ergebnisse von Adsorptionsexperimente zeigten, dass *E. faecium* in der Lage ist, SIV zu adsorbieren. Die erzielten *in vitro* Ergebnisse zeigen, dass die untersuchten probiotischen Mikroorganismen Influenzaviren auf zellulärer Ebene über mindestens zwei Mechanismen hemmen können, die direkte physikalische Interaktion (Adsorption) und mittels Stärkung der natürlichen Immunität. Mit Hilfe der *in vivo*-Studien sollte geprüft werden, ob das Probiotikum *Enterococcus faecium* NCIMB 10415 sowie Zinkoxid (ZnO) als Futterzusätze geeignet sind, die SIV-Impfung von Ferkeln und damit die Infektion der Tiere mit SIV positiv zu beeinflussen. Hierzu erhielten 72 Absatzferkel Futter, dem entweder *E. faecium* oder eine hohe Dosis ZnO (2,500 ppm) beigemischt war, oder das als Kontrolle kein weiteres ZnO enthielt (natürlicher Zinkgehalt 50-80 ppm). Die Hälfte der Tiere in jeder der Fütterungsgruppen wurde zweifach mit einem inaktivierten, trivalenten SIV-Impfstoff intramuskulär geimpft (VAC). Anschließend wurden alle Tiere intranasal mit H3N2 SIV infiziert. Vor der SIV-Infektion wurde in den mit *E. faecium*-Zusatz gefütterten Tieren eine gesteigerte wöchentliche Gewichtszunahme beobachtet. Nach der SIV-Infektion nahm das Gewicht in den mit der hohen Zink-Dosis und *E. faecium* supplementierten Ferkeln zu während die Tiere, die das Kontrollfutter erhielten, an Gewicht verloren. Mit Hilfe der ELISA-Technik wurden in der *E. faecium*+VAC-Gruppe signifikant erhöhte Konzentrationen von H3N2-spezifischen Antikörpern zwei Tage vor sowie am Tag der Challenge-Infektion und 4 und 6 Tage nach der Infektion nachgewiesen. Erhöhte Hämagglutinations-Inhibitionstiter (HI) wurden ebenfalls in the Zn+VAC sowie der *E. faecium*+VAC Gruppe an den Tagen 0, 1 and 4 nach der Infektion bestimmt. Allerdings waren zwischen den Fütterungsgruppen keine signifikanten Unterschiede in der Virusproduktion nachzuweisen. Verglichen mit der Kontrollgruppe fanden sich in der hoch dosierten Zn- sowie der *E. faecium*-Fütterungsgruppe zu verschiedenen Zeiten nach der Infektion bei der durchflußzytometrischen Analyse des Blutes signifikant erhöhte Mengen an CD4+CD8+ und CD8+ (zytotoxische T Lymphozyten, CTL). Zusammen genommen legen die erzielten

## Zusammenfassung

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Ergebnisse den Schluss nahe, dass die Zufütterung hoher Konzentrationen von Zinkoxid und besonders die Supplementierung mit *E. faecium* durch Steigerung der humoralen Abwehr die Überwindung der SIV-Infektion geimpfter Tiere positiv beeinflussen kann. Allerdings wurden keine Hinweise auf eine fütterungsbedingte Hemmung der Virusausscheidung und der Pathologie der Lunge verzeichnet.



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## PUBLICATIONS

### A. From the present work

1. **Zhenya Wang**, Weidong Chai, Michael Burwinkel, Sven Twardziok, Paul Wrede, Christiane Palissa, Bettina Esch, Michael F. G. Schmidt. (2013) Inhibitory influence of *Enterococcus faecium* on the propagation of swine influenza A virus *in vitro*. *PLoS One* 8(1): e53043
2. Weidong Chai, Michael Burwinkel, **Zhenya Wang**, Christiane Palissa, Bettina Esch, Sven Twardziok, Paul Wrede, Michael F. G. Schmidt. (2012) Antiviral effects of a probiotic *Enterococcus faecium* strain against transmissible gastroenteritis coronavirus. *Arch Virol.* 158(4): 799-807
3. **Zhenya Wang**, Michael Burwinkel, Weidong Chai, Elke Lange, Ulrike Blohm, Angele Breithaupt, Sven Twardziok, Pawel Janczyk, Robert Pieper, Klaus Osterrieder. Influence of dietary *Enterococcus faecium* and Zinc on Swine Influenza Virus infection in pigs. (Accepted).
4. Weidong Chai, Michael Burwinkel, **Zhenya Wang**, Pawel Janczyk, Sven Twardziok, Ulrike Blohm, Nikolaus Osterrieder. Effect of dietary zinc oxide on porcine reproductive and respiratory syndrome virus (PPRSV) infection in pigs. (Submitted).
5. Weidong Chai, Silke S. Zakrzewski, Dorothee Günzel, Robert Pieper, **Zhenya Wang**, Sven Twardziok, Pawel Janczyk, Nikolaus Osterrieder, Michael Burwinkel. High-dose dietary zinc oxide mitigates infection with transmissible gastroenteritis virus in piglets. (Accepted).

### B. From previous work

1. Yan-Bin Wang\*, **Zhen-Ya Wang\***, Hong-Ying Chen, Bao-An Cui, Ya-Bin Wang, Hong-Ying Zhang, Rui Wang. (2009) Secretory expression of porcine interferon- $\gamma$  in baculovirus using HBM signal peptide and its inhibition activity on the replication of porcine reproductive and respiratory syndrome virus. *Veterinary Immunology and Immunopathology* 132: 314–317 (\*Joint first author)

2. Hong-Ying Chen, Li Zhao, Zhan-Yong Wei, Bao-An Cui, **Zhen-Ya Wang**, Xin-Sheng LI, Ping-An Xia, Jin-Peng Liu. (2010) Enhancement of the immunogenicity of an infectious laryngotracheitis virus DNA vaccine by a bicistronic plasmid encoding glycoprotein B and interleukin-18. *Antiviral Res.* 87(2): 235-41
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5. **Zhenya Wang**, Yanbin Wang, Hongying Chen, Panfeng Shao, Xiaodong Ning, Na Pan, Lijing Han, Baoan Cui. (2011) Expression of porcine interleukin-18 in baculovirus/insect cells. *Chinese Journal of Biotechnology.* 27(1): 118-23
6. **Zhenya Wang**, Rui Wang, Yanbin Wang, Hongying Chen, Zhanyong Wei, Panfeng Shao, Houwei Li, Baoan Cui. (2009) A RT-PCR detection of chicken infectious bronchitis. *Chinese Husbandry and Veterinary.* 36: 134-135

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## Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe.

Ich versichere dass ich ausschließlich die angegebenen Quellen und Hifen in Auspruch genommen habe.

Berlin, 19.12.2013

Zhenya Wang