Unmasking the microbicidal and immunostimulatory properties of warthog fecal microbiota *in vitro* and *in vivo*

Jinya Zhang

PhD Thesis

Bellaterra (Barcelona), 2020

Desenmascarando las propiedades microbicidas e inmunoestimuladoras de la microbiota fecal del facóquero *in vitro* e *in vivo*

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Doctoral thesis presented by **Jinya Zhang** to obtain the Doctoral degree under the programme of Animal Medicine and Health at Faculty of Veterinary Medicine from Universitat Autònoma de Barcelona, under the supervision of **Fernando Rodríguez**, **Florencia Correa-Fiz** and **Jorge Martínez.**

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Tesis doctoral presentada por **Jinya Zhang** para acceder al grado de Doctora en el marco del programa de Doctorado en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, bajo la dirección de **Fernando Rodríguez**, **Florencia Correa-Fiz** y la tutoría de **Jorge Martínez.**

Bellaterra, 2020

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Certify:

That the research studies done in the doctoral tesis work "***Unmasking the microbicidal and immunostimulatory properties of warthog fecal microbiota in vitro and in vivo***", presented by **Jinya Zhang** to obtain the Doctoral Degree in Medicina i Sanitat Animals was done under supervision, and authorize its presentation to be evaluated by the corresponding commission.

We signed this certificate for the records in Bellaterra (Barcelona) 9th October, 2020.

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Certifican:

Que los trabajos de investigación desarrollados en la memoria de tesis doctoral "***Unmasking the microbicidal and immunostimulatory properties of warthog fecal microbiota in vitro and in vivo***", presentados por la licenciada **Jinya Zhang** para la obtención del Grado de Doctor en Medicina i Sanitat Animals se ha realizado bajo la dirección y tutoría, y autorizan su presentación a fin de ser evaluada por la comisión correspondiente.

Y porque así conste y tenga los efectos que correspondan, firman el presente certificado.

Bellaterra (Barcelona), 9 de Oct de 2020
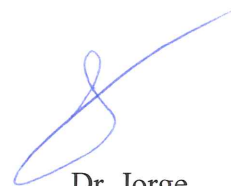
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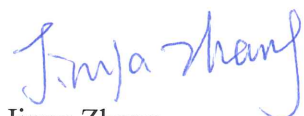
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Doctoranda

May the world peace.

May the human salud.

May the animals health.

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LIST OF ABBREVIATIONS

ADWG	Average daily weight gain
AWG	Average weight gain
AMPs	Antimicrobial peptides
AMR	Antimicrobial resistant
ANCOM	Analysis of composition of microbiomes
ARDB	Antibiotic Resistance Genes Database
ASF	African Swine Fever
ASFV	African Swine Fever Virus
BC	Before Christ
Bcl-2	B-cell lymphoma 2
BHI	Brain heart infusion
BLCA	Bayesian Lowest Common Ancestor
BSL-3	Biosafety level 3
CARD	Comprehensive Antibiotic Resistance Database
CDI	*Clostridium difficile* infection
CFU	Colony forming unit
CO2	Carbon dioxide
CRE	A cyclic AMP response element
CREP	cAMP-response element binding
CTL	Cytotoxic T cells
CXCL	C-X-C motif chemokine ligand
DC	Dentric cells
DF	Dietary fibers
DIVA	Differentiating Infected from Vaccinated Animals
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked ImmunoSorbent Assay
ELISpot	Enzyme-linked immune absorbent spot
ERIC-PCR	Enterobacterial repetitive intergenic consensus -PCR
FBS	Fetal bovine serum
FMT	Fecal Microbiota Transplantation
GALT	Gut-associated lymphoid tissue
GALT	Gut-associated lymphoid tissue
GF	Germ-free
GI	Gastrointestinal
HAD	Hemadsorption dose
HAU	Hemagglutination units
HBSS	Hanks' Buffered Salt Solution

HE	Hematoxylin and eosin
HIF1α	Hypoxia-inducible factor 1-alpha
IBD	Inflammatory bowel disease
IECs	Intestinal epithelial cells
IFN	Interferon
IFNI	Interferon I
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgE	Immunoglobulin E
IL	Interleukin
ILFs	Isolated lymphoid follicles
iNKT	Invariant natural killer T
LAB	Lactic acid bacteria
LAV	Live attenuated virus
LP	Lamina propria
LPS	Lipopolysaccharide
MEGA	Molecular Evolutionary Genetics Analysis
MGF	Multigene family
MHC	Major histocompatibility complex
ML	Maximum likelihood
MLNs	Mesenteric lymph nodes
mTOR	Mammal target of Rapamycin
NF-AT	Nuclear factor of activated T cells
NF-κB	Muclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing
NKT	Natural killer T
NLRs	Nucleotide-binding oligomerization domain-like receptors
NOD	Nucleotide-binding oligomerization domain
NR	Non-redundant
OD	Optical density
OIE	World Organization for Animal Health
ORF	Open Read Frame
OTUs	Operational taxonomic units
PAMPs	Pathogen-associated molecular patterns
PATRIC	Pathosystems Resource Integration Center
PBS	Phosphate buffered saline
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PCV-2	Porcine circovirus type 2
Pen/Strep	Penicillin /Streptomycin
PERMANOVA	Permutational analysis of the variance
PP	Peyer's patches
PRRs	Pattern-recognition receptors

PRRSV	Porcine reproductive and respiratory syndrome
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
RCDI	Recurrent CDI
RDP	Ribosomal Database Project
RIG-I	Retinoic acid-inducible gene I
RLRs	Retinoic acid-inducible gene I (RIG-I)-like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Rectal temperature
SCFAs	Short-chain fatty acids
sIgA	Secretory IgA
SPF	Specific pathogen free
TGF	Transforming growth factor
Th1	T helper 1 cell
Th17	T helper 17 cell
TLRs	Toll-like receptors
TNF	Tumor necrosis-factor
Treg	Regulatory T cell
tRNA	Transfer RNA
UC	Ulcerative colitis
VACV	Vaccinia virus
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
YCFAwo	Yeast extract-Casein hydrolysate-fatty acids without short fatty acids

Abstract

African swine fever virus (ASFV) is the number one threat for the pig industry. Until today, there is no commercial vaccine or treatment available, thus complicating the control and eradication of African swine fever (ASF). ASFV can infect domestic pigs and Eurasian wild boars (both being *Sus Scrofa*), resulting in different clinical disease courses, varying from acute ASF with up to 100% mortality rate to chronic infection. Conversely, ASFV can infect African wild pigs, including warthog (*Phacochoreus africanus*), bushpig (*Potamochoerus larvatus*) and giant forest hog (*Hylochoerus meinertzhageni*), without causing apparent disease.

The work here presented is based on two observations preliminary obtained in our laboratory. On one hand, we could demonstrate that specific-pathogen-free (SPF) pigs infected with attenuated ASFV strains, developed acute ASF dying in a matter of two weeks, while conventional domestic pigs inoculated with same ASFV strains perfectly overcame the infection, despite sharing identical genetic background than the SPF pigs. These results definitively demonstrated that, together with genetic differences, environmental factors could also play a role in ASF susceptibility. On the other hand, fecal microbiota comparisons performed in our laboratory between two swine species (pigs and warthogs), grown in diverse environmental conditions, confirmed that microbiota composition also vary depending on genetic and environmental factors.

With this data at hand and taking into account that gut microbiota is one of the key players driving body homeostasis equilibrium, immune system maturation and pathogen resistance, the **main objective** of this thesis was to investigate the potential role that warthog fecal microbiota might play in ASF resistance.

To achieve this general goal, in the present study we proposed four specific objectives: 1) To establish a fecal microbiota transplantation (FMT) model in domestic pigs using microbiota from domestic pigs or warthogs. 2) To use this animal model to compare the ASF susceptibility after experimental challenge with virulent or attenuated ASFV strains. 3) To isolate individual bacteria from the fecal microbiota for further characterization of their *in vitro* microbicidal or immunostimulatory capabilities, and

4) to inoculate *in vivo* domestic pigs with selected components of the *in vitro* characterized microbiota, aiming to mimic the effects observed after FMT.

The main findings obtained during this thesis can be summarized as follows: (1) Transplantation of fecal microbiota from warthog is not harmful to domestic weaned piglets. (2) FMT from warthog modifies the microbiota composition of domestic weaned piglets. (3) FMT from warthog improves the mucosal immunity of transplanted domestic pig, with higher levels of total secretory IgA in sera. (4) FMT from warthog to domestic pigs confers partial protection against intramuscular infection with E75CV1, an attenuated strain of ASFV. Thus, pigs transplanted with fecal microbiota from warthog, showed a very significant reduction of virus in serum, nasal shedding and clinical signs, while FMT from pigs to pigs did not. No effect was observed against intramuscular challenge with E75, a highly virulent strain of ASFV. (5) The isolation of individual bacteria from warthog feces allowed the characterization of individual microbiota components *in vitro*. Therefore, some bacteria showed beneficial properties on pig ileum and colon organoids growth, others showed microbicidal properties against different pig pathogenic bacteria, including: *Clostridium perfringens* (type B)*, Salmonella enterica, Salmonella enterica* serovar *Typhimurium* monophasic variant *Escherichia coli* K88, *Streptococcus suis* (virulent and apathogenic strains). We also found several bacteria able to stimulate *in vitro* the secretion by gut associated lymphoid tissues (GALTs) of a key cytokine involved in ASFV protection, IFNγ. (6) The intragastric inoculation of 15 bacterial strains selected according to their *in vitro* properties, improved the mucosal immunity in the recipient animals denoted by the increase in total IgA production in sera and the ASFV-specific IgA found in both serum and nasal swabs upon E75CV1 challenge.

The results obtained during the present doctoral thesis will open new avenues for the future fighting not only against ASFV, but also against other pathogens. The unmasking of the biological role of warthog fecal microbiota and warthog-fecal-originated bacteria might be of high benefit for the future.

Resumen

El Virus de la Peste Porcina Africana (VPPA) es, a día de hoy, la amenaza número uno para la industria porcina. La ausencia de vacunas o tratamientos eficaces frente al virus complica el control y erradicación de la enfermedad que este virus provoca: la Peste Porcina Africana (PPA). El VPPA infecta eficazmente tanto a cerdos domésticos (*Sus scrofa domesticus),* como a jabalíes euroasiáticos (*Sus Scrofa)*, siendo ambos igualmente susceptibles a la enfermedad. Dependiendo entre otros factores, de la cepa de VPPA circulante, la PPA cursa con distintos cuadros clínicos, que varían desde la PPA aguda o hiperaguda, con tasas de mortalidad de hasta el 100%, hasta la infección crónica. A pesar de que el VPPA es capaz de infectar también a los cerdos salvajes africanos, incluyendo al facóquero (*Phacochoreus africanus*), al potamoquero (*Potamochoerus larvatus*) y al hiloquero o cerdo gigante del bosque (*Hylochoerus meinertzhageni*), el virus no provoca signos clínicos aparentes en estos huéspedes convirtiéndose de hecho en reservorios naturales del VPPA, capaces de excretar virus durante periodos de tiempo muy prolongados.

La Tesis aquí realizada se ha sustentado en dos observaciones realizadas con anterioridad en nuestro laboratorio. Por un lado, pudimos demostrar que la infección de cerdos libres de patógenos específicos (SPF del inglés), con una cepa atenuada de VPPA, provocaba un cuadro agudo de PPA incluyendo la muerte de los animales en menos de dos semanas, mientras que esa misma infección resultaba prácticamente inocua para los cerdos domésticos crecidos en granjas convencionales. El hecho de que dos poblaciones de una misma especie (genéticamente idénticas) tuviera una susceptibilidad tan distinta al VPPA demostraba que la resistencia a la PPA podría venir marcada tanto por factores genéticos, como ambientales. Por otro lado, estudios comparativos centrados en dos especies de cerdos con muy distinta susceptibilidad a la VPPA: *Sus scrofa* y *Phacochoreus africanus,* permitieron demostrar que la composición de su microbiota fecal, venía marcada igualmente tanto por factores genéticos como ambientales.

Ambos resultados, junto con el conocimiento fehaciente que existe sobre el papel clave de la microbiota fecal en el mantenimiento del equilibrio homeostático del organismo, en la maduración del sistema inmunológico y en la resistencia a los

patógenos, nos condujo a plantear como **objetivo general** de esta tesis, investigar el papel potencial que podría desempeñar la microbiota fecal de facóquero en su resistencia a la PPA.

Para lograr este objetivo general, en el presente estudio nos propusimos cuatro objetivos específicos: 1) Establecer un modelo de trasplante de microbiota fecal (TMF) de facóqueros en cerdos domésticos, trasplantando en paralelo heces de cerdos domésticos en cerdos domésticos como control del ensayo; 2) utilizar este modelo animal para comparar su susceptibilidad a la PPA, tras la infección experimental con cepas virulentas o atenuadas del VPPA; 3) aislar bacterias individuales de la microbiota fecal del facóquero para caracterizar en lo posible, tanto su capacidad inmunoestimulatoria como microbicida *in vitro*, y 4) inocular cerdos domésticos *in vivo* con componentes seleccionados de la microbiota caracterizada *in vitro*, con el objetivo de mimetizar los efectos observados después de TMF.

Los principales hallazgos obtenidos durante esta tesis se resumen a continuación: (1) El TMF del facóquero no es perjudicial para los lechones domésticos recién destetados. (2) El TMF de facóquero modifica la composición de la microbiota de los lechones domésticos recién destetados. (3) El TMF de facóqueros mejora la inmunidad de mucosas del cerdo doméstico trasplantado, como demuestra la presencia de concentraciones más elevadas de inmunoglobulina A secretora (IgA) total en suero que en los cerdos control. (4) El TMF de facóqueros a cerdos domésticos, confiere protección parcial contra la infección intramuscular con E75CV1, una cepa atenuada del VPPA. Así, los cerdos trasplantados con microbiota fecal de facóquero, mostraron una reducción muy significativa del virus en suero, excreción nasal y signos clínicos, en comparación con el grupo control. Sin embargo, el TMF de facóquero no resultó eficiente frente la infección intramuscular con E75, una cepa altamente virulenta del VPPA. (5) El aislamiento de bacterias individuales a partir de heces de facóquero permitió la caracterización de componentes individuales de su microbiota *in vitro*. De modo resumido, se pudieron caracterizar bacterias de facóquero con propiedades beneficiosas sobre el crecimiento *in vitro* de organoides del íleon y del colon del cerdo. Del mismo modo, se pudieron caracterizar un grupo muy amplio de bacterias con propiedades microbicidas, capaces de inhibir el crecimiento *in vitro* de diferentes bacterias patógenas del cerdo, entre ellas: *Clostridium perfringens* (tipo B), *Salmonella*

enterica, *Salmonella enterica* serovar *Typhimurium* variante monofásica, *Escherichia coli* K88 y *Streptococcus suis* (cepas virulentas y apatogénicas),. Finalmente, se pudo caracterizar una aislado bacteriano de facóquero capaz de estimular la secreción *in vitro* de IFNγ por parte de células del tejido linfoides asociados al intestino (GALT, del inglés), siendo ésta una citoquina clave implicada en la protección del VPPA. (6) La inoculación intragástrica de 15 cepas bacterianas seleccionadas de acuerdo con sus propiedades *in vitro*, mejoró la inmunidad de mucosas en los animales receptores indicada por un aumento en la producción total de IgA en el suero y tras la infección con E75CV1, cepa atenuada del VPPA, de la IgA específica frente al VPPA, tanto en suero como en hisopo nasal.

Los resultados obtenidos durante la presente tesis doctoral han abierto una nueva vía de investigación en la búsqueda de estrategias que permitan luchar no sólo contra el VPPA, sino también contra otros patógenos del porcino. El desenmascaramiento del papel biológico de la microbiota fecal de los facóqueros y de las bacterias existentes en sus heces podría suponer de gran beneficio para el futuro.

PART I

General introduction, hypothesis and objectives

Chapter 1

General introduction

1.1 African Swine Fever

African Swine Fever (ASF) is a devastating disease of domestic pigs (*Sus scrofa domesticus*) and wild boars (*Sus scrofa scrofa*), caused by African swine fever virus (ASFV). ASF is a notifiable disease for the World Organization for Animal health (Arias *et al.*, 2017) and today it is considered the most serious constraint for pig production. ASF control is especially difficult, overall due to the lack of a safe and efficient vaccine available. The reasons explaining the lack of a vaccine available are multiple. In one hand, ASFV is one of the most complex viruses described in nature, and depending on the geographic scenario, ASFV can circulate in several hosts, including wildlife, complicating its control. In the other hand, the nature of ASFV requires working under high biosecurity confinement, therefore reducing the number of research groups working in this problem.

1.1.1 ASFV molecular and structural studies

ASFV is a member of the *Asfivirus* genus (Dixon *et al.*, 1995) from *Asfarviridae* family, and the only known linear double-strained DNA arbovirus. ASFV is a nucleocytoplasmic large DNA enveloped virus of approximately 260 to 300 nm in diameter (Wang *et al.*, 2019) with a genome size of 170-193 kbp (Dixon *et al.*, 2013) that can encode more than 150 different genes (Malmquist *et al.*, 1960). The ASFV particle poses a very complicated multilayered structure and an overall icosahedral morphology (Andrés *et al.*, 1997; Breese *et al.*, 1966; Carrascosa *et al.*, 1984). Intracellular ASFV has a dense genome-containing nucleoid (core, the first layer), which is surrounded by a thick protein layer (Andrés *et al.*, 2002), the core shell (second layer) . The core shell is wrapped by an inner capsid, an icosahedral protein capsid (the third layer), an inner membrane (the fourth layer), and an outer capsid (the fifth layer) (Andrés *et al.*, 2020). And finally, ASFV gains an outer envelope (the sixth layer) as it buds through the plasma membrane (Breese *et al.*, 1966). Sixty-eight structural proteins have been identified so far, in purified extracellular virus (Figure 1) (Alejo *et al.*, 2018).

Two polyprotein precursors pp220 and pp62, are encoded by two open read frames (ORFs) *CP2475* and *CP530R* genes, respectively. Both polyproteins are expressed late in infection and are post-translationally processed by the viral cysteine proteinase pS273R (Andrés *et al.*, 2001). Protein pp220 is cleaved to yield the mature

virion proteins p150, p37, p14, and p34. Protein pp62 can be proteolytically cleaved into the mature virion proteins p35 and p15 (Andrés *et al.*, 2002). The inner capsid is composed of mature proteins derived from pp220 and pp62, which are similar to the core shell (Andrés *et al.*, 2020). The proteins p54 and p30 are important antigenic structural proteins involved in viral entry, which are encoded by *E183L* and *CP204L* genes, respectively. The p72 protein, encoded by the gene *B646L* (VP72) is the major structural protein of ASFV, and it is the crucial antigenic protein. This protein, p72, with its highly antigenic and immunogenic character, serves as the major component of viral icosahedrons. It is also very important in forming the outer capsid in late stage expression of virus infection (Neilan *et al.*, 2004). CD2v protein, also named pEP402R, resembles the T-lymphocyte surface adhesion receptor CD2 (Borca *et al.*, 1994), and is involved in cell-cell adhesion, virulence enhancement and immune response modulation. The p14.5 protein, also called pE120R, is encoded by the *E120R* gene. This protein, p14.5, is synthesized during the late phase of viral infection, and is the necessary protein involved in transferring virions from viral factories to the plasma membrane (Andrés *et al.*, 2001).
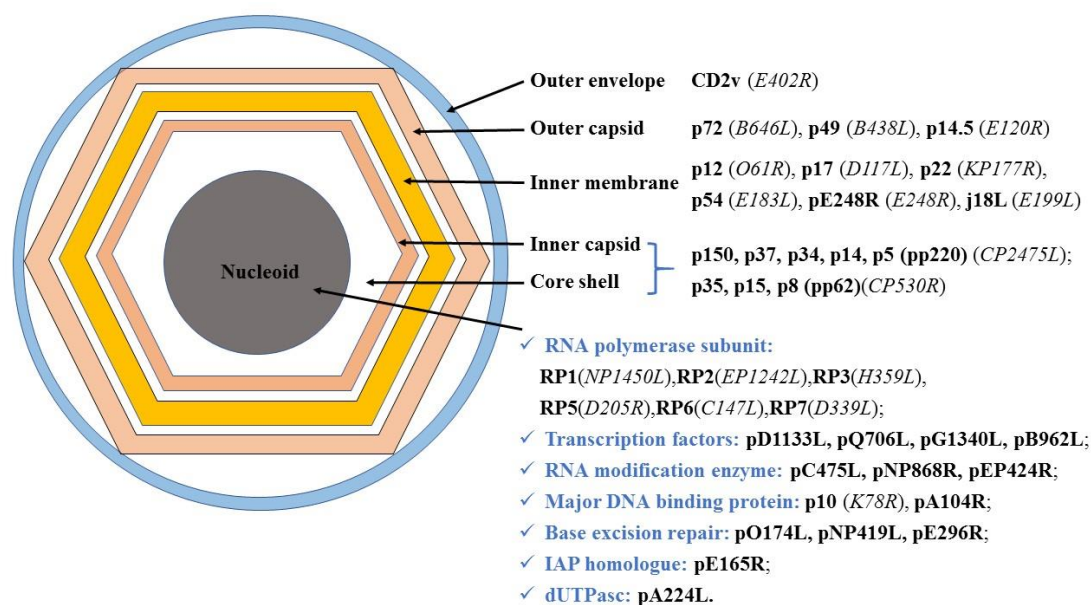
Figure 1. Graphic representation of the structural composition of an ASFV particle. The structural proteins found in each particular virus' layer are indicated. There are six layers: nucleoid, core shell, inner capsid, inner membrane, outer capsid and outer envelope, from the inside to outside. Protein names are in bold, and gene names in italic font. The known functions of the nucleoid proteins are in blue (ASFV structure has been adapted from Andrés *et al.*, 2013).

General introduction

ASFV molecular polymorphism has been identified by partially sequencing *B646L,* which encodes the major capsid protein p72 (Bastos *et al.*, 2003), and there are 24 different genotypes identified until now (Quembo *et al.*, 2018). In addition, *E183L* (Rowlands *et al.*, 2008) and *CP204L* genes (Gallardo *et al.*, 2009) encoding proteins p30 and p54 respectively together with the central variable region (CVR) within the ORF *B602L* (Gallardo *et al.*, 2009), have also been used to differentiate closely-related ASFV isolates more precisely.

The complex structure of the ASFV particle partially explains its ability to survive under a variety of environment conditions and its highly resistance to inactivation in the presence of organic material (Sánchez-Vizcaíno *et al.*, 2012). Infected pigs harbor high amount of virus in the blood, saliva, tears, nasal secretions, urine, feces, and secretions from the genital tract (Beltrán-Alcrudo *et al.*, 2017). An experimental study showed that up to $10^{8.7}$ HAD$_{50}$/ml (50 percent haemabsorbing doses per ml), 10^4 HAD$_{50}$/ml, 10^4 HAD$_{50}$/ml virus could be detected in blood, nasal fluid and rectal fluid, respectively from infectious pigs (Guinat *et al.*, 2014). It could survive for 1000 days in frozen meat (Sánchez-Vizcaíno *et al.*, 2009), in feces for at least 11 days when stored in the dark (Montgomery, 1921). In feces and urine ASFV remain infectious for 8.48 and 15.33 days on average at 4°C, and 3.71 and 2.88 days at 37°C, respectively (Davies *et al.*, 2017). ASFV is also reported to be able to persist for 1.5 years in blood, 15 weeks in putrefied blood, or approximately 5 months in boned meat at 4°C, and 140 days in salted dried hams. ASFV may persist for several years in frozen carcasses and in pig pens for at least 1 month (Abraham *et al.*, 2000). Unpublished findings from a report in the 1960s indicated that at least small amounts of infectious virus might persist in forest soil for nearly 4 months, in freshwater for up to 7 weeks in summer and approximately 6 months in winter, and on wooden boards or bricks buried in dirt for 2-3 months (Panel *et al.*, 2010a).

Some disinfectants are effective against ASFV showing that the virus could be inactivated at 57ºC for 70 min or 60ºC for 20 min. ASFV could be inactivated in serum-free medium with very low (pH < 3.9) or very high pH (pH >11.5). ASFV is susceptible to ether and chloroform and to many solvents that disrupt lipid bilayers and commercial disinfectants (1% formaldehyde in 6 days, 2 % NaOH in 1 day).

1.1.2 Global Distribution and Epidemiology of ASFV

General introduction

ASF was first recognized as a distinct disease in 1910 in Kenya and first published in 1921 as an acute hemorrhagic fever that caused death of most infected domestic pigs (Montgomery, 1921). From Africa, ASFV (genotype I) was introduced into Portugal in 1957 but was controlled rapidly. In 1960, ASFV was re-introduced to Portugal and spread to Spain, remaining endemic in the Iberian Peninsula for more than 30 years until it was eradicated in the mid-1990s. Between 1960s and 1980s, ASFV occurred sporadically in several European countries, including Andorra (1975), Malta (1978), Italy (1967, 1980), France (1964, 1967, 1974), Belgium (1985), the Netherlands (1986) and Caribbean and South American countries (Cuba, the Dominican Republic, Haiti, and Brazil) (Costard *et al.*, 2009). While all these countries eradicated ASFV, Sardinia (Italy) remained as the only endemic area in Europe (genotype I still circulating today) until 2007. It was in this year when a new ASFV strain (genotype II) was imported from Africa, emerging in Georgia this time, in the Caucasian region. Later, ASFV spread from Caucasus to neighboring countries: Armenia, Azerbaijan and Russian Federation. In 2012, Ukraine reported its first case, Belarus in 2013, and Lithuania reported ASFV for the first time in January 2014 in wild boars. Then, it spread to Poland in 2014, Estonia (2014), Latvia (2014), Lithuania (2014), Moldova (2016), Czech Republic (2017), Romania (2017), Hungary (2018), Bulgaria (2018), Belgium (2018) and Slovakia (2019). Compared to Europe, where the virus has circulated relatively slowly, mostly associated to wild boars (Cwynar *et al.*, 2019; Martínez-Avilés *et al.*, 2020), ASFV in Asia has moved extremely fast being most of the times associated with domestic pigs. Thus, since China reported its first ASFV outbreak in domestic pigs in Shenyang city, Liaoning province in August in 2018, ASFV spread to other countries in Asia: Mongolia, Democratic People's Republic of Korea, Republic of Korea, Philippines, Vietnam, Lao People's Democratic Republic, Cambodia, Myanmar, Indonesia and Timoe-Leste. Recently, Serbia in January 2020, Greece in February 2020, Papua New Guinea in March 2020, India in May 2020 (World Organisation for animal health (OIE), 2020a) and Germany in September 2020 (World Organisation for animal health (OIE), 2020b) (World Organisation for animal health (OIE), 2020a) reported their first ASFV outbreak. In summary, the current distribution of African swine fever (ASF) extends across more than 60 countries (Figure 2) in four continents (Africa, Asia, Ocean island and Europe) (World Organisation for animal health (OIE), 2020a).
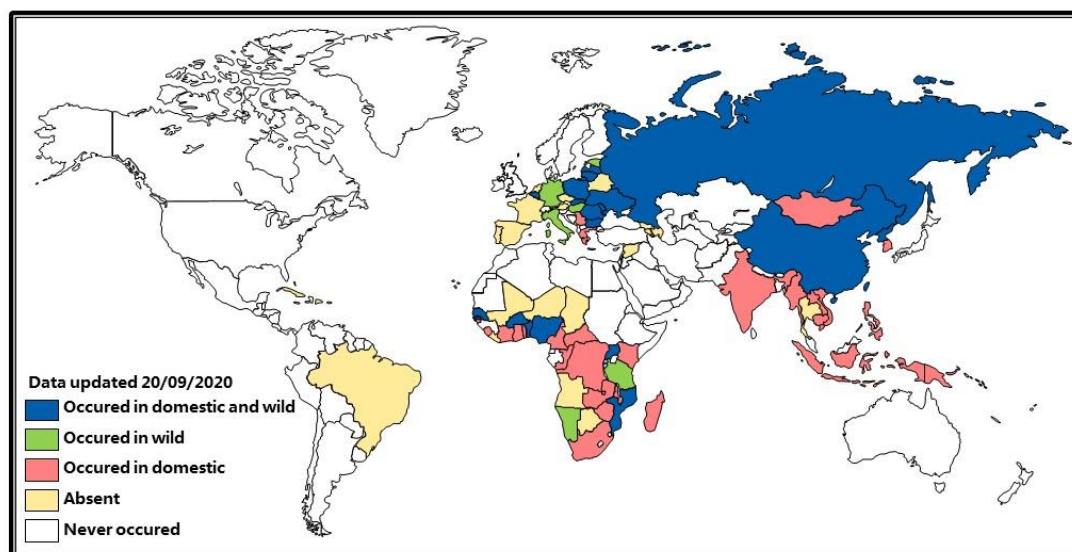
Figure 1. Map of the worldwide distribution of ASF until 20/09/2020. The information of occurrence of ASF in domestic pigs or wild pigs comes from the OIE website: https://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statuslist. ASFV occurs mainly in Africa, Asia, Ocean island and Europe. Countries that never reported ASFV are colored in white. Countries reported ASFV occurrence before, while there is no ASFV nowadays were colored in yellow. When ASFV occurred only in domestic pigs, the infected countries are colored in pink. In green, the countries where ASFV was reported only in wild boars. If ASFV occurred in both domestic pigs and wild boars, they are colored in blue. For Italy, ASFV was endemic only in Sardinia.

1.1.3 Clinical signs and lesions of ASF

Depending on the virulence of the ASFV isolate, the route of infection, the dose of virus and the situation of the host, ASFV can produce different clinical signs and lesions ranges from chronic or subclinical to subacute, acute and peracute, resulting the latter in up to 100% mortality to naïve pigs (Sánchez-Vizcaíno *et al.*, 2015b). Similarly, ASFV isolates are classified as highly virulent, moderately virulent and low virulent (Pan *et al.*, 1984). Peracute and acute forms of the disease are normally induced by highly virulent ASFV strains, characterized by high fever (body temperature 41-42℃), loss of appetite, inactivity, hyperpnoea and cutaneous hyperaemia. Animals suffering the peracute form, usually die suddenly 1-4 days after the onset of clinical signs without evident lesions in any organs.

In the acute form of ASF, which is induced by highly or moderately virulent viral strains, animals display fever (40-42℃) and a tendency to crowd together, loss of appetite, inactivity, apathy and early leucopenia (Pan *et al.*, 1984; Sánchez *et al.*, 2012).

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Severe pulmonary edema (foam) is generally observed around the mouth and nose (Carrasco *et al.*, 1996; Sierra *et al.*, 1990). Affected pigs show erythema and cyanosis of the skin, almost 90-100 % of pigs with these signs will die within 6-9 days for highly virulent strains, or within 11-15 days for moderately virulent virus (Beltrán-Alcrudo *et al.*, 2017). In necropsy, animals present with the characteristic hyperemia, hemorrhages in lymph nodes (mainly gastro-hepatic and renal nodes), petechial hemorrhages in kidneys, mucosa of the urinary bladder, epicardium, endocardium and pleura (Charles, 1988); and also excess of hydropericardium with yellowish fluids in the heart and body cavities (hydrothorax and ascites). Abortion is also observed in pregnant sows at all stages of pregnancy.

Subacute forms of the disease are caused by moderately virulent isolates and may occur in endemic regions. Pigs usually die within 7-20 days, with lethality rate ranging from 30 to 70%. The survivors may recover after one month. Clinical signs are similar (although generally less intense) to those observed in the acute form, except for the more pronounced vascular changes, mainly hemorrhages and edemas. Fluctuating fever, accompanied by depression and loss of appetite, are also common. Walking may appear painful and the joints are often swollen with accumulated fluid and fibrin. Pregnant sows may abort (Arias *et al.*, 1986). Due to concomitant bacterial infections, there may be signs of dyspnea, pneumonia and serous or fibrinous pericarditis (Beltrán-Alcrudo *et al.*, 2017).

Chronic ASF has been associated with infection by moderate-to-low virulence isolates (McVicar, 1984) which were only described in Spain, Portugal and the Dominican Republic when ASF infection in these areas was endemic. This form of the infection is not currently circulating out from Africa (Sánchez-Vizcaíno *et al.*, 2015b), but cannot be discarded in the future if ASFV become endemic for long periods of time.

1.1.4 Prevention and control of ASF

Since there is no vaccine and treatment available for ASFV infection, the prevention strategy to protect the pigs from infection and stop the disease spreading is very important. In 1939 in southern Africa, ASFV was eradicated from Cape Province by a policy of complete slaughter and quarantine (Pini *et al.*, 1975). In Europe, ASFV was successfully eradicated from Europe in the late 20th century through strict animal

movement control and implementation of culling policies, besides improving biosecurity on farms and increased disease awareness of pig farmers.

Delays in recognizing ASF in Georgia resulted in its spreading to neighbor countries, with wild boars playing a key role during transmission. Later on, illegal transportation of infected pigs and contaminated pigs' products resulted in the explosion of ASFV in China and other Asia countries. These failures highlight how important it is to conduct strict prevention and control measures if willing to protect the pig industry. A quick response, and isolation and culling of infected animals are vital for containing outbreaks in ASFV-free regions (Spickler, 2019). In ASF-free areas, strict high biosafety measures should be taken to prevent the introduction of virus: presence of physical barriers, minimal and controlled people traffic and access to the farm, strengthened import controls especially by proper disposal of waste food from aircraft/ships coming from infected countries, etc. Prevention and applicable measures are also based on classical disease control methods, including intensive surveillance and designation of protection and surveillance zones. All successful eradication programs should be approved by all the role players (farmers, veterinarians and policy makers), involving the rapid diagnosis, tracing and stamping out of infected herds, epidemiological investigations and adequate financial support (Bech-Nielsen *et al.*, 1993). Appropriate disinfectants and procedures should be used to achieve complete disinfection of contaminated areas and materials when developing ASF contingency and eradication plans because of great persistence of ASFV (Panel *et al.*, 2010b). Current regulations in the EU allow pig farms to be restocked as soon as 40 days after cleaning and disinfection if an ASF outbreak occurs in the absence of vectors, but the minimum quarantine is 6 years if vectors are thought to be involved in transmission (Spickler, 2019).

1.1.5 Mechanisms involved in immune protection and ASF vaccine development

1.1.5.1 Immunological mechanisms involved in protection against ASFV

The evidence available indicates that immune protection against ASFV involves antibody-mediated and cell-mediated mechanisms (Oura *et al.*, 2005; Takamatsu *et al.*, 2013), adaptive immune response that in turn required the efficient triggering of the

innate immune system (Leitão *et al.*, 2001; Takamatsu *et al.*, 2013). Learning about ASFV has allowed identifying potential weaknesses. Thus, several genes from ASFV contribute to surviving strategies, including the evasion of the host's innate immune defenses, by using different molecular mechanisms (Reis *et al.*, 2017). Blocking the crucial component of the innate response to viral infection (Golding *et al.*, 2016; Zhang *et al.*, 2010), type I IFN (IFNI, IFNα and IFNβ) in ASFV-infected macrophages, is probably one of the smartest strategies used by virulent ASFV strains. Type I-interferon evasion is provoked by the expression of several members from the multigene families (MGF) 360 and 505/530 (Afonso *et al.*, 2004; Burrage *et al.*, 2004; O'Donnell *et al.*, 2015). Deletion of these members from the ASFV genome, yielded highly attenuated ASFV strains with vaccine potential (O'Donnell *et al.*, 2015), confirming IFNI-pathway as an efficient antiviral arm. ASFV can also manipulate the host innate immune system by using other proteins, such as A238L, which could inhibits the activation of TNFα by modulating NF-Kb (nuclear factor kappa-light-chain-enhancer of activated B cells), NF-AT (nuclear factor of activated T cells), and c-Jun *trans* activation through a mechanism that involves cAMP-response element binding (CREB) protein/p300 function (Granja *et al.*, 2006). CD2v, encoded by the *EP402R* gene, is a very interesting virulence factor that is involved in multiple functions during ASFV infection. In one hand, it is responsible for the ASFV particle attachment to the red blood cells (Borca *et al.*, 1994), complicating its recognition by the immune system and facilitating the virus spreading through the body. In the other hand, the carboxy-terminal end of CD2v can block several pathways blocking the recognition of the infected cells for the immune system. As described for the MGFs, deletion of *EP402R* gene from the BA71 virulent strain, lead to highly attenuated virus with very promising cross-protective potential (Borca *et al.*, 1998; Monteagudo *et al.*, 2017; Rowlands *et al.*, 2009), that is currently being transferred to a multinational company for future commercialization (Monteagudo *et al.*, 2017).

ASFV is also capable to manipulate the cell apoptosis in infected cells, always in its own advantage (Banjara *et al.*, 2017; Dixon *et al.*, 2017; Granja *et al.*, 2004; Hurtado *et al.*, 2004; Rodríguez *et al.*, 2004), some ASFV genes are able to inhibit the apoptosis, including *A179*L, a Bcl-2 family member; *A224L*, an inhibitor of apoptosis proteins family member; *EP153R*, a C-type lectin; and *DP71L*; while other genes activate the apoptosis *(E183L/p54)*, both *in vitro* and *in vivo*.

1.1.5.2 ASF vaccine development

Due to the complexity of ASFV itself, limited understanding of ASFV virulence factors and lack of correlates with protection, complicate the development of a successful vaccine. Inactivated virus or recombinant proteins failed in providing solid protection against ASFV experimental challenges so far (Arias *et al.*, 2017; Revilla *et al.*, 2018). DNA and peptide-based vaccines induce specific antiviral immune responses, including virus specific antibodies and/or T-cells, but show only low or partial protection after virulent ASFV challenge, with sometimes inconsistent results (Argilaguet *et al.*, 2011; Lacasta *et al.*, 2014; Sánchez *et al.*, 2019). Conversely, live attenuated virus (LAV) obtained either from nature or by tissue culture adaptation, have proved to stimulate the innate and adaptive immune system and efficiently confer homologous protection from homologous challenge with parental virulent ASFV strains (Chen *et al.*, 2020; Gallardo *et al.*, 2019; García-Belmonte *et al.*, 2019; King *et al.*, 2011; Leitão *et al.*, 2001; Monteagudo *et al.*, 2017; O'Donnell *et al.*, 2017, 2015; Sánchez-Cordón *et al.*, 2017).

The use of LAV vaccines have presented safety concerns derived from their inherent infectious nature, complicating their field implementation (Dixon *et al.*, 2019). Massive vaccination with unsafe LAVs in the seventies, seemed to ocassionally provoke the appearance of ASFV carriers developing chronic lesions. Pigs were exposed to multiple infection and re-infections with heterologous viruses from the in-field experience of Spain and Portugal, that in the long term complicated the eradication of the disease from the Iberian Peninsula (Ribeiro *et al.*, 1963; Sánchez, 1963). Other strategy involved the immunization of pigs with the naturally attenuated ASFV strains OURT88/3 or NH/P68 (Leitão *et al.*, 2001; Sánchez-Cordón *et al.*, 2017). Immunized pigs were protected against challenge with homologous virulent strains, albeit partial cross-protection has been shown against heterologous viruses.

Recombinant technology has allowed improving the generation of LAV by deleting single or multiple genes in the genome. Today, we count on half a dozen prototypes with very promising possibilities to become the first commercial ASF vaccines in the short-medium term. In this regard, it is worthy to mention a vaccine prototype developed in our laboratory, in collaboration with the CBMSO-CSIC, BA71ΔCD2 a recombinant LAV obtained by targeted deletion of the gene encoding

CD2v (the hemagglutinin protein). Pigs vaccinated with BA71ΔCD2 are protected not only against the homologous virus (BA71), but also (unique for this prototype) against heterologous ASFV strains from the same and different genotype, including the currently circulating Georgia 2007/1 ASFV strain (Monteagudo *et al.*, 2017). This characteristic, together with the advantageous ability of BA71ΔCD2 to grow in the stablished cell line (Monteagudo *et al.*, 2017) (ASFV only grows in primary macrophages) and its ability to induce differential immune responses that allow Differentiating Infected from Vaccinated Animals (DIVA), has facilitated its transfer to the private sector to initiate the registration process. The arbitration of national and international regulatory agencies will ensure that any vaccine to be commercialized will accomplish the efficacy and safety requisites required for field implementation.

1.1.6 ASFV-host interactions and transmission cycles

ASFV transmission among hosts can be classified as different cycles, depended on the hosts involved (Figure 3).

1.1.6.1 Boar-habitat cycle

Conversely to that described for African wild pigs, domestic pigs and wild boars (both *Sus scrofa*) are equally susceptible to ASFV. In Europe, ASFV was detected in domestic pigs of all production sectors, and also in wild boars in most of the affected countries. In South Korea, ASFV was reported in a dead wild boar in October, 2019 (Kim *et al.*, 2020). Wild boars are identified as one of the main factors in introduction and subsequent local spreading and promoting the continued spread of ASFV, overall in Europe (Iglesias *et al.*, 2018; Pejsak *et al.*, 2014; Ståhl *et al.*, 2019). When wild boar is infected with ASFV, this virus establishes self-sustaining cycles within the wild boar population (Pikalo *et al.*, 2019). Furthermore, wild boars have a very high density in central Europe, and its movement could not be affected by the application of strict sanitary and biosecurity measures. Thus, the presence of infected wild boars surrounding the farms, exponentially increases the transmission risk from the wild to domestic pigs (Claire Guinat *et al.*, 2016; Woźniakowski *et al.*, 2016).
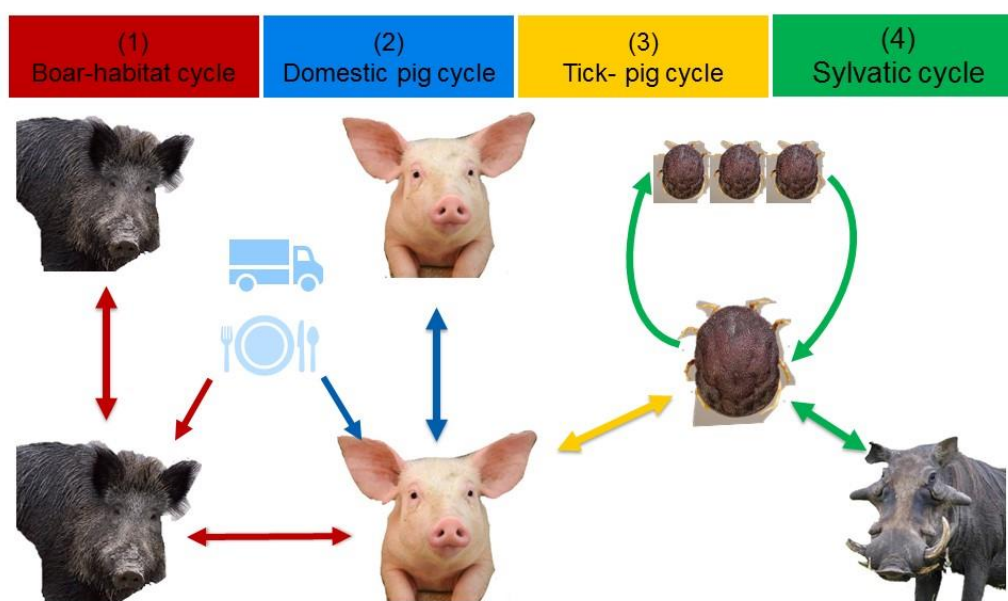
Figure 3. The epidemiologic cycle of African swine fever and the main transmission agents. (1) The Boar–habitat cycle: wild boar (*Sus scrofa*), pig-, and wild boar–derived products and carcasses, and the habitat. (2) The domestic pig cycle: domestic pigs and pig-derived products. (3) The tick–pig cycle: soft ticks (*Ornithodoros spp*) and domestic pigs (*Sus scrofa domesticus*). (4) Sylvatic cycle: the common warthog (*Phacochoerus africanus*) and soft ticks. Figure adapted from Chenais *et al.*, 2018.)

1.1.6.2 Domestic pig cycle

Once the domestic pigs are infected with ASFV, the virus can circulate in the domestic pigs at local, regional and international level (Costard *et al.*, 2013b). Direct contact with infected pigs, infected pig products and/or fomites (people and vehicles) result in virus transmission (Chenais *et al.*, 2019; C. Guinat *et al.*, 2016; Montgomery, 1921; Rowlands *et al.*, 2008). Direct contact with blood from infected animals is the most efficient way of virus transmission, without the presence of the tick vector (Chenais *et al.*, 2019). Movement of pigs and lack of biosecurity practices highly contribute to the local spread of ASF in endemic areas.

1.1.6.3 Tick-domestic pig cycle

The presence of the soft tick *Ornithodoros erraticus* in the Iberian Peninsula, played an important role in maintaining and spreading ASFV in domestic pigs in 1970s (Boinas *et al.*, 2011; Oleaga-Pérez *et al.*, 1990; Pérez-Sánchez *et al.*, 1994; Wilkinson *et al.*, 1988). There is no proof that *O. erraticus* plays an important role in the endemic

situation in Sardinia (Sánchez-Vizcaíno *et al.*, 2015a). Interestingly, *O. erraticus* are unlikely to be capable vectors of ASFV strains currently circulating in Eurasia (de Oliveira *et al.*, 2019). Five species of ticks under the genus *Ornithodoros* are described in China till now (Sun *et al.*, 2019; Zhao, 2018), which are *O. tartakovskyi* (Xinjiang), *O. papillipes* (Xinjiang, Qinghai, Shanxi, Shaanxi), *O. lahorensis* (Gansu and Xinjiang), *O. capensis* (Taiwan) and *O. huajianensis* (Gansu). Studies showed that *O. huajianensis* is close to *O. moubata* in the phylogenetic tree based on 16s rRNA (Sun *et al.*, 2019). The role of *Ornithodoros* ticks in ASFV transmission during the current global outbreaks, with the exception of Africa, has been neglected mainly due to their absence in most affected areas and/or for the lack of enough scientific evidences. Thus, Arthropods and other non-susceptible animals have been suggested as potential mechanical transporters for ASFV, although probably playing a minor role in ASFV transmission.

1.1.6.4 Sylvatic cycle

ASFV circulates in a natural sylvatic cycle in South and East Africa, which includes the *Ornithodoros moubata* (*O. moubata*), common warthogs (*Phacochoerus africanus)* and bushpigs (*Potamochoerus larvatus*) (Costard *et al.*, 2013a; Sánchez-Vizcaíno *et al.*, 2012), however, the role of the bush pig in the sylvatic cycle remains unclear. ASF transmission occurs repeatedly in warthog burrows, between infected soft ticks *O. moubata* and neonatal warthogs that develop high levels of viremia and very mild or subclinical disease, while sufficient to infect naive ticks that feed on them (Charles, 1988; Thomson *et al.*, 1980). Older warthogs, although persistently infected, have low viremia and are generally asymptomatic (Thomson, 1985). There is no evidence of horizontal or vertical transmission in the warthog or of direct transmission from warthogs and domestic pigs, maintenance of the virus within warthog populations and infection of domestic pig in these area are dependent on the soft tick *O. moubata* which inhabits warthog burrows (Pini *et al.*, 1975; Plowright, 1981; Plowright *et al.*, 1969). Domestic pig in Africa, can be alternatively exposed to infected warthogs carcasses (Wilkinson, 1989), albeit this route of infection has not been experimentally confirmed (Penrith *et al.*, 2004). Oura and Anderson proved in 1998 that bushpigs could transmit ASFV to feeding ticks and to in-contact pigs under experimentally conditions (Anderson *et al.*, 1998; Oura *et al.*, 1998). Bushpigs are unlikely to play a significant role in the maintenance and transmission of ASFV in Madagascar (Ravaomanana *et al.*,

2011). Indirect interactions between domestic pigs and wild pigs (common warthog and bushpig) are frequent, particularly during the dry season at water sources, and this may pose an opportunity for ASFV transmission in Uganda (Kukielka *et al.*, 2016). However, very little information is available about the role of bushpigs as reservoir hosts in Africa and Madagascar because of limited studies.

1.1.7 ASFV-susceptibility: genetics and environment

One of the main questions still unanswered is why pigs infected with ASFV develop different disease outcomes. Thus, African wild pigs are resistant to ASF while domestic pigs and wild boars, both *Sus scrofa*, are equally susceptible to ASF (Simões *et al.*, 2019). Bushpigs and warthogs are clustered together and distant from Europe wild boars and commercial breeds from population structure. Local breeds from Africa testing ASFV negative had significantly (P = <0.0001) higher local African ancestry, (54 % and above) compared to the ones testing positive (Mujibi *et al.*, 2018), leading to the conclusion that genetic factors might be the key to explain the differential susceptibility between African and Eurasian pigs (Mujibi *et al.*, 2018). Together with genetics, environmental factors might also contribute to ASF susceptibility. Thus, certain authors claim that indigenous domestic pigs grown in semi-liberty conditions in certain regions of Africa are more resistant to ASF than their conventional partners (Thomas *et al.*, 2016), albeit these studies were not conclusive. Additional data obtained in our laboratory, demonstrated that specific-pathogen-free (SPF) pigs were extremely susceptible to attenuated ASFV strains than genetically identical pigs grown in conventional conditions (Lacasta *et al.*, 2014), definitively confirming that environmental condition do influence ASF susceptibility. Today, we do know that the environment affects multiple physiological parameters, being one the most important ones the gut microbiota, that in turns modify intestinal homeostasis, immune system. Coinciding with this evidence, the gut microbiota of warthogs (ASF resistant), indigenous pigs, conventional and SPF-pigs do share a microbiota core but also can differentiate between species-specific cores and environmental cores (Correa-Fiz *et al.*, 2019).

1.2. Gut microbiota plays multiple beneficial roles in intestinal homeostasis

Gut microbiota plays important roles in maintaining intestinal homeostasis, promoting immune system maturation, regulating innate and adaptive immune responses to commensal flora, pathogenic bacteria and also viruses.

1.2.1 Gut barrier

The intestinal tract has the largest barrier tissue in the human body with a surface area of about 300 m^2 in adults (Lazar *et al.*, 2018). In pigs, the length of small intestine is half, though diameter is comparable in the two species (Hatton *et al.*, 2015). Gut barrier is composed by gut microbiota, the mucosal epithelium, the immune cells harboring in the submucosa and the bidirectional interactions between all these layers (Figure 4). Delicate and complex interactions among these layers determine mucosal homeostasis. Three major lines of defense provide complete protective function of gut barrier, which integrates:

(a) Biological barrier represented by gut microbiota. Normal intestinal flora forms a microbial buffer which limits the access by those that are not part of consortium, by establishing robust and interlinked both metabolic and nutrient networks, producing biofilms.

(b) Immune barrier mainly includes intestinal mucosa lamina propria (LP) with its gut-associated lymphoid tissue (GALT): Peyer's patches (PP) of the distal ileum, isolated lymphoid follicles (ILFs), cryptopatches (small aggregates of lineage-negative cells) and mesenteric lymph nodes (MLNs). MLNs and cryptopatches are inductive sites for adaptive immune responses to gut-derived antigens. There are in total 71499 (± 22976) ILFs in the total gastrointestinal (GI) tract of pig (Merchant *et al.*, 2011).

(c) Mechanical barrier, consisting of a relatively impenetrable but highly responsive epithelium, forms an important interface between the body interior (mucosal tissues) and exterior (intestinal lumen), and maintains the function of nutrient uptake.
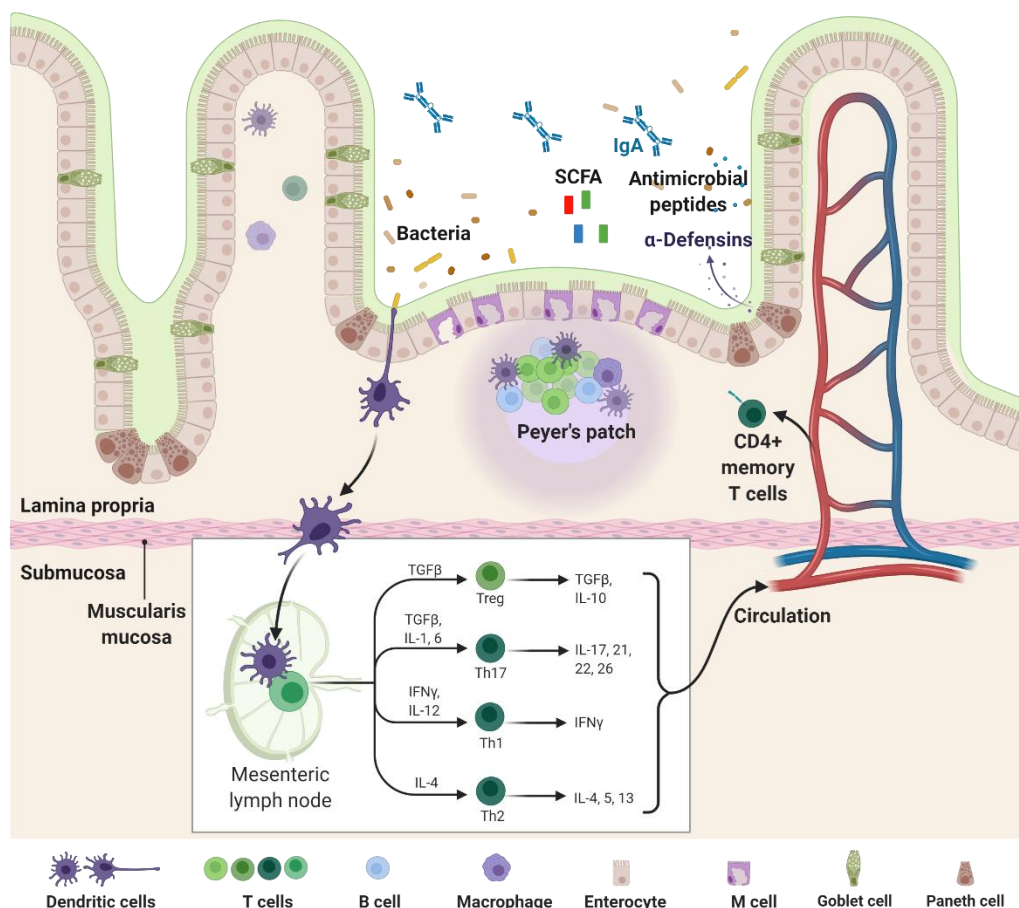
Figure 4. Gut barrier and interaction with intestinal microbiota**.** Gut barrier is composed with gut microbiota in the lumen, epithelial cells, lamina propria and GALT cells. Gut microbiota mainly the bacteria, bacteria metabolites can interact with GALTs and regulate the local and systemic immune response. Under the stimulation of bacteria and its product, gut tissue could produce IgA, antimicrobial peptides and defensins, playing important role in mucosal response. (Figure is adapted from "Intestinal Immune System (Small Intestine)", by BioRender.com (2020). Retrieved from https://app.biorender.com/biorender-templates.)

1.2.2 How the gut barrier balances homeostasis

1.2.2.1 Epithelial Regulation of Innate Immunity through PRPs

Intestinal epithelial cells (IECs) and immune cells in the intestinal tract express a diverse range of germline-encoded pattern-recognition receptors (PRRs), which are able to detect the pathogen-associated molecular patterns (PAMPs) expressed by the resident microbiota and pathogens such as lipopolysaccharide, flagellin, bacterial DNA and RNA. Thus, immune cells can differentiate between commensals and pathogens. Most PRRs fall into three families: transmembrane Toll-like receptors (TLRs), cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs),

and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Hevia *et al.*, 2015).

PRR signaling has significant roles in intestinal homeostasis, by regulating the number and composition of commensal bacteria. Epithelial PRR signaling mainly promotes mucosal protection through induction of pathways that leads to cell proliferation and survival, or cytoprotection in response to mucosal injury and mucosal permeability in response to epithelial injury. In addition, PRR signaling in immune cells from the lamina propria may be involved in inflammatory cytokine production in response to invasion of pathogens.

1.2.2.2 Microbe-Epithelial Cell Regulation of Intestinal Secretory immunoglobulin A (IgA)

The gut is by far the largest antibody-producing organ in the body, since more than 80% of the activated B cells reside in the mucosal tissues in humans (Brandtzaeg *et al.*, 1999). Gut microbiota can induce the mucosal immune system to produce IgA) and antimicrobial peptides, which are released in the intestinal lumen in large amounts limiting local bacterial colonization, providing a crucial defense against pathogens and also playing a role in shaping the ecology of the microbiota (Bekeredjian-Ding *et al.*, 2009; Peterson *et al.*, 2007; Salzman *et al.*, 2007).

1.2.3 Bacterial diversity in the gut

There are about 100 trillion microorganisms in the human GI tract. The intestinal microbiome is considered as a virtual organ of the body (Valdés *et al.*, 2018). Human genome contains around 23,000 genes, while the microbiome contains approximately 3 million genes which is as much as 150 times of the human genome genes (Valdés *et al.*, 2018).

Gut microbiome is complex and dynamic, including not only bacteria but also yeast, mold, virus, parasites and bacteriophages. During the last decades, the study of the complex network of microorganisms living in symbiotic relationship in the gut has increases noticeably due to the new technologies. Through next generation sequencing (NGS) of the 16s rRNA marker gene, the myriad of bacteria inhabiting this (and other) body sites can be detected without culturing them (Wu *et al.*, 2012).

Coinciding with humans, a common set of 4,430 non-redundant (NR) genes out of 7,685,872 NR genes, is known to be shared by 100% of the 287 pig samples tested,

suggesting the existence of a core of genes, species and functions in the gut microbiome of pigs (Xiao *et al.*, 2016). Additionally, different breeds own specific microbial composition. *Firmicutes* and *Bacteroidetes* represented around 70 -90 % of the gut microbiota in the pig feces at phylum level (Costa *et al.*, 2014; Mach *et al.*, 2015; Ramayo-Caldas *et al.*, 2016). Other abundant phyla found were *Proteobacteria, Spirochaetes, Actinobacteria, Synergistetes, Tenericutes, Verrucomicrobia,* and *Planctomycetes*, but with low relative abundance (Correa-Fiz *et al.*, 2019; Zhao *et al.*, 2015). Forty-four genera and 1127 operational taxonomic units (OTUs) were identified in the feces from 518 piglets at 60 days of age, the most abundant genera were *Prevotella* and *Roseburia* (Ramayo-Caldas *et al.*, 2016). Based on the microbiota composition, these 518 piglets were divided into two enterotype-like groups, which were dominated by either *Ruminococcus* and *Treponema* genera, or *Prevotella* and *Mitsuokella* genera. A significant effect of enterotype-like cluster assignment on body weight and average daily gain during the post weaning time were revealed by statistical analysis (Ramayo-Caldas *et al.*, 2016). Gut microbiota changed from aerobes to strict anaerobes over the time from neonates to adults (Kim *et al.*, 2011). Longitudinal analysis of fecal microbiota from 20 pigs revealed that the fecal microbiota changed during the time from 10 days after born to 22 days after born, and microbiota changes towards a more similar state, furthermore animal bacterial diversity variation was affected by less abundant bacterial component of feces (Kim *et al.*, 2011). In total 175 genera were found in the fecal microbiota of these 20 piglets (Kim *et al.*, 2011). Microbiota composition dynamic is affected by different factors, such as diet, antibiotic, stress or pathogenic infections (Isaacson *et al.*, 2012).

1.2.4 Microbiome-immune interaction

1.2.4.1 Microbiome colonization is crucial in initiating and educating immune system

The absence of microbiota impacts most, if not all, aspects of the immune system (Sender *et al.*, 2016). Immune maturation is likely influenced directly and/or indirectly by the presence of commensal microbes (Geuking *et al.*, 2011; Rakoff-Nahoum *et al.*, 2008). Maturation of the intestinal mucosa and its GALT is initiated by and contingent on intestinal colonization (Maynard *et al.*, 2012).

General introduction

Germ-free (GF) mice model not only led to the discovery that gut microbiota is required for the normal generation and maturation of GALT, but also facilitated the investigation of the effect of gut microbiota on the immune system. The absence of commensals leads to defected structural and limited function of several lymphoid tissues, including but not limited to spleen, thymus, and lymph node (Bauer *et al.*, 1963). Smaller PPs and MLNs were observed in GF animals (Macpherson *et al.*, 2004). GF mice possess few IgA-expressing B cells in the small intestine, low number of Th1 cell and T helper type 17 (Th17) cells. Colonization with normal flora or certain microbiome in adult GF mice could restore ILF numbers and rescue IgA production by B cells in the small intestine (Crabbé *et al.*, 1968; Hapfelmeier *et al.*, 2010; Macpherson *et al.*, 2004; Mosconi *et al.*, 2013). GF mice exhibit also a lower expression of major histocompatibility complex (MHC) class II on IECs that can be rescued through colonization with microbiota, an IFNγ–dependent event (Matsumoto *et al.*, 1992).

1.2.4.2 Window of opportunity for colonization of certain bacteria

Early exposure to microbes may have durable consequences for the host that may extend into adult life (Gensollen *et al.*, 2016). Restoring a few cellular defects occurring because of absence of microbiota is age dependent (El-Aidy *et al.*, 2013). For example, colonization of adult (> 5 weeks of age) GF mice with a complex microbiota does not influence the number or activity of colonic invariant natural killer T (iNKT) cell populations. However, if the colonization occurs when GF mice are neonates, the number of iNKT cells is reduced and their later activation is well-controlled (Rakoff-Nahoum *et al.*, 2008). Immunoglobulin E (IgE) production in adult mice is dependent on intestinal bacterial diversity in neonates rather than on colonization with specific bacterial species, since a low diversity of microbiota in the early life is not sufficient to normalize IgE levels in adult life (Cahenzli *et al.*, 2013).

1.2.4.3 Colonization resistance of commensal bacteria to enteric pathogen

The commensal microbiota ensures the mechanical integrity of the mucosal barrier, thereby offering protection against harmful pathogenic microbes. The microbiota promotes colonization resistance against pathogens in different ways.

(a)   Commensal bacteria can directly inhibit intestinal pathogens through adhering to both nutrient-based niches which consumes limited resources, and physical space (lumen, or in the outer mucus layer, or more rarely at the epithelial surface),

which competitively inhibit the adhesion of enteropathogens.

(b) Certain bacteria can produce a large number of bactericidal molecules which act towards other members of intestinal commensal and enteric pathogens. Most of which are bacteriocins, consist of small polypeptides or ribosomal proteins, could inhibit other related or unrelated microorganisms. Most bacteriocins act against taxonomically close related bacteria, albeit some have a wider spectrum of activity. Lactic acid bacteria (LAB) such as *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and some *Streptococcus* are examples of bacteria that normally produce bacteriocins.

(c) Intestinal microbiota can produce specific metabolites which could modulate the intestinal environmental conditions, compromising pathogen growth and/or virulence. Bacterial species that feed on non-digestible dietary fibers (DF) can produce metabolites that exert positive effects on the intestinal mucosa. For instance, short-chain fatty acids (SCFAs) can be produce from DF by anaerobic fermentation in the intestine. SCFAs could exert positive effects on the intestinal mucosa and intestinal epithelial cells, thus increasing epithelial barrier function, antimicrobial peptides (AMPs) production, cell proliferation, maintaining the balance of inflammatory and anti-inflammatory T cell subsets (Corrêa-Oliveira *et al.*, 2016; Donohoe *et al.*, 2011; Gijs *et al.*, 2013). Members of the *Bacteroidetes* mainly producing acetate and propionate, while *Firmicutes* mostly produce butyrate in the human gut (Louis *et al.*, 2017, 2009). Interestingly, human IBD (inflammatory bowel disease) patients not only show reduced levels of dominant SCFAs-producing bacteria (e.g. *Faecalibacterium prausnitzii* and *Roseburia intestinalis*) in intestinal mucosa and feces, but lower levels of SCFAs compared to healthy controls (Joossens *et al.*, 2011; Pascal *et al.*, 2017).

1.2.4.4 Gut microbiota and extra-intestinal organ immune symbiosis

Gut microbiota have a profound influence on systemic immune responses. Intestinal microbiome and microbiome-associated metabolites/products can translocate in different organs through the circulatory system, subsequently inducing tissue-specific local immune responses in lung, liver, brain, and other organs (Carabotti *et al.*, 2015; Zheng *et al.*, 2020). There are increasing evidence that support that extra-intestinal mucosal surfaces can modulate organ-specific immune responses. The inoculation of specific microbes to GF mice is sufficient to induce arthritis (Maeda *et al.*, 2019), while

a depletion of microbiota after broad-spectrum antibiotic treatment increased the mice's susceptibility to lymphocytic choriomeningitis or influenza virus (Abt *et al.*, 2012). Translocation of peptidoglycan, a major component of bacterial wall, from the gut to bone marrow, can enhance bone marrow-derived neutrophils functions and systemically prime the innate immune system, enhancing the killing of two important pathogens: *Streptococcus pneumoniae* and *Staphylococcus aureus* (Clarke *et al.*, 2010). Intestinal pathogens can also activate DCs and NKT cells, exacerbating immunological hepatic injury in the liver (Chen *et al.*, 2014). On the other hand, bacterial lipopolysaccharide (LPS) can stimulate hepatic stellate cells, mainly through TLR4 signaling, resulting in upregulation of multiple chemokines and adhesion molecules in liver (Paik *et al.*, 2003). Moreover, diet-derived SCFAs produced by bacteria, contribute to microglia homeostasis and promotes regulatory T cells to counter-regulated autoimmunity in the central nervous system (Zheng *et al.*, 2020).

1.2.4.5 Gut microbiota and viral infections

Commensal bacteria can influence various pathogenic viral infections either hindering or promoting, sometimes even aggravating the disease (Karst, 2016; Wilks *et al.*, 2013, 2012). Enteric viruses use commensal bacteria to enhance viral infectivity directly or indirectly. Some viruses increase their viral fitness through bacterial stabilization of the viral particle or enhanced binding to the surface of the target host cell by interacting with microbiota and their products directly (Fouts *et al.*, 2012; Kuss *et al.*, 2011; Qin *et al.*, 2011; Robinson *et al.*, 2014). Poliovirus use LPS to promote its attachment to the surface of permissive cells through direct facilitation of viral binding to the poliovirus receptor (Guo *et al.*, 2013; Kuss *et al.*, 2011). Mouse Mammary Tumor Virus-bound LPS triggered TLR4 and subsequently induce the inhibitory cytokine Interleukin (IL)10, inducing an immune evasion pathway and facilitating the establishment of a persistent viral infection (Kane *et al.*, 2011).

Microbiota can inversely benefit the host depending on the viral agent on different body sites. For example, the presence of the microbiota is essential for an effective immune response against the *Vaccinia virus* (VACV) (Lima *et al.*, 2016). GF and immunosuppressed mice models show similar profile of systemic infection of VACV, whereas conventional mice were refractory (Lima *et al.*, 2016). A microbiota-mediated protection was observed for influenza A virus in mice, microbiota

composition regulates virus-specific CD4 and CD8 T cells' generation when exposed to influenza virus (Ichinohe *et al.*, 2011). Additionally, commensal bacteria can calibrate the activation threshold of innate immunity, which revealed an interplay between commensal and antiviral interferon signaling pathways in macrophages, involved in responses to Influenza A virus (Wang *et al.*, 2013).

On the other side, a viral infection can affect the gut microbiota composition, as well. Respiratory influenza infection can cause digestive diseases such as intestinal immune injury, which may have resulted from an altered intestinal microbiota composition mediated by IFNγ produced by lung-derived T-cells and recruited into the small intestine (Wang *et al.*, 2014). Influenza infection increases the host susceptibility to *Salmonella* intestinal colonization and dissemination during secondary *Salmonella*-induced colitis through suppression of host intestinal immunity mediated influenza-induced type I interferons induced in the pulmonary tract (Deriu *et al.*, 2016). Respiratory syncytial virus or influenza virus infection in the lung could induce inappetence associated with CD8 T-cells, which in turn alters the gut microbiome and metabolome (Groves *et al.*, 2020).

1.2.5 Fecal Microbiota Transplantation

Fecal Microbiota Transplantation (FMT), also called "feces transplantation" and "fecal bacteriotherapy", is the transfer of the fecal microbiota from a healthy, screened donor to a recipient (McCormack *et al.*, 2018). FMT aims to restore a disrupted microbiota and amend imbalances through establishment of a stable, complex microbiota.

1.2.5.1 History of Fecal Microbiota Transplantation

The first written record on the oral use of fecal matter was contained in one of the oldest Text of Chinese Medicine excavated in an ancient tomb in Middle China, called "Fifty-two Treatment Formulae" (Shi, 2017). which was estimated that the document was written in 770 BC (Before Christ). In the 4th century, Ge hong, a well-known Taoist healer, described many medicinal items and formulae which contained human, chicken, dog, cattle and horse feces (Ge, 341AD). The next recorded FMT was in the 16th century during the Ming Dynasty by Li Shizhen, fresh or fermented fecal suspensions, dry feces or infant feces applied as effective treatment for constipation,

diarrhea, vomiting and abdominal pain (Li, 1596). The ancient fecal transplantation did not have any evidence related with the modern life science, however, the traditional Chinese medicine doctors observed that fecal matter could provide unexpected and favorable outcomes (Leung *et al.*, 2019). The first four cases of fecal transplantation in modern medicine for the control of pseudomembranous enterocolitis was reported in 1958 (Eiseman *et al.*, 1958). FMT was recorded for treating pseudomembranous enterocolitis patients in 1981 (Bowden *et al.*, 1981). In 1989, FMT was applied to treat ulcerative colitis (UC), the exchange of bowel flora on a refractory UC patient showed full and lasting clinical recovery (McEvoy, 1989). Since then, a lot of FMT trials to cure gastrointestinal disease, *Clostridium difficile* infection (CDI), recurrent CDI (RCDI) or IBD (Cui *et al.*, 2015; Zhang *et al.*, 2017), were performed (Brandt *et al.*, 2012). Since 2013, FMT is the only indication approved for CDI treatment by the United States Food and Drug Administration.

1.2.5.2 FMT investigation in pigs

Research via gastrointestinal microbiome manipulation to improve outcomes in pigs started very recent with few available publications. A very interesting example comes from the experiments done by Diao *et al.* in 2018, where Duroc x Landrace x Yorkshire sucking piglets (3-day old) were transplanted with feces from different donor breeds: Yorkshire, Rongchang and Tibetan. Compared with the control group, FMT from Yorkshire and Rongchang pigs had adverse effects on gut development and function, whereas FMT from Tibetan pigs showed some positive effects on intestinal health and function (Diao *et al.*, 2018). FMT to suckling piglets with maternal feces could affect microbiota colonization in stomach, ileum and colon in piglets. The metabolite profile of piglets by day 7 post transplantation seemed to indicate a more efficient energetic metabolism and a more active protein synthesis, albeit the impact of these changes on the health of the recipients was not clear (Lin *et al.*, 2018). One study with pigs (age from 14 days post birth to 70 days post birth), implied that members from *Prevotella* genus was positively correlated with luminal secretory IgA concentrations (Mach *et al.*, 2015).

On the other hand, several studies described positive effects from FMT on bacteria infection. It could reduce the negative impact of *Escherichia coli* K88 infection on the gastrointestinal epithelium of piglets (Hu *et al.*, 2018). In another study,

transferring fecal microbiota from healthy native Chinese breed into commercial crossbred piglets by oral administration prior to early weaning, conferred diarrhea resistance, which was related with gassericin A, a bacteriocin secreted by *Lactobacillus gasseri* LA39 and *Lactobacillus frumenti* (Jun *et al.*, 2018). Moreover, FMT has been used successfully also to fight porcine viral diseases. Thus, FMT reduced clinical signs and pathology associated with Porcine circovirus associated disease in piglets, including a reduction in virus load and increased viral antigen-specific antibodies, although no impact of FMT on bacterial diversity or global changes in bacterial composition was detected (Niederwerder *et al.*, 2018).

As mentioned above, gut microbiota composition changes play important role in maintaining intestinal health and function, but also facilitate the defense against pathogenic bacterial and viral infections in pigs. Regarding ASFV, On the one hand, different swine species: domestic pigs, SPF pigs, warthogs and indigenous pigs in Africa showed different susceptibility to ASFV. On the other hand, fecal microbiota composition was compared among ASF-sensitive pigs: SPF pigs and domestic pigs from the same bred, indigenous domestic pigs from a backyard farm in Kenya, and ASF- resistant warthogs (warthogs from Africa and Barcelona zoo) (section 1.7). African animals showed the highest microbial diversity while the SPF pigs the lowest. Forty-five OTUs was shared in the core microbiota from warthogs and domestic pigs, while 6 OTUs were exclusively present in resistant animals, including members of the *Moraxellaceae* (family), *Pseudomonadales* (order) and *Paludibacter*, *Anaeroplasma*, *Petrimonas*, and *Moraxella* (genera). However, the effects of microbiota composition on the ASFV pathology to hosts was not addressed (Correa-Fiz *et al.*, 2019). These studies lead, in fact, to the thesis here presented, aiming to confirm the importance of gut microbiota in ASF susceptibility.

Chapter 2

Hypothesis and objectives

Warthogs (*Phacocaerus africanus*) are natural reservoirs for ASFV in Africa, being resistance to develop clinical signs of ASF, independently of the strain virulence. On the other hand, commercial domestic pigs (*Sus scrofa)* develop different clinical signs of ASF, reaching up to 100% mortality rates when infected with highly virulent strains. Previous work in our laboratory demonstrated that SPF pigs were much more susceptible to attenuated strains of ASFV than conventionally raised pigs, despite sharing identical genetic background. These results clearly demonstrated that both genetic and environmental factors play a role in ASF resistance. Finally, recent work performed in our laboratory confirmed that warthog and domestic pig fecal microbiota composition varied depending on both genetic and environmental factors.

With this knowledge regarding the crucial role of gut microbiota in body homeostasis, immune response and pathogen resistance, we **hypothesized that the warthog fecal microbiota might have a potential role in ASF resistance.** To demonstrate this hypothesis, we defined four specific objectives: **1)** To establish a fecal microbiota transplantation (FMT) model in domestic pigs using fecal microbiota from domestic pigs or from warthogs. **2)** To use this animal model to compare the ASF susceptibility after experimental challenge with virulent or attenuated ASFV strains. **3)** To isolate individual bacteria from warthog fecal microbiota for further characterization of their *in vitro* microbicidal or immunostimulatory capabilities. **4)** To inoculate *in vivo* selected components of the *in-vitro* characterized microbiota, aiming to mimic the effects observed after FMT.

Advancing these goals will facilitate a new scope for future anti-ASFV strategies and widen the knowledge of warthog fecal-microbiota' biological role.

PART II

Studies

Chapter 3
Study I

Fecal microbiota transplantation from warthog to pig confirms the influence of the gut microbiota on African swine fever susceptibility.

Abstract

African swine fever virus (ASFV) is the causative agent of a devastating hemorrhagic disease (ASF) that affects both domestic pigs and wild boars. Conversely, ASFV circulates in a subclinical manner in African wild pigs, including warthogs, the natural reservoir for ASFV. Together with genetic differences, other factors might be involved in the differential susceptibility to ASF observed among Eurasian suids (*Sus scrofa*) and African warthogs (*Phacochoerus africanus*). Preliminary evidence obtained in our laboratory and others, seems to confirm the effect that environmental factors might have on ASF infection. Thus, domestic pigs raised in specific pathogen-free (SPF) facilities were extremely susceptible to highly attenuated ASFV strains that were innocuous to genetically identical domestic pigs grown on conventional farms. Since gut microbiota plays important roles in maintaining intestinal homeostasis, regulating immune system maturation and the functionality of the innate/adaptive immune responses, we decided to examine whether warthog fecal microbiota transplantation (FMT) to domestic pigs affects host susceptibility to ASFV. The present work demonstrates that warthog FMT is not harmful for domestic weaned piglets, while it modifies their gut microbiota; and that FMT from warthogs to pigs confers partial protection against attenuated ASFV strains. Future work is needed to elucidate the protective mechanisms exerted by warthog FMT.

Keywords: ASFV; fecal microbiota; fecal microbiota transplantation; pig immunity; warthog

Study I--FMT influence on ASFV susceptibility

Introduction

African Swine Fever (ASF) is a devastating disease of domestic pigs and wild boars, caused by African swine fever virus (ASFV). ASF is a notifiable disease to the World Organization for Animal health (OIE)[1] and today it is considered the most serious constraint for pig production. The current distribution of African swine fever extends across more than 50 countries from African, Asian and European continents and more recently, also from Oceania[2].

ASFV is a large enveloped virus of approximately 260 to 300 nm in diameter[3] with a genome size between 170 and 193 kbp[4] encoding at least 150 different proteins[5] and the only known DNA arbovirus. ASF was described for the first time in 1921 as a new disease affecting domestic pigs in Kenya[6]. Before domestic pigs were introduced into Africa, ASFV was circulating following a sylvatic cycle between soft ticks (*Ornithodoros)* and African warthogs (*Phacochoerus africanus*). Warthogs and bushpigs (*Potamochoerus porcus*) act as ASFV reservoirs in the wild[7]. Depending on the viral isolate, domestic pigs infected with ASFV can develop a disease that ranges from chronic or subclinical to subacute and hyper-acute[8], resulting the latter in up to 100 % mortality in naïve pigs[9]. The mechanisms of ASF-resistance showed by warthogs and bush pigs has not yet been elucidated, albeit both genetic[6,10] and environmental factors could be involved. Preliminary experimental evidences described local pigs in Africa as less susceptible to infection with certain ASFV genotypes[10]. On this regard, it is worth to mention that specific-pathogen-free (SPF)-pigs were more susceptible to infection with ASFV attenuated strains than genetically identical pigs raised in conventional farms[11], allowing to hypothesize that, together with genetics, warthog microbiota could contribute to ASF-resistance.

The intestinal microbiota affects multiple facets of organism homeostasis through its influence on the innate immune system[12,13]. The gut microbiota of the animal species mentioned above, i.e. warthogs and both SPF- and domestic pigs, have been recently unveiled, showing relevant differences in composition[14]. Fecal microbiota transplantation (FMT) is a delivery of donor microbiome to a recipient in order to establish or restore intestinal homeostasis, or populate the gastrointestinal tract with potentially beneficial bacteria[15]. Interest on the novel FMT for the prevention and treatment of intestinal disorders has been increasing in human medicine, for example,

to control *Clostridium difficile* infection[16] or inflammatory bowel disease[17]. More and more clinical applications of FMT have provided convincing proofs that modification of the intestinal microbiota is an effective therapy for intestinal dysbiosis-related diseases[18]. In pigs, published studies using FMT are scarce, but they provide evidence of the ability to reprogram the porcine intestinal microbiota via FMT, altering immune phenotype of the host[19].

In the present work, weaned piglets were first, transplanted with fecal microbiota from either warthogs or domestic pigs, and second, they were challenged with a virulent or an attenuated ASFV strain to compare their disease outcomes. No differences were observed after intramuscular challenging with E75, a virulent ASFV strain, independently of the FMT. Conversely, a very significant reduction of virus in serum, nasal viral shedding and clinical signs were observed when pigs transplanted with warthog feces were intramuscularly challenged with E75CV1, an attenuated ASFV strain, when compared with pigs transplanted with domestic pig feces. Far from understanding the mechanisms involved in the protection afforded, we provide here evidences showing the protective potential to ASF of warthog microbiota.

Results

FMT modifies the gut microbiota diversity in transplanted pigs

Forty-eight 21-day-old animals divided into four groups were orally inoculated over three consecutive days with either a pool of warthog feces (WF group), a pool of domestic pig feces (PF group) or PBS (control group). The fourth group of pigs was treated with a cocktail of antibiotics one day before inoculation with a pool of warthog feces (AWF group), aiming to facilitate the warthog microbiota transplantation. Fecal samples from 5 pigs from each group (PF, WF, AWF and PBS) were collected at 15 days post fecal transplantation (dpft) and their fecal microbiota was compared. The 16S ribosomal DNA (rDNA) was sequenced individually from all these samples. After quality trimming processes, 730,952 high-quality sequences were obtained for 20 feces samples. The read count for each sample ranged from 1,425 to 60,027, with a mean frequency of 36,548. Since the read count from one animal (PF#13) was too low (1,425) in comparison with the mean frequency, it was discarded from the analysis (Supplementary Table S1).

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The observed taxa at different taxonomic levels for different groups are shown in Figure 1. The classified taxa were distributed in 15 phyla, 60 families, and 77 genera. *Firmicutes* and *Bacteroidetes* were the two dominant phyla for all the groups, followed by *Spirochaetes* or *Proteobacteria*. In relative terms, the most abundant family in all the groups was *Ruminococcaceae*, while the second most abundant family was *Prevotellaceae* for all transplanted groups (PF, WF and AWF) and *Erysipelotrichaceae* for PBS. *Prevotella* was the dominating genus in PBS, WF and AWF while *Sporobacter* was dominant for PF group (Supplementary Table S2).
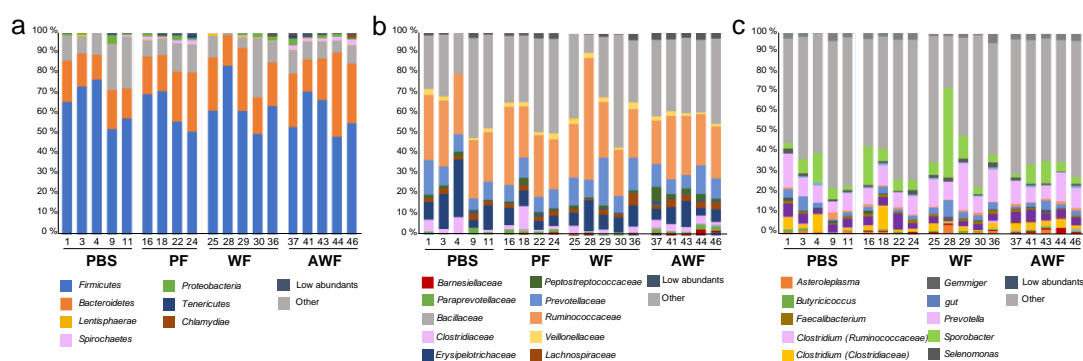
Figure 1. Fecal microbiota composition at 15 days post-fecal-transplantation (dpft) in WF, AWF, PF and PBS groups. Each bar represents the relative abundance of taxa found in feces at different taxonomical levels: phylum (A), family (B), genus (C). The taxa found with less than 1% of relative abundance were collapsed as 'low abundants'. The ten most relatively abundant taxa are shown in the legend. For a full list of the taxa composing the fecal microbiota composition, please refer to Supplementary Table S2.

Alpha diversity was estimated for all the groups at 15 dpft through Chao1 index that considers richness (Fig. 2a) and Shannon index that considers both richness and evenness (Fig. 2b). The richness (Fig. 2a), showed a tendency to be higher in both PF and AWF in comparison with PBS ($P = 0.08$ and $P = 0.07$ respectively), while proved to be statistically higher than WF ($P = 0.01$ and $P = 0.009$ respectively). This increase in richness was accompanied with an increase in diversity, only for AWF (Fig. 2b), showing a Shannon index statistically higher than WF ($P = 0.009$) and a tendency to be higher than PBS ($P = 0.07$).

The spatial changes in bacterial communities among groups was explored through PCoA. Beta diversity analysis was done using weighted (Fig. 2c) or unweighted Unifrac phylogenetic (Fig. 2d) distances. PCoA was performed to visualize the differences in Unifrac distances for all samples at 15 dpft. The beta diversity

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analysis showed a distinct clustering comparing the different groups, explaining 28.44 % of the differences in the quantitative analysis (weighted, $P = 0.007$) and 21.87 % in the qualitative analysis (unweighted, $P = 0.021$). Interestingly, pig AWF#44 showed a dissimilar microbiota composition (Fig. 2b) with the highest mean distance (0.241 ± 0.02) compared to the mean of the AWF group (0.199 ± 0.03).
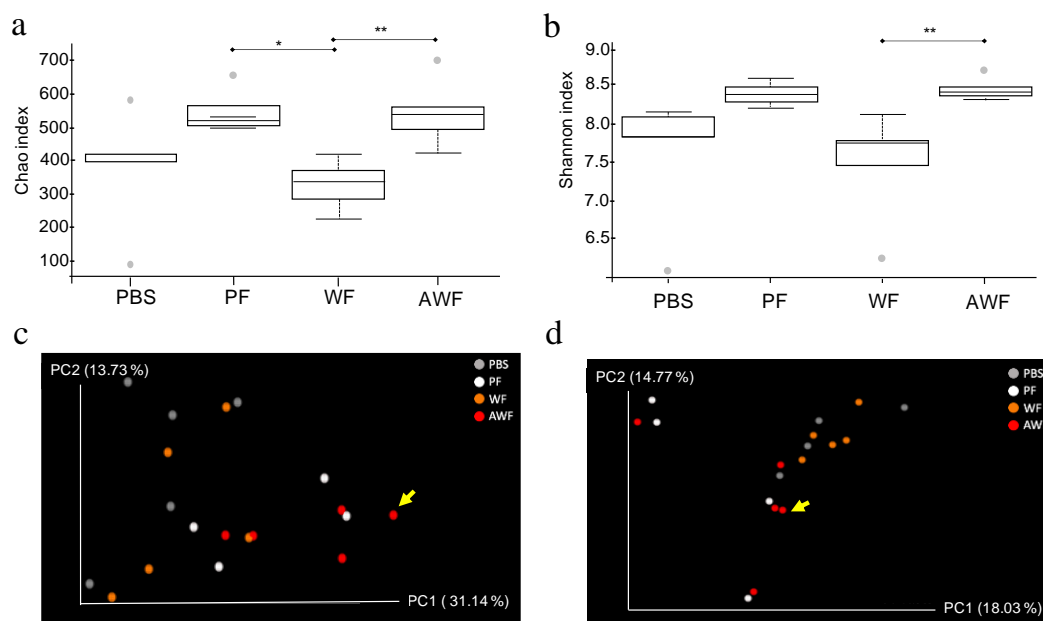
Figure 2. Alpha and beta diversity on rarefied fecal samples at 15 days post-fecal-transplantation (dpft) in WF, AWF, PF and PBS groups. Alpha diversity computed through Chao1 index (a) or Shannon-Wiener's metrics (b). Dotted lines represent the standard deviation and outliers are indicated with grey circles. * stands for $P < 0.05$ and ** $P < 0.01$. Beta diversity was calculated through weighted (c) and unweighted (d) Unifrac distances at 15dpft. The principal axes are shown with the percentage of variation explained between brackets. Arrows indicate the microbiota composition for animal #44.

Warthog fecal microbiota transplantation is not harmful

Transplantation was done using weaned piglets (21 days-old) and clinical parameters were recorded for 30 days. Despite the delicate transition suffered during weaning, warthog fecal transplantation did not harm the animals. The diarrhea observed in the AWF group lasted for 2-3 days, starting with the antibiotic treatment and finishing the third and last day of FMT.

No significant differences were observed between pigs transplanted with warthog

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feces (WF and AWF) and the PBS group in terms of mean weight or average daily
weight gain (ADWG) from 0 dpft to 30 dpft (Fig. 3a, b). The mean weight of PF group
was lower than the AWF group at 15 dpft and was the lowest from all other groups at
30 dpft (Fig. 3a). The ADWG increased significantly in time for WF, PBS and PF
groups when compared the first and the second fortnights (Fig. 3b). While the AWF
group showed the same trend of weight gain during the period, WF group showed
different growth dynamics since the animals within this group gained less weight during
the first 15 days, but their growth improved dramatically in the second fortnight. On
the other hand, PF pigs gained less weight than PBS control group during the whole
observation period (Fig. 3b), a group that showed diarrhea, coinciding also with the
shortest colon crypt depths among all the groups (Supplementary Table S3).
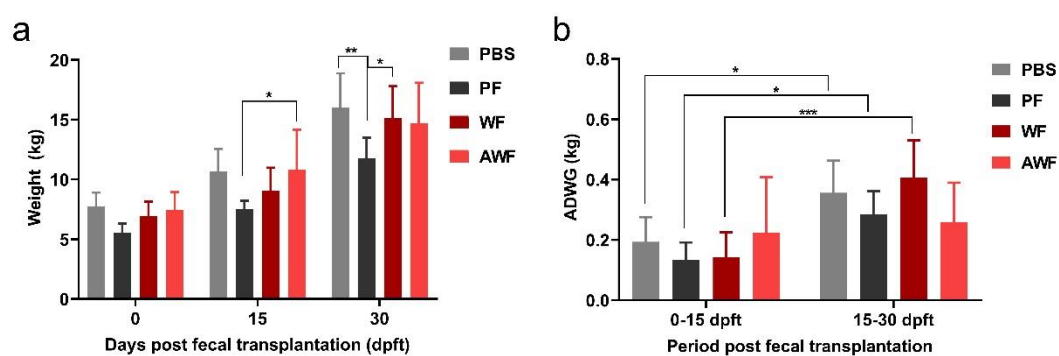
Figure 3. Warthog FMT do not harm the domestic pigs in terms of weight and average daily weight
gain (ADWG). Comparison of average weight at 0, 15 and 30 dpft (a) and ADWG calculated for two
periods: 0 to 15 dpft and 15 to 30 dpft in PBS, PF, WF and AWF groups. * stands for $P < 0.05$, ** $P
< 0.01$ and *** $P < 0.005$.

Some transplanted pigs (PF#13, PF#18, WF#28, WF#29, WF#33 and AWF#46),
showed a brief increase in the rectal temperature (RT) in the period from 4 to 8 dpft
(Fig. 4b, c, d), not observed in PBS animals (Fig. 4a). Conversely, from 15 dpft to 30
dpft, RT remained normal and constant in WF, AWF and PF pigs, while PBS pigs' RT
showed evident oscillations.
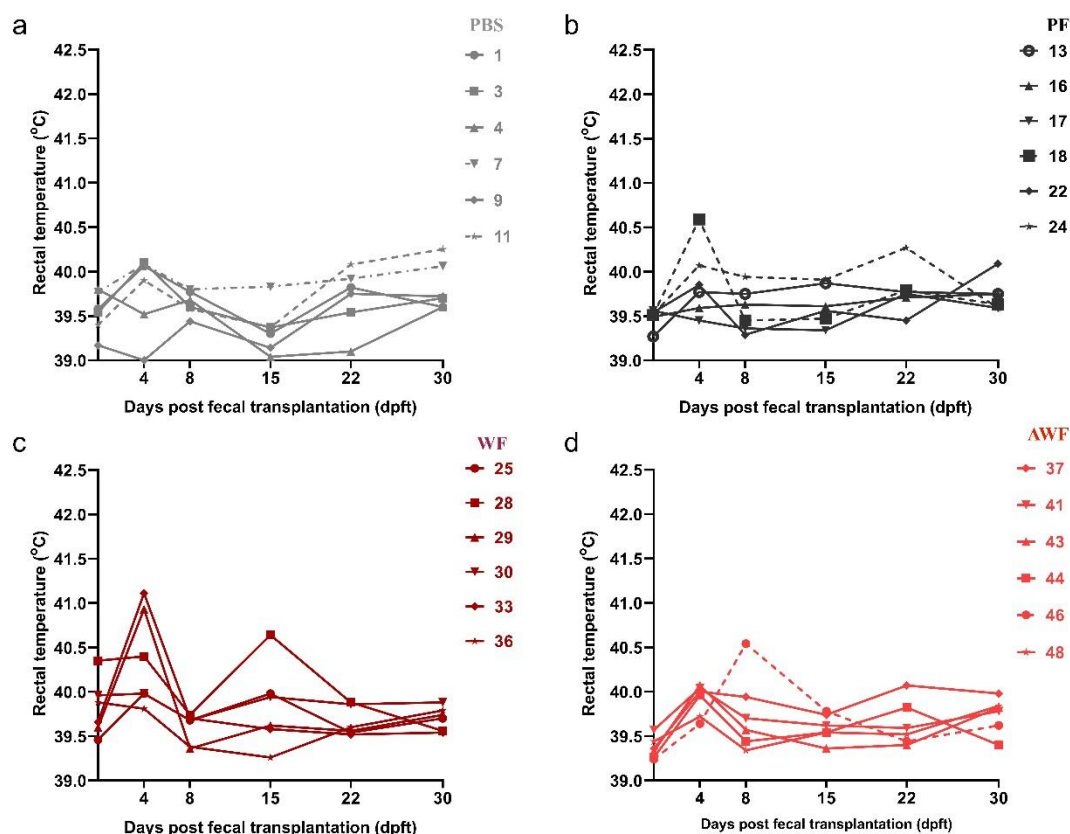
Figure 4. Comparison of rectal temperatures between groups at different days post-fecal transplantation (dpft). Rectal temperature was taken on 0, 4, 8, 15, 22 and 30 dpft for PBS (a); PF (b); WF (c); and AWF groups (d). Transplanted pigs (PF, WF and AWF) showed constant rectal temperature in the late period after FMT.

Warthog fecal microbiota transplantation confers partial protection against ASF *in vivo*.

To evaluate the effects of warthog FMT on ASF susceptibility, transplanted domestic pigs were infected with either the E75 virulent strain or the E75CV1 cell culture-adapted strain[20]. Thirty days after FMT, the animals were transported to BSL3 facilities. PF and AWF were intramuscularly challenged with 100 hemagglutinin units (HAU) of the attenuated E75CV1 strain, facilitating the observation of potential anti-viral effects of the warthog microbiota; while PBS and WF pigs were infected with 10^4 HAU of the parental virulent E75 virus, a more severe ASFV acute lethal challenge. Animals were observed daily according to a welfare schedule to monitor their health

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status and to record the clinical signs after the infection of ASFV (Supplementary Table S4). Correlating with previous observations, after intramuscular inoculation of 100 HAU of E75CV1[20], 50 % of the PF (#13, #16, #24) showed consistent elevated rectal temperatures late after infection (Fig. 5a), starting at 15 dpi (days post infection) and prolonged until day 23 dpi, with only PF#13 showing fever by the end of the experiment (24 dpi). Conversely, only AWF#44, showed fever comparable to that observed for PF pigs (Fig. 5a). Mild clinical signs compatible with chronic ASF were observed (Fig. 5b), perfectly matching with the fever profile observed, with pigs PF#13, PF#16, PF#24 and AWF#44, showing testicular and joint inflammation late after infection. With the exception of AWF#48 that accidentally died while blood sampling at 13 dpi, no other significant clinical findings were recorded in AWF group. At 24 dpi, all animals were euthanized but no gross or microscopic lesions were observed in any animal.
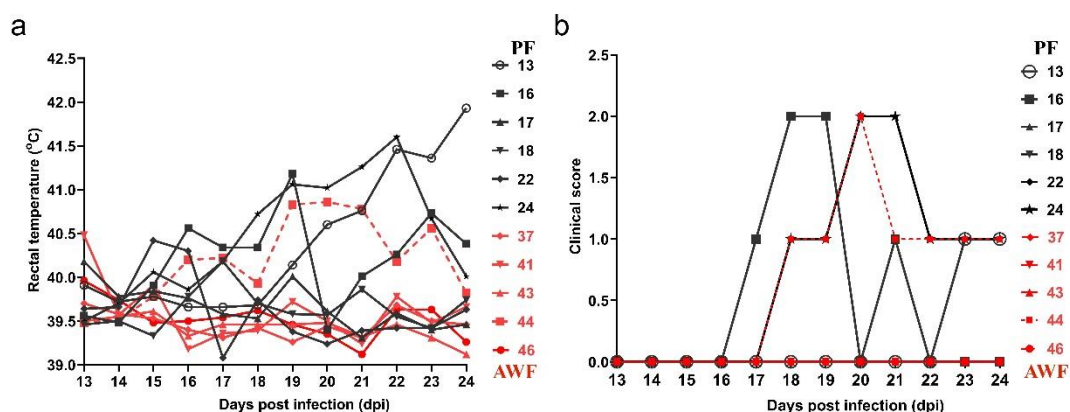
Figure 5. Comparison of rectal temperatures (a) and chronic ASF-compatible clinical signs (b) from PF (in black) and AWF (in red) groups after infection with attenuated E75CV1 strain. Clinical scores were calculated considering body condition, behavior, digestive respiratory and other significant clinical signs including arthritis, dermatitis, testicular tumefaction.

Additionally, the presence of significant virus in serum (Fig. 6a) and virus shedding (Fig. 6b) were also high for PF#13, PF#16, PF#24 and AWF#44, and paralleled with fever and clinical signs in the infected pigs (Fig. 5). PF and AWF pigs, independent of the origin of the transplantation material, mounted detectable cellular and humoral responses against ASFV. The amount of ASFV specific antibodies found in the serum of E75CV1 infected pigs seemed to correlate with the virus titers found in serum and nasal swabs, with PF#13, PF#16, PF#24 and AWF#44 showing the highest antibody titers by day 24 dpi (Fig. 6c). No differences were observed for the ASFV-

specific T-cell responses, independent of the fecal microbiota origin. ASFV-specific T-cells were detectable in all pigs by IFNγ-ELISPOT as early as at 13 dpi and remained present until the end of the experiment (Fig. 6d).
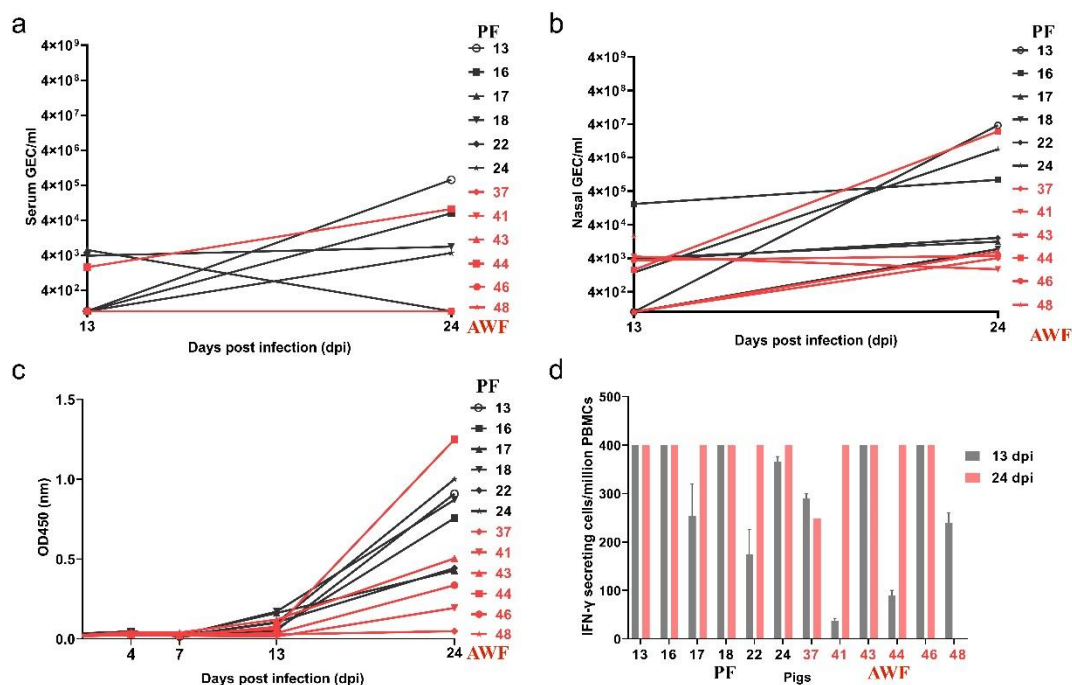
Figure 6. Comparison of virus titration in serum (a), nasal viral excretion (b), ASFV-specific antibodies production detected by ELISA (c) and specific T-cell responses measured in an IFNγ-ELISPOT (d) between PF (in black) and AWF (in red) groups after infection with attenuated E75CV1 strain. The limit of detection of the qPCR is 1 gene equivalent copies (GEC) of ASFV genome/µl of serum or nasal swab homogenate, while the maximum number of positive spots quantifiable in the ELISPOT is 400 spots/million PBMCs.

As above-mentioned, the protection failure observed in pig AWF#44, seemed to correlate with a different microbiota composition at 15 dpft than the rest of the pigs (Fig. 2c, d). Confirming this observation, the *Firmicutes/Bacteroidetes* ratio, commonly used as a health indicator[21-24], gave a value of 1.16 for the AWF#44 at 15 dpft, much lower than the rest of the pigs within the AWF group that showed an average ratio of 2.323 (Table 1).

Table 1. *Firmicutes/Bacteroides* ratio along time in PF and AWF groups after FMT

	PF					AWF				
time	#13	#16	#18	#22	#24	#37	#41	#43	#44	#46
0 dpft	3.333	2.742	1.429	0.654	1.674	1.449	1.428	1.774	1.693	1.219
8 dpft	2.130	2.029	3.372	1.496	1.000	2.076	1.137	1.192	0.838	1.439
15 dpft	NA	3.718	3.962	2.251	1.739	2.003	4.502	3.235	1.160	1.875

NA, not available (sample excluded from analysis). PF: pig feces group; AWF: antibiotic-treated warthog feces group. dpft: days post-fecal transplantation.

Despite the absence of direct and solid *in-vitro* correlates for ASFV protection, once more confirmed in here, we found that warthog FMT might enhance mucosal immunity. Thus, the total IgA found in sera from pigs transplanted with warthog feces /WF and AWF groups), showed a tendency to increase when compared with that found in PF and PBS groups (Figure 7). The amount of ASFV-specific IgA found in the sera of E75CV1-infected pigs parallel that observed for the IgG and with the virus titers found in serum and nasal swabs.
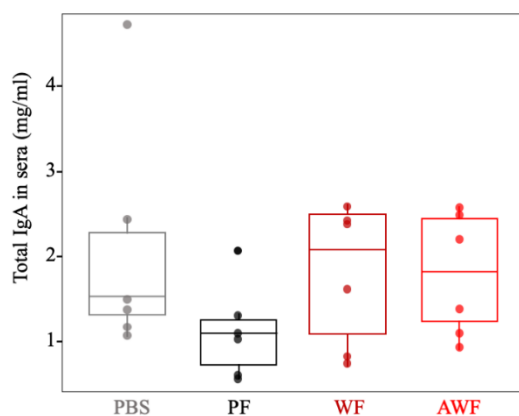
Figure 7. Effect of the FMT on the mucosal immunity. An increased tendence of IgA levels was found in pigs transplanted with warthog feces (WF and AWF) when compared with pig-feces transplanted animals (PF) or non-transplanted control pigs (PBS). No statistically difference was found between groups (Kruskal Wallis, *P*=0.3). Plots were generated using ggplot2 package[81] in R Studio software[82] (Version 1.2.5033).

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In order to evaluate more carefully the anti-viral potency of warthog fecal microbiota, two additional groups of pigs (PBS and WF) were inoculated with a lethal dose of 10^4 HAU of the E75 strain. As expected from our previous study[20], PBS pigs developed acute clinical signs of ASF from 4 dpi (Fig. 8a, 8b), including anorexia, depression, redness and petechiae in the skin and high rectal temperature. WF pigs had clinical signs identical to those described for the PBS pigs (Fig. 8a, 8c). PBS pigs and WF pigs were humanely euthanized at 7 dpi. At necropsy, pigs showed similar lesions among groups consisting of multiple hemorrhages on serosal surfaces, mild ascites, interstitial edema of the lung and the mesentery, moderate to marked splenomegaly and hemorrhages in the gastro-hepatic lymph node. There was no difference in virus replication rate in both serum and nasal swabs (Fig. 8c, 8d).
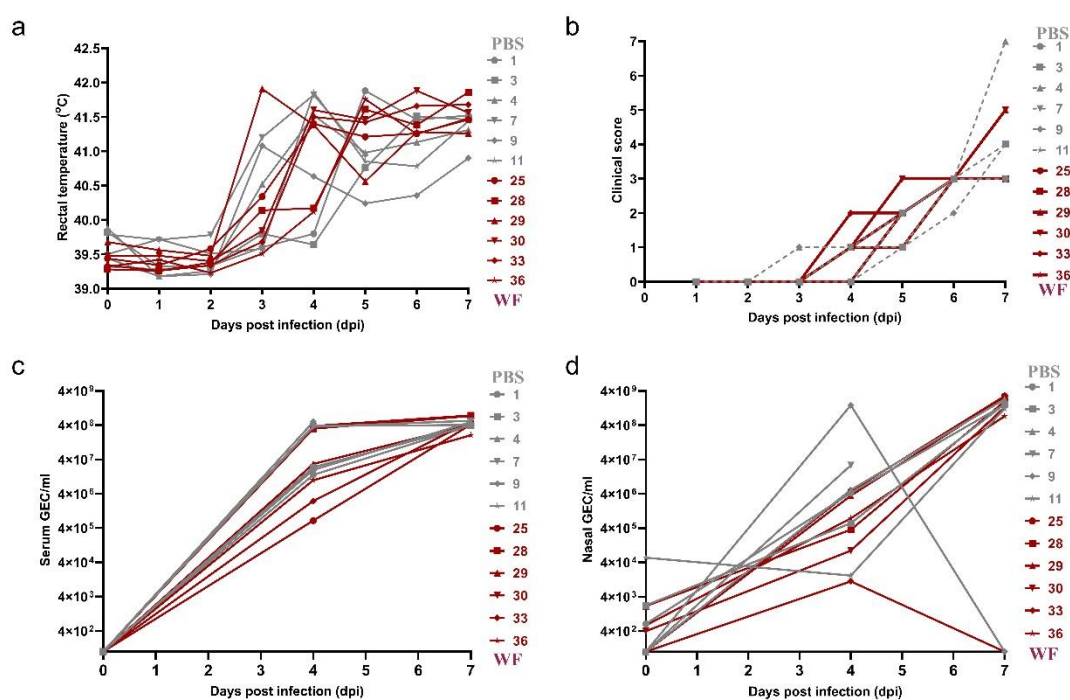
Figure 8. Comparison of rectal temperatures (a), clinical signs compatible with acute disease (b), virus titration in serum (c) and nasal swabs (d) between PBS (in grey) and WF (in dark red) groups after infection with virulent E75 strain. Clinical scores were calculated following a guide previously published by the group[76].

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Longitudinal characterization of the PF and AWF microbiota composition

Based on the results above described, we decided to complete the characterization of the fecal microbiota changes observed along time in both the AWF (E75CV1-resistant) and PF (E75CV1-sensitive) groups. Since the 16S rDNA from AWF and PF fecal samples was already characterized at 15 dpft (Fig. 1 and 2), additional samples from the same animals from these two groups (AWF and PF), collected before FMT (0 dpft) and at 8 days post-fecal-transplantation (8 dpft), were subjected to 16S ribosomal DNA sequencing and analysis. After quality trimming processes, 808,856 high-quality sequences were obtained for these 20 feces samples (Supplementary Table S1). The longitudinal microbiota analysis of fecal samples obtained at 0, 8 and 15 dpft from AWF and PF, showed that AWF richness, decreased between 0 and 8 dpft ($P = 0.08$), and recovered between 8 and 15 dpft (Fig. 9a). A similar pattern was observed regarding AWF diversity (Fig. 9b), when comparing 0 and 8 dpft ($P = 0.1$), showing also recovery between 8 and 15 dpft ($P = 0.1$). PF diversity showed a tendency to increase over time, although it was not statistically significant (0 to 8 dpft, $P = 0.6$; and 8 to 15 dpft, $P = 0.08$). The ANCOM (quantitative) analysis comparing the microbiota composition of AWF and PF at 15 dpft, identified four differential taxa present in AWF: *Mycoplasma* and *Chlamydia*, together with one unidentified genus from *Enterobacteraceae* family and another from the *Proteobacteria* phylum. No quantitative differences were found through ANCOM, either at 0 or 8 dpft.

Beta diversity analysis using weighted Unifrac distances, additionally demonstrated that the microbiota composition changed along the time (0, 8 and 15 dpft, Fig. 9c, 9d). Thus, the microbiota composition of AWF at 0 dpft was different from the composition at 8 dpft (PERMANOVA, $P = 0.05$) and at 15 dpft ($P = 0.01$). The differences for PF group only became evident when comparing 0 and 15 dpft (PERMANOVA, $P = 0.02$).
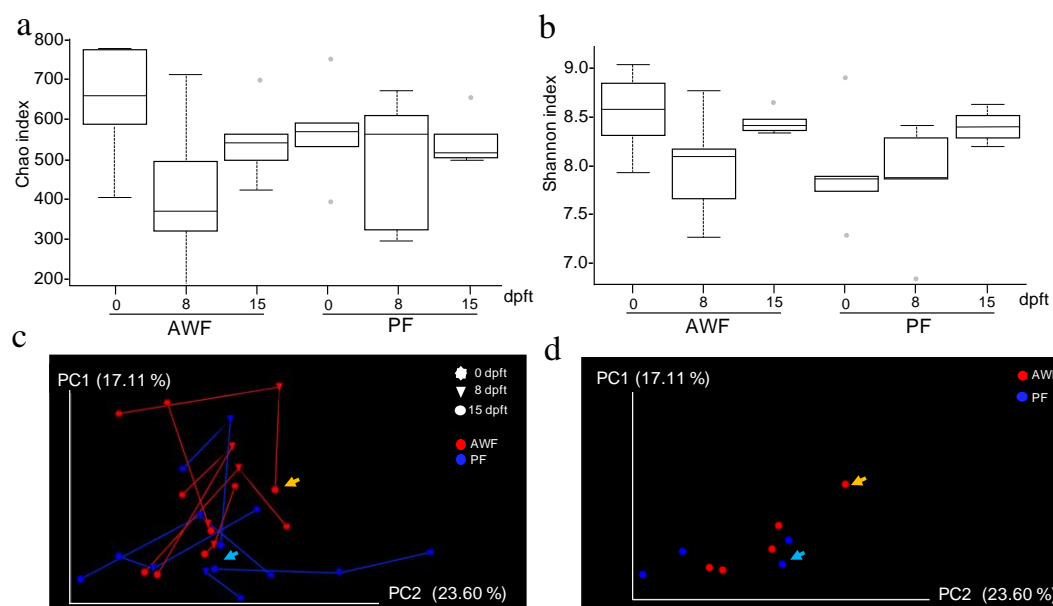
Figure 9. Longitudinal analysis on rarefied fecal samples from AWF and PF groups. Alpha diversity was computed through Chao1 index (a) or Shannon-Wiener's metrics (b) at 0, 8 and 15 days post-fecal-transplantation (dpft). Dotted lines represent the standard deviation and outliers are indicated with grey points. Beta diversity was estimated through weighted Unifrac distances comparing AWF and PF at 0, 8 and 15 dpft (c). The microbiota composition of each animal at each timepoint is represented with triangles for 0 dpft, spheres for 8 dpft and hexagons for 15 dpft. The lines join the microbial composition for each animal at different timepoints (in red for AWF and blue for PF). The beta diversity for AWF and PF at 15 dpft is represented by disabling the visibility of other timepoints (0 and 8 dpft) for better visualization (d). The arrows indicate the microbial composition of AWF#44 and PF#22 at 15 dpft in orange and light blue, respectively. The principal axes are shown with the percentage of variation explained between brackets. * stands for $P < 0.05$ and ** $P < 0.01$.

Finally, in an attempt to find qualitatively different taxa among groups we determined first, the core taxa found in AWF and PF at different times post-transplantation: 0, 8 and 15dpft and compared with the taxa composition of the warthog FMT inoculum (pWF) and the porcine FMT (pPF) inoculum used for the transplantation (Supplementary Table S1). As depicted in Table 2, six genera were present in pWF but absent pPF: *Desulfovibrio Roseburia, Ruminococcus, Actinobacillus, Faecalibacterium, Butyricicoccus*. After fecal transplantation, only *Faecalibacterium* was detected in AWF core at 8 and 15 dpft, while *Roseburia* and *Desulfovibrio* were detected only at 15 dpft. Four genera were detected only in pPF i.e. *Asteroleplasma, Paraprevotella Gemmiger* and *Treponema*. With the exception of *Gemminger*, all these genera were found in both transplanted groups at 15 dpft with similar relative abundance but *Treponema,* that was found in lower relative abundance

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in the AWF group. When we compared the core composition of the two transplanted groups at 15 dpft, we found four genera as exclusive members of the PF core microbiota: *Campylobacter, Clostridium* (from *Lachnospiraceae* family*), Actinobacillus* and *Succinispira*. While *Barnesiella*, *Desulfovibrio*, *Anaerorhabdus* and *Roseburia,* were part of the AWF core at 15 dpft. Finally, *Barnesiella* and *Anaerorhabdus,* they seemed to preferentially colonize pigs in the context of pWF FMT, since they were found exclusively in the AWF core at 15 dpft, although they were present with the same relative abundance in both the pPF and the pWF original inocula. Detailed information about the core taxa are shown in Supplementary Table S5.

Table 2. Mean relative abundance of core genera from the inoculum used and the different groups at different timepoints after FMT

Taxa		Inoculum		PF			AWF		
Family	**Genus**	**pPF**	**pWF**	**0 dpft**	**8 dpft**	**15 dpft**	**0 dpft**	**8 dpft**	**15 dpft**
Barnesiellaceae	*Barnesiella*	1.114	0.772	4.018	1.717		2.347	3.06	1.217
Campylobacteraceae	*Campylobacter*	0.05	0.021			0.15			
Clostridiaceae	*Clostridium*	0.823	1.38	4.499		3.969	3.641		2.943
Desulfovibrionaceae	*Desulfovibrio*		0.12	0.177					0.065
Erysipelotrichaceae	*Unclassified*	0.976	0.741		0.821	2.161	0.945	1.211	2.428
Erysipelotrichaceae	*Asteroleplasma*	0.371				1.096		0.302	0.893
Erysipelotrichaceae	*Anaerorhabdus*	0.438	0.421		0.483		0.253		0.241
Lachnospiraceae	*Clostridium*	0.248	2.841			0.227		0.369	
Lachnospiraceae	*Defluviitalea*	0.155	0.082				0.094		
Lachnospiraceae	*Roseburia*		0.958	0.248					0.539
Lachnospiraceae	*Ruminococcus*		0.415	0.482					
Paraprevotellaceae	*Paraprevotella*	1.961			2.263	0.923	1.267	2.949	1.023
Pasteurellaceae	*Actinobacillus*		0.023			0.078			
Peptostreptococcaceae	*Clostridium*	0.143	0.532				2.191		
Prevotellaceae	*Prevotella*	4.951	3.015	9.536	9.566	8.428	19.217	17.029	9.916
Ruminococcaceae	*Sporobacter*	2.617	1.25	2.48	4.346	7.694	2.783	5.897	6.109
Ruminococcaceae	*Clostridium*	4.247	8.514	4.111	5.255	4.127	3.036	5.178	4.275
Ruminococcaceae	*Papillibacter*	0.222	0.273	0.285	0.321	0.217	0.378		0.224
Ruminococcaceae	*Faecalibacterium*		0.303			1.303		1.147	0.791
Ruminococcaceae	*Butyricicoccus*		0.994	0.358		0.51	0.414		
Ruminococcaceae	*Gemmiger*	0.248						0.546	
Ruminococcaceae	*Bacteroides*	0.203	0.192	0.219					
Spirochaetaceae	*Treponema*	1.24				0.481			0.217
Veillonellaceae	*Selenomonas*					1.283			0.869
Veillonellaceae	*Succinispira*	0.529	0.462	0.575		0.485	0.349	0.37	

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Discussion

Fecal microbiota transplantation has proven beneficial in the treatment of human and animal viral diseases[25], chronic liver diseases[26], ulcerative colitis[27] and mainly, to fight multi resistant *Clostridium difficile* infections in humans[28,29]. FMT has confirmed the critical role that gut microbiota plays in early weaned-piglets in conferring diarrhea resistance[25,30], using not fully understood mechanisms[31,32].

Feeding animals with feces from the same or different species[33] has been a traditional fattening practice which vanished from industrial farming due to sanitary problems, especially when using feces from the same animal species. The diarrhea observed in the PF group might be due to the unwanted transplantation of enteric pathogens present in the transplanted material, despite being selected from healthy sows. *Clostridium perfingens* type B has been isolated from sick animals, albeit most probably, other enteric pathogens might be responsible of the diarrhea observed. The fact that WF and AWF groups did not show any morbidity when compared with PBS control pigs, seems to confirm the innocuous nature of warthog feces for pigs.

Weaning is one of the most delicate periods in the pig's life, when many stressful events occur contributing to intestinal and immune system dysfunctions that result in impaired pig health, reduced growth and feed intake, particularly during the first week after weaning[34]. Early intervention with FMT improved the maturation of the immune system alleviating weaning stress and reduced morbidity and mortality associated to porcine circovirus type 2 (PCV-2)[35]. Compared with weaned pigs from PBS group, WF animals showed a significant increase in their daily weight gain between 15 and 30 dpft. The PF group, however, rendered poor results compared to the rest of the groups, most probably due to accidental transplantation of specific swine enteric pathogens, as mentioned above. The prior use of antibiotics makes it difficult to compare AWF with the rest of the groups. Conversely to PBS, the increase on ADWG of WF became evident from 15dpft and not at early times, concurring with the early and transient peak of fever. Coinciding with the early RT increase, transplanted pigs showed higher percentages of monocytes in their blood at 4 dpft than those found in the PBS group (Supplementary Table S6), inverting this relationship by 15 dpft, most probably reflecting an early and transient inflammatory reaction, already described in the literature after fecal transplantation[36,37]. Conversely, the uniform and constant

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temperatures found between 15 and 30 dpft in transplanted pigs, independently of the feces origin (domestic pigs or warthogs), might reflect a synchronization in their circadian rhythms, as described in humans[38]. In this regard, pigs and warthog microbiota share with humans several common intestinal microbiota components, such as *Enterobacter aerogenes*, a bacteria that is sensitive to the pineal and gastrointestinal melatonin hormone, precisely working in circadian rhythms of 24h between the 26°C and 40°C[39]. As expected, PBS control group animals showed differences in their body temperatures, within the physiological range.

Despite the changes described herein, transplantation of warthog feces did not dramatically change the microbiota of the transplanted piglets, or at least, there is not any enrichment in the few taxa previously described as unique from the warthog species[14]. These results coincide with that described in many reports, dissociating beneficial effects of FMT from easily detectable changes in host microbiota29, allowing hypothesizing with a beneficial stimulation of the immune system. Comparing ASF clinical score, ASFV in serum and nasal shedding of pigs from PF and AWF groups, AWF controlled E75CV1 infection better than PF, except for AWF#44 that has an anomalous microbiota composition compared with the rest of the animals within the AWF group. However, animals from both groups showed similar humoral and cellular responses, at least measured by ELISA and IFNγ-ELISPOT, respectively. This result confirms, once more, these measures[40], as bad protection correlates (unfortunately, no correlates of ASF protection have been described so far), pointing towards more subtle differences in the innate/adaptive immune responses induced between both groups. In this regard, work performed in the last decades have allowed identifying T-cell responses as crucial in ASFV protection[20]. In particular, a direct correlation seems to exist between protection, Th1-like responses and the induction of specific CD8$^+$ T-cells (cytotoxic T-lymphocytes) against ASFV[40]. Experimental immunization with attenuated strains also shows that this inflammatory response comes together with the induction of regulatory T-cells that tightly controls any excessive inflammation and that this equilibrium dictates somehow the safety and the efficacy and long-term duration of the vaccine[20,41]. Interestingly, most of the bacteria specifically found in transplanted pigs at 15 dpft, just before ASFV challenge, have been associated with anti-inflammatory states. This is the case for *Faecalibacterium,* considered a constitutive marker of a healthy gut for human[42] and is associated with

anti-inflammatory properties[43]. Interestingly, *Faecalibacterium* was the core taxa for AWF pigs at 8 dpft and 15 dpft and the relative abundance of *Faecalibacterium* for AWF#44 was different compared with the rest pigs under the same treatment, perhaps contributing to its susceptibility to ASFV infection. *Roseburia*[44] is another member of the AWF core taxa at 15dpft, a bacteria capable to produce short-chain fatty acids (SCFA)[45-47], metabolic mediators balancing of inflammatory and anti-inflammatory T cell responses subsets and antimicrobial peptides (AMPs) production[48]. From the other genera found exclusively in the AWF core at 15 dpft, *Barnesiella*[49], has been directly involved in the reduction of the pathogenic vancomycin-resistant *Enterococcus* leading the microbiota reconstitution after FMT in humans. Interestingly, many of the genera found in the PF core at 15 dpft included genera that have different species commonly associated to swine diseases, such as *Campylobacter*[50], *Clostridium*[51] and *Actinobacillus*[52]. Together with the already-mentioned exclusively members, each one of the core genera found in at least in AWF, deserve further investigation to elucidate the potential role in ASF resistance, since the complexity of the whole microbial network and their interactions[53] may be essential to promote this effect.

Transplantation of microbiota components from one species to another is also a human ancient practice, since our species has been feeding with a complex community of bacteria composing the milk from diverse animals[54]. A more sophisticated and modern microbiota transplantation in humans is the intake of probiotics such as *Bifidobacterium* or *Lactobacillus* isolated from different animal species[55]. Considering this information, we propose identifying individual components of the warthog microbiota to characterize their protective potential against ASFV. If confirmed the benefits of their administration, we should be able to unravel the mechanisms of action and their potential future use in pigs as probiotics.

As described for humans, FMT improves intestine metabolism, epithelial barrier integrity[30], performance on suckling pigle[34], and mucosal immunity even in distal places[56,57]. In brief, the alpha diversity showed a significant increase at 15 dpft for AWF, suggesting a better gut health status in this group[15,24]. The increase in total IgA found in pigs transplanted with warthog feces support this observation. The fact that IgA and IgA+ plasma cells play key roles not only in mucosal immunity and gut microbiota composition[58], but also regulating the innate and adaptive T-cell immunity[59], could explain the protection afforded against E75CV1 infection. As an example, IgA+ cells

are capable of inducing the expression of IFNγ in a TNFα dependent manner, both cytokines known to play important roles in protection against ASFV[60]. More recent results obtained in the laboratory confirms that transplantation of warthog fecal microbiota components specifically stimulates mucosal immunity in the respiratory track (not shown). With this new evidence at hand, in the near future we plan to change our intramuscular ASFV-challenge model to an in-contact infection protocol, therefore increasing the options to control ASFV infection at the entry site, using either attenuated or virulent ASFV strains.

Methods

Animals and animal housing

Forty-eight piglets (Landrace x Large White) were acquired in a commercial farm that was negative for PRRSV, Aujezsky's disease virus, *Pasteurella multocida* and *Brachyspira hyodysenteriae*. At weaning, 3-week-old animals were vaccinated against PCV2 and *Mycoplasma hyopneumoniae* (Porcilis PCV M hyo, MSD Animal Health) and transferred to the animal facilities from the Servei de Granges i Camps Experimentals of the UAB. In these facilities, animals were separated in 4 groups of 6 piglets, placed in 4 independent boxes with individual ventilation and maintained in a 23-25ºC atmosphere. The experiment had a duration of 30 days and all personnel changed clothes, boots and gloves before entering in each box and handling the animals. Piglets were daily inspected for clinical signs. Water and feed were supplied *ad libitum*. A commercial feed for weaned piglets was provided (P-120, LA GIRONINA, Spain), with essential requirements of ZnO (110 mg/Kg) and without antibiotics.

Fecal microbiota transplantation

To prepare the FMT, fresh feces were collected from a colony of eleven 4-to-8 years-old warthogs (*Phacochoerus africanus*), from the Barcelona zoo. The colony was originated mating 2 sows with 2 different boars. Warthogs from the zoo were fed with commercial cereal-based feed complemented with apples, potatoes and carrots. No antibiotic treatment was used at least three months prior to the collection of feces. Five different feces droppings were collected in sterile containers from the pen ground within the hour after defecation and stored at 4ºC. Next, 12 g of feces per animal were immersed in 40 ml of buffer protective solution (PBS 2x, glycerol 15% and cysteine 0.1%), and stored at -80ºC.

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Fresh feces were also collected from 5 healthy domestic adult sows from a PRRS-negative PCV2-vaccinated commercial farm. Fecal collection, dilution and storage was done as previously described. Warthog and domestic pig feces were confirmed negative for PRRSV by using a commercial qRT-PCR system (LSI VetMAX PRRSV EU/NA 96 Real-Time PCR Kit, THERMOFISHER).

All the animals were orally inoculated during 3 consecutive days with freshly prepared fecal material processed as follows. Pools of feces from 5 warthogs and from 5 domestic pigs (2g/animal) were mixed in 40 ml sterile PBS using a commercial vortex machine (IUL) and maintained in special bag containers (stomacher lab system) until homogenized. The fecal slurry was filtered in sterilized gauze to remove larger particles and the filtered feces suspension was dispensed in 50 ml aliquots and kept at 4 ℃ until inoculation. Ten ml of PBS or freshly prepared feces resuspension were administered into the esophagus through the mouth, by using a 10 cm-long plastic cannula. One group was inoculated with PBS as a control (PBS), the second group was inoculated with domestic pig feces supernatant (pPF) and the third group was inoculated with warthog feces supernatant (pWF). One day before inoculation with warthog feces supernatant, a fourth group (AWF, #37-48) was orally given a dose of a cocktail of antibiotics composed of colistine (CEVA Sante animale; 700,000 UI/kg), neomicine (S.P. Veterinaria; 420 UI/Kg), bacitracine (Alpharma ZOETIS; 420 UI/kg), oxitetraciclina (MAYMO; 0.28 g/kg), and the following day they were inoculated with warthog feces supernatant during 3 consecutive days.

Fecal sample collection

Fecal samples were collected at 0, 8, 15 days post fecal microbiota transplantation (dpft) and kept at -80 °C for further processing. Pigs were weighted at 0, 15 and 30 dpft. Rectal temperature was recorded at 0, 4, 8, 15, 22 and 30 dpft. Blood samples were collected in EDTA tubes at 4 and 15 dpft for whole blood cell counting from all pigs. At 30 dpft, 6 animals per group were selected and moved to the BSL-3 facility to perform the ASFV challenge.

16S rRNA analysis from fecal samples

DNA was extracted from feces (300 mg per sample) collected from pools of warthog and domestic pig feces used as inoculum (pWF and pPF, respectively), and from feces collected from the WF group (#25, #28, #29, #30, #36) and PBS group (#1,

#3, #4, #9, #11) at 15 dpft. Moreover, DNA was extracted from feces collected on 0, 8 and 15 dpft for groups PF (#13, #16, #18, #22, #24) and AWF (#37, #41, #43, #44, #46). Briefly, frozen feces were suspended in 900 µl PBS by vortex. After centrifuging at 12,000 rpm/min for 10 min, 200 µl of the supernatant were submitted to genomic DNA extraction using Machinery Nigel Kit (GmbH & Co, Düren; Germany). Purified DNA was eluted in a final volume of 50 µl elution buffer. The quality and quantity of genomic DNA was evaluated on a BioDrop DUO (BioDrop Ltd).

The V3-V4 region of the 16S rRNA gene (~460 bp) was targeted[61] to perform amplification and sequencing using Illumina pair-end 2 x 250 bp sequencing with MiSeq, following the manufacturer instructions (MS-102-2003 Miseq Reagent Kit v2,500 cycle). Sequence reads were submitted to quality control using FastQC software[62]. The QIIME[63,64] software package (version 2019.10) was used to process the reads and infer the microbiota composition. Denoising and trimming was done with DADA2[65] under the default parameters to exclude both primers and low-quality reads from the sequences. Taxonomic classification was done with the machine learning Pyhton library scikit[64-66] using the pre-trained naïve Bayes classifier trained against Greengenes[67] (gg-13-8-99-nt-classifier) provided by qiime2 project (available at **https://docs.qiime2.org/**). The core-taxa was calculated with *in-house* scripts to find the taxa present in all animals from a group at a particular timepoint. Phylogeny was built aligning reads using MAFFT[68] masking reads to remove not-conserved positions and building a tree rooted with FastTree2[69]. Rarefaction was done to evaluate the depth of sampling. Alpha and beta-diversity metrics were calculated at maximum depth. Shannon[70] and Chao indexes[71] were estimated as measurements of the alpha diversity and richness of the samples, respectively. Alpha diversity between groups was compared through two-sample non-parametric t-tests (Monte Carlo method) at maximum depth in rarefied samples (with 999 permutations). Unifrac weighted and unweighted distances were calculated to assess differences across samples[72,73]. Principal coordinates analysis (PCoA) was done to visualize the distances or dissimilarities matrices. Venn graphs were done using Venn diagram software (available at http://bioinformatics.psb.ugent.be/webtools/Venn/).

Hematology

Blood collected in EDTA tubes was analyzed for whole blood count in the Servei

d'Hematologia Clínica Veterinària of the UAB (ADVIA 120, Siemens). Laboratory Reference Values were according to previous reports[74].

Histopathology

A total of fifteen pigs: four from the PBS group (#6, #8, #10, #12), five from the WF group (#26, #27, #31, #34, #35), three from the PF group (#14, #19, #20), and three from the AWF group (#39, #45, #47), were sacrificed 30 dpft for a comparative morphometric study using both small intestine and colon samples. The procedure followed is based on a methodology previously described[75] with slight modifications. Briefly, tissues were dehydrated and embedded in paraffin wax, sectioned at 3 µm, and stained with hematoxylin and eosin (HE). Villi height and crypt depth from ileum and crypt depth from colon were assessed on 10 well-oriented villi and crypts for each animal. Villus:crypt ratio was assessed by dividing villus height by crypt depth. Sections were analyzed under the light microscope in a blind-fashion manner by one only person.

Quantification of total IgA

The concentration of secretory IgA was measured in pig sera using the porcine ELISA kit following the manufacter's recommendations (E101-102; Bethyl Laboratories, Inc., Montgomery).

African swine fever virus experimental infection after FMT

At the end of the FMT experiment, 6 pigs from each group were selected for the ASF experimental infection and moved into a BSL-3 facility. After a three-day period of adaptation, pigs from PBS and WF were intramuscularly inoculated with the E75 virulent strain (10^4 HAU); while pigs from PF and AWF were intramuscularly injected with 100 HAU of the attenuated E75CV1 strain, aiming to facilitate the observation of any potential antiviral effect[20]. Water and feed were supplied *ad libitum* with a commercial feed for growing piglets (Feed N. 555 growing pigs, Corporación alimentaria Guissona S.A., Lleida, Spain), which contained ZnO (94 ppm/kg) as an addition.

Temperature and clinical signs were daily recorded. Clinical scores were calculated following a guide previously published by the group[76]. Serum and nasal swabs were taken at 0, 4, 7, 13 and 24 days post-infection (dpi). Serum were used for

checking specific antibody responses against ASFV by ELISA[20], serum and nasal swabs were checked by qPCR for the virus excretion[20]. PBMCs were extracted on 13 and 24 dpi for monitoring specific T‑cell responses by ELISPOT[20].

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS Inc, Chicago, USA). Differences in hematology, animal weight and daily weight gain during FMT were analyzed by one-way ANOVA.

The significant differences in alpha diversity were evaluated through Kruskal-Wallis test[77]. The percentage of variation between grouped samples was measured by R2, using Adonis function of the vegan package in R software[78]. Estimation of P values was done through Monte Carlo test with 999 random permutations of the data set. Permutational analysis of the variance (PERMANOVA) was performed to compare beta diversity matrices over the treatments under study with 999 permutations (*q2 diversity beta-group-significance*)[79]. To identify differentially abundant taxa from the microbiota from different groups and timepoints, analysis of composition of microbiomes (ANCOM)[80] was done using the *qiime composition ancom* plugin from QIIME.

When *P values* were $P < 0.05$ (*) or $P < 0.01$ (**) they were considered significantly different, $P < 0.005$ (***) was considered highly different, while $P < 0.10$ referred to a trend of showing statistically difference.

Ethics Statement

All experiments were performed in the Servei de Granges i Camps Experimentals of the Universitat Autònoma de Barcelona (UAB) and the Biosafety Level 3 facilities of the Centre de Recerca en Sanitat Animal (IRTA-CReSA, Barcelona). Animal care and experiments were performed in accordance to relevant guidelines and regulations, including the Good Experimental Practices (GEP) guidelines. All procedures were done under the approval of the Ethical and Animal Welfare Committee of the UAB (Permit Number: CEEAH 3166).

Data availability

The entire sequence dataset is available in the NCBI database, BioProject PRJNA625746 and BioSamples SAMN14608419 -14608463.

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Fecal microbiota transplantation from warthog to pig confirms the influence of the gut microbiota on African swine fever susceptibility.

Supplementary data

Study I--FMT influence on ASFV susceptibility

Supplementary Table S1. Number of sequences per sample obtained after filtering used for this study.

Sample ID (#animal_dpft)	Sequence Count
13D15	1,425
1D15	39,774
3D15	34,841
4D15	11,643
6D15	48,778
9D15	62,010
16D15	44,153
18D15	51,097
22D15	55,039
24D15	23,711
25D15	26,415
28D15	30,556
29D15	43,867
30D15	36,541
36D15	27,450
37D15	23,849
41D15	53,813
43D15	67,027
44D15	29,913
46D15	19,050

Sample ID (pools)	Sequence Count
pWF	81,614
pPF	53,689

Study I--FMT influence on ASFV susceptibility

Sample ID (#animal_dpft)	Sequence Count
13D0	47,310
16D0	50,364
18D0	24,635
22D0	39,719
24D0	45,343
37D0	38,801
41D0	46,035
43D0	43,323
44D0	30,032
46D0	38,076

Supplementary Table S1. Number of sequences per sample obtained after filtering used for this study (cont.).

Sample ID (#animal_dpft)	Sequence Count
13D8	25,989
16D8	44,081
18D8	32,428
22D8	40,595
24D8	15,640
37D8	19,232
41D8	38168
43D8	16,850
44D8	10,054
46D8	26,878

Supplementary Table S2. Relative abundance (%) of taxa assigned at different levels (Phyla, Family and Genera) for each animal sample and mean relative abundance (%) for each group of animals.

Kingdom	PF_1 5dpft					WF_1 5dpft						AWF_1 5dpft						PBS_ 15dpft					
	16	18	22	24	AVERAGE	25	28	29	30	36	AVERAGE	37	41	43	44	46	AVERAGE	1	3	4	9	11	AVERAGE
Bacteria Actinobacteria	0,000	0,149	0,220	0,169	0,134	0,000	0,000	0,217	0,000	0,204	0,084	0,117	0,000	0,179	0,057	0,037	0,078	0,000	0,000	0,000	0,000	0,103	0,021
Bacteria Bacteroidetes	18,590	17,825	24,712	29,147	22,568	26,163	15,038	30,996	17,952	21,322	22,294	26,399	15,649	20,486	41,484	29,291	26,662	20,395	16,128	12,282	19,267	14,780	16,570
Bacteria Chlamydiae	0,000	0,168	0,000	0,000	0,042	0,000	0,383	0,000	0,000	0,521	0,181	0,583	0,106	0,145	0,204	1,475	0,502	0,221	0,000	0,000	0,000	0,116	0,067
Bacteria Chloroflexi	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,016	0,003	0,000	0,000	0,000	0,000	0,000	0,000
Bacteria Cyanobacteria	0,283	0,618	0,325	0,198	0,356	0,000	0,121	0,552	0,501	0,197	0,274	0,021	0,136	0,275	0,000	0,100	0,106	0,128	0,218	0,000	0,935	0,137	0,284
Bacteria Deferribacteres	0,116	0,061	0,000	0,017	0,048	0,129	0,000	0,000	0,000	0,342	0,094	0,109	0,098	0,136	0,097	0,000	0,088	0,000	0,000	0,000	0,357	0,060	0,083
Bacteria Elusimicrobia	0,000	0,029	0,031	0,000	0,015	0,011	0,000	0,000	0,071	0,120	0,041	0,000	0,000	0,000	0,007	0,315	0,064	0,000	0,000	0,000	0,000	0,035	0,007
Archaea Euryarchaeota	0,000	0,000	0,124	0,257	0,095	0,000	0,000	0,000	0,000	0,058	0,012	0,294	0,000	0,069	0,057	0,278	0,139	0,000	0,000	0,000	0,000	0,024	0,005
Bacteria Fibrobacteres	0,000	0,029	0,000	0,101	0,033	0,000	0,000	0,098	0,000	0,000	0,020	0,000	0,279	0,064	0,000	0,152	0,099	0,000	0,000	0,000	0,000	0,189	0,038
Bacteria Firmicutes	69,123	70,624	55,619	50,673	61,510	61,064	83,178	60,932	49,646	63,333	63,631	52,891	70,446	66,267	48,130	54,908	58,528	65,309	73,029	76,226	52,015	57,191	64,754
Bacteria Fusobacteria	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Bacteria Lentisphaerae	0,358	0,131	0,104	0,000	0,148	1,049	0,000	0,506	0,213	0,244	0,402	0,084	0,210	0,149	0,000	0,110	0,111	0,000	0,583	0,000	0,154	0,000	0,147
Bacteria OD1	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Bacteria Planctomycetes	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Bacteria Proteobacteria	1,649	0,742	2,353	1,101	1,461	0,216	0,507	1,040	2,230	2,200	1,239	3,489	0,433	1,599	1,464	0,766	1,550	0,832	0,944	0,000	4,506	0,848	1,426
Bacteria Spirochaetes	1,619	0,613	1,446	2,029	1,427	0,541	0,000	0,513	0,364	0,066	0,297	2,252	1,646	1,513	1,936	2,835	2,036	0,028	0,057	0,000	0,119	0,703	0,181
Bacteria Synergistetes	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Bacteria Tenericutes	0,000	0,086	0,233	1,485	0,451	0,053	0,000	0,000	0,000	0,109	0,032	1,413	0,299	0,179	0,114	0,115	0,424	0,040	0,043	0,000	0,086	0,010	0,036
Bacteria TM7	0,231	0,646	0,603	0,789	0,567	0,000	0,000	0,000	0,000	0,761	0,152	0,138	0,983	0,315	0,528	0,667	0,526	0,644	0,373	0,000	0,310	0,221	0,309
Bacteria Unclassified	7,698	7,910	13,470	13,466	10,636	10,774	0,772	4,938	28,965	10,226	11,135	11,497	8,375	8,153	5,770	8,934	8,546	12,222	7,818	11,492	22,112	25,472	15,823
Bacteria Unclassified	0,152	0,110	0,420	0,333	0,254	0,000	0,000	0,210	0,057	0,295	0,112	0,021	0,544	0,136	0,154	0,000	0,171	0,181	0,772	0,000	0,139	0,077	0,234
Bacteria Verrucomicrobia	0,181	0,258	0,342	0,236	0,254	0,000	0,000	0,000	0,000	0,000	0,000	0,692	0,795	0,336	0,000	0,000	0,365	0,000	0,034	0,000	0,000	0,034	0,014

Supplementary Table S2. Relative abundance (%) of taxa assigned at different levels (Phyla, Family and Genera) for each animal sample and mean relative abundance (%) for each group of animals(cont.).

Study I--FMT influence on ASFV susceptibility

Supplementary Table S2. Relative abundance (%) of taxa assigned at different levels (Phyla, Family and Genera) for each animal sample and mean relative abundance (%) for each group of animals (cont.).

Study I--FMT influence on ASFV susceptibility

Supplementary Table S3.

Crypt depth and villi height in ileum by group (μm)

	PBS group		PF group		WF group		AWF group	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
Villi Height	344.250	17.115	334.333	10.793	336.200	8.861	334.333	12.426
Crypt Depth	217.750	7.574	221.667	12.309	223.400	7.807	224.000	10.368
V/C	1.649	0.111	1.662	0.111	1.603	0.073	1.580	0.087

V/C: Villi height/Crypt Depth ratio

Crypt depth in colon by group (μm)

	PBS group*		PF group *		WF group		AWF group	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
Crypt Depth	495	7.425	466.000	14.016	489.000	6.222	474.667	13.128

* *P* value (0.01-0.05)

Supplementary Table S4.

Score	Body condition		Behavior	Digestive signs	Respiratory signs	Other signs
	Vertebra	**Ribs**	**Signs**	**Signs**	**Signs**	**Dermatitis, testicular tummefaction, lameness**
0	Non visible	Non visible	Active, alert, plays	Perianal skin clean	Non apparent	Non apparent
1	Visible in the dorsum	Non visible	Sadness, quite	Perianal skin dirty	Mild dispnea	Mild
2	Prominent	Visible	Mild depression, alert	Hind limbs dirty	Evident dyspnea	Moderate
3	Very marked	Individually	Depression, stillness, postration	Red/blotchy skin lesions in the back	panting, difficult breathing	Severe

Study I--FMT influence on ASFV susceptibility

Supplementary Table S5. Core taxa list from different sampling times in PF and AWF groups and Venn diagrams representing the shared taxa.

Groups	total	family	Genera
PF_0dpft PF_8 dpft PF_15dpft	4	*Ruminococcaceae*	*Clostridium*
		Prevotellaceae	*Prevotella*
		Ruminococcaceae	*Sporobacter*
		Ruminococcaceae	*Papillibacter*
PF_0dpft PF_8dpft	1	*[Barnesiellaceae]*	*Barnesiella*
PF_0dpft PF_15dpft	3	*Veillonellaceae*	*Succinispira*
		Clostridiaceae	*Clostridium*
		Ruminococcaceae	*Butyricicoccus*
PF_15dpft PF_8dpft	2	*Erysipelotrichaceae*	*gut*
		Paraprevotellaceae	*Paraprevotella*
PF_0dpft	4	*Desulfovibrionaceae*	*Desulfovibrio*
		Ruminococcaceae	*Bacteroides*
		Lachnospiraceae	*Roseburia*
		Lachnospiraceae	*Ruminococcus*
PF_8dpft	1	*Erysipelotrichaceae*	*Anaerorhabdus*
PF_15dpft	7	*Spirochaetaceae*	*Treponema*
		Ruminococcaceae	*Faecalibacterium*
		Veillonellaceae	*Selenomonas*
		Pasteurellaceae	*Actinobacillus*
		Lachnospiraceae	*Clostridium*
		Campylobacteraceae	*Campylobacter*
		Erysipelotrichaceae	*Asteroleplasma*
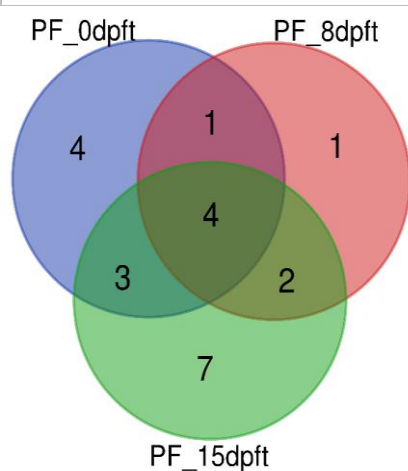
73

Supplementary Table S5. Core taxa list from different sampling times in PF and AWF groups and Venn diagrams representing the shared taxa (cont.).

Groups	total	Family	Genera
AWF_0 dpft AWF_8 dpft AWF_15 dpft	**6**	*Ruminococcaceae*	*Clostridium*
		Prevotellaceae	*Prevotella*
		[Barnesiellaceae]	*Barnesiella*
		Erysipelotrichaceae	*gut*
		Ruminococcaceae	*Sporobacter*
		Paraprevotellaceae	*Paraprevotella*
AWF_0 dpft AWF_8 dpft	**1**	*Veillonellaceae*	*Succinispira*
AWF_0 dpft AWF_15 dpft	**3**	*Erysipelotrichaceae*	*Anaerorhabdus*
		Clostridiaceae	*Clostridium*
		Ruminococcaceae	*Papillibacter*
AWF_8 dpft AWF_15 dpft	**2**	*Ruminococcaceae*	*Faecalibacterium*
		Erysipelotrichaceae	*Asteroleplasma*
AWF_0dpft	**3**	*Peptostreptococcaceae*	*Clostridium*
		Ruminococcaceae	*Butyricicoccus*
		Lachnospiraceae	*Defluviitalea*
AWF_8dpft	**2**	*Ruminococcaceae*	*Gemmiger*
		Lachnospiraceae	*Clostridium*
AWF_15dpft	**4**	*Desulfovibrionaceae*	*Desulfovibrio*
		Spirochaetaceae	*Treponema*
		Veillonellaceae	*Selenomonas*
		Lachnospiraceae	*Roseburia*
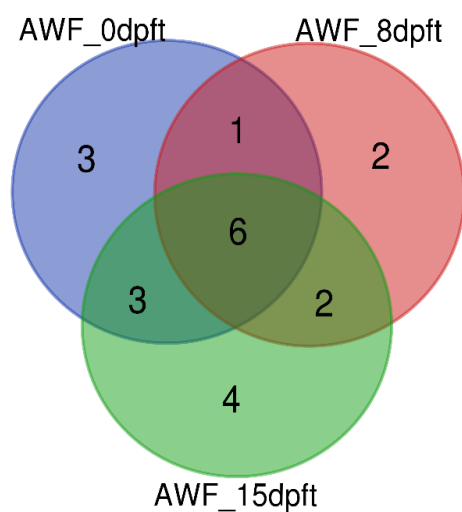
Study I--FMT influence on ASFV susceptibility

Supplementary Table S6. Average haematology parameters in pigs at 4 dpft and 15 dpft.

	4 dpft				15 dpft				Reference
	PBS	PF	WF	AWF	PBS	PF	WF	AWF	
WBC (x10E3)	21.46	22.89	24.22	23.25	24.93*	31.8	34.12*	29.8	11.0 - 22.0
RBC (x10E6)	5.93	5.79	5.92	5.64	6.45	5.99	6.17	5.84	6.8 -12.9
LYM (x10E3)	7.9	7.6	7.88	8.98	9.58	9.71	10.36	10.75	4.3 - 13.0
% LYM	43.27	40.1	37.25	39.88	40.91	33.41	34.53	37	
MONO (x10E3)	0.73	0.84	0.98	0.88	1.09	1.22	0.95	0.92	0.2 - 2.2
% MONO	3.7	3.84	4.54	3.93	4.54	3.83	3.13	3.15	
EOS (x10E3)	0.7	0.28	0.36	0.31	0.5	0.59	0.6	0.51	0.05 - 2.4
% EOS	3.88	1.38	1.48	1.34	2.06	1.93	1.78	1.76	
NEU (x10E3)	11.91	13.93	14.84	12.82	13.52*	20.06	22.00*	17.38	3.1 - 10.5
%NEU	48.14	53.48	56	53.68	51.47	60	59.85	57.26	
BASO (x10E3)	0.04	0.05	0.04	0.05	0.07	0.06	0.06	0.05	
% BASO	0.22	0.22	0.19	0.21	0.28	0.21	0.21	0.17	

*P value < 0.05. PBS: non-transplanted group; PF: pig feces group; WF: warthog feces group; AWF: antibiotic + warthog feces group.

Study I--FMT influence on ASFV susceptibility

Chapter 4

Study II

Microbicidal and immunostimulatory capabilities of
isolated bacteria from warthog fecal microbiota.

(*Manuscript in preparation*)

Study II—Microbicidal and immunostimulatory capabilities of BFW

Abstract

African warthogs (*Phacochoerus africanus*) act as African swine fever virus (ASFV) reservoirs in the wild without apparent signs of disease. This virus is the causative agent for a deadly disease of pigs, African swine fever (ASF). Fecal microbiota transplantation from warthogs to domestic pigs could partially protect them from ASF in an experimental infection with E75CV1, a live attenuated virus, while FMT with pig feces does not. Intestinal bacteria and their metabolic products play important roles in maintaining local and systemic homeostasis, through many different mechanisms, including pathogen inhibition and immune host regulation. Here, we isolated and identified bacteria from warthog feces and investigated both their microbicidal potential *in vitro* and their immunostimulatory capabilities *in vivo* using weaned piglets and the ASFV infection model. A group of bacteria isolated from warthog fecal microbiota in aerobic and anaerobic conditions were classified attending to their 16S rRNA and then, they were selected based on the evolutionary distance to the ones isolated from pigs; they also demonstrated lack of cytotoxicity on ileum and colon organoid cells. Next, their microbicidal properties were tested *in vitro* using a panel of pathogenic bacteria, including *Clostridium perfringens* (type B), S*treptococcus suis* S10, *Escherichia coli* K88*, Salmonella enterica*, *Salmonella enterica* serovar *Typhimurium* monophasic variant (antigenic formula 4,12:i:-), and a nonpathogenic S*treptococcus suis* T15. Fifteen bacteria from warthog microbiota were finally selected to be characterized regarding the *in vitro* and *in vivo* stimulatory capabilities, the later using weaned piglets. Interestingly, GALT cells specifically secreted IFNγ in response to one of the selected bacteria. The immunostimulatory capabilities showed by warthog feces bacteria *in vitro* correlated *in vivo* with a higher average weight gain in the inoculated pigs and an enhancement in the total IgA found in thesera. Moreover, inoculated pigs showed an enhancement in the mucosal immunity induced against ASFV, detectable form day 14 after intramuscular challenge with E75CV1, an attenuated strain of ASFV. The observed microbicidal and immunostimulatory capability of particular isolates from warthog microbiota opens promising expectations for their future usage as potential probiotics.

Key words: warthog, microbiome, microbicidal, probiotics, immunostimulatory capability, ASFV

Introduction

African Swine Fever (ASF) is a devastating disease of domestic pigs and wild boars, caused by African swine fever virus (ASFV). ASF is a notifiable disease to the World Organization for Animal health (OIE)[1], and today it is considered the most serious constraint for pig production. The current distribution of ASF extends across more than 60 countries from African, Asian and European continents and more recently, also from Oceania[2]. African warthogs (*Phacochoerus africanus*) act as ASFV reservoirs in the wild without apparent signs of disease[3]. Conversely, domestic pigs infected with ASFV develop a disease, that depending on the viral isolate, ranges from chronic or subclinical to subacute and hyper-acute[4], resulting the latter in up to 100% mortality in naïve pigs[5]. The mechanisms of ASF-resistance showed by warthogs has not yet been elucidated, albeit both genetic[6] and environmental factors[7,8] could be involved. Recent experiments performed in our lab proved that fecal microbiota transplantation (FMT) from warthog could partially protect domestic weaned pigs from experimental infection with E75CV1, a live attenuated virus (LAV), while FMT with pig feces did not[9]. Microorganisms residing in the digestive tract contribute not only to food digestion, but also play important roles in maintaining the intestinal homeostasis including education of the immune system, improving gut immune barrier function, thus protecting the host from pathogenic microorganisms[10,11]. However, the microbiota components and the mechanisms involved in the protection afforded are unknown. Thanks to anaerobic cultivation techniques developed by Hungate[12] and the incorporation of molecular techniques about a dozen years ago, bacteria have been isolated and identified from different organs, including the gastrointestinal tract of humans, pigs and other animals and even from the environment[13,14], despite less than 1% of environmental bacterial species are considered cultivable using standard methods. Although several efforts have been dedicated to culture all the bacteria from a niche, not all gastrointestinal bacteria can be cultured, even with alternative and creative cultivation approaches[15], complicating the complete understanding of the microbiota-host interactions.

In the present work, we isolated spore-forming and non-spore-forming bacteria from warthog feces in both aerobic and non-strict anaerobic conditions. Bacteria isolated from pig or warthog feces were identified by sequencing their 16S rRNA and their relationship was assessed through phylogenetic analysis. We selected 21

representative bacteria isolated from warthogs that clustered apart from the pig isolates for further studies. The cytotoxicity of these bacteria was evaluated on ileum and colon organoid cells. Moreover, their microbicidal properties were studied using a panel of bacterial strains: *Clostridium perfringens* type B (*C.perfringens*)*, Streptococcus suis* (S10 and T15) (*S.suis* S10, *S.suis* T15) *Escherichia coli* (*E.coli*) K88*, Salmonella enterica* (*S.enterica*) and *Salmonella enterica* serovar *Typhimurium* monophasic variant (monophasic *S. Typhimurium*) (antigenic formula 4,12:i:-). Additionally, their immunostimulatory potential was also studied *in vitro*. From all these results, 15 bacteria were selected for *in vivo* inoculation of weaned pigs. Interestingly, the average weight gain (AWG) was higher for inoculated pigs than for control animals, coinciding with the fact that inoculated pigs showed an enhancement in the mucosal immunity induced, showing an enhancement in both the total IgA present in the sera of inoculated pigs and the ASFV specific IgA induction upon ASFV-challenge. The potentiality of these and alternative warthog gut microbiota components will be here discussed.

Material and methods

Animals and animal safety

Twenty three-week-old weaned male pigs (Landrace x Large White), negative for PRRSV, Aujezsky's disease virus, *Pasteurella multocida* and *Brachyspira hyodysenteriae,* and vaccinated against porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* (Porcilis® PCV Mhyo, MSD Animal Health), were transferred to the BSL3 facilities from CReSA and hosted in one box divided in two identical spaces by a double fence. Water and feed were supplied *ad libitum.* Animal care and procedures were carried out in accordance with the guidelines of the Good Experimental Practices and under the supervision of the Ethical and Animal Welfare Committee of the Institut de Recerca en Tecnologia Agropecuaria-IRTA (Spain).

Fecal sampling and processing

Fecal sampling was performed as recently described[9]. Briefly, five different feces drops were freshly collected from the warthogs' (*Phacochoerus africanus*) pen ground of Barcelona Zoo, hosting eleven 8-10 years-old animals. Fecal material from five individual domestic pigs (Landrace x Large White) were additionally taken. Feces were collected in sterile containers, transported to the laboratory in non-strict anaerobic

conditions (GENbox, Biomerieux) and immediately processed. Fecal processing was performed as previously described (Zhang *et al.*, 2020) and diluted in aerobic dilution (PBS 1X) from 10^{-1} to 10^{-9}, and 200 µl-aliquots from each dilution were plated in both BHI (Brain Heart Infusion) and polymyxin-BHI agar plates (35 or 70 ng/µl). For isolating the spore-forming bacteria, a heat-shock treatment was done to both kill vegetative bacteria and stimulate the spore germination at the same time, replicates were incubated in aerobic and anaerobic condition at 37℃, respectively for up to 72 h. Polymyxin was added to isolate gram positive bacteria[16].

Isolation and identification of bacteria

Colonies were re-plated in new BHI agar plates until pure colonies were obtained, being named according to their origin: warthogs (W) or pigs (P), followed by aerobic (AE) or anaerobic (AN) referring to the isolation condition. If requiring additional heat-shock treatment, bacteria were named according to their origin followed by SA (shock aerobic) or SN (shock anaerobic).

Each pure isolation was finally suspended in 500 µl sterile PBS and kept at 20℃ until DNA extraction. Bacterial genomic DNA was extracted from 250 µl bacterial PBS suspensions using Chelex based Instagene™ Matrix (Bio-Rad) following manufacturer's instructions. Preliminary identification of isolates was performed by partial sequencing of the 16S rRNA gene using primers 358F (5'- CTACGGG AGGCAGCAGT-3') and 907R (5'- CCGTCWATTCMTTTGAGTTT-3')[17]. Sequences were analyzed by blasting against the Ribosomal Database (http://rdp.cme.msu.edu). Isolates identified from same genera were genotyped by ERIC-PCR[18] with primers ERIC-1F (5'- ATGTAAGCTC CTGGGGATTCA*C*-3') and ERIC-2R (5'- AAGTAAGTGACTGGG GTGAGCG-3')[19]. One isolate from each fingerprinting profile was selected for further analysis. Finally, the isolates were identified by sequencing the full-length 16S rRNA gene. This gene was amplified using universal primers 27F (5'- AGAGTT TGATCCTGGCTCAG -3') and 1492R (5'- CGGTTACCTTGTTACGACTT -3')[20] and subjected to Sanger sequencing (ABI 3730XL, Macrogen Europe). The chromatograms were inspected and trimmed of low-quality sequences (Q < 40) with Finch TV. Then, the consensus sequences were obtained by aligning forward and reverse reads with ClustalW software. The consensus sequences were classified using RDP classifier (v2.11)[21] and Bayesian Lowest

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Common Ancestor (BLCA)[22].

A maximum likelihood (ML) tree was obtained using the substitution model test with the lowest BIC score (MEGAX)[23,24]. The Kimura 2-parameter with a discrete Gamma distribution was used to model evolutionary rate differences among sites and construct the phylogenetic tree with bootstrapping (1000). The phylogenetic tree was further edited using iTol software (vs 5.6.3)[25–28].

WST-1 cytotoxicity assay on ileum and colon organoids

WST-1 cytotoxicity assay is an efficient test for cell viability measurement, that relies on the cellular reduction of tetrazolium salts to their formazan products[29]. The total amount of formazan produced is directly proportional to the number of viable cells in the culture[30]. Here, we examined the viability of organoids cells when stimulated with bacteria culture supernatant.

Ileum and colon organoids plated in fresh Matrigel matrix droplets and grown in basal culture medium were generated from pig intestinal tissue, as described before[31]. Briefly, the intestinal crypts from fresh intestinal sections were suspended in Matrigel, which was diluted 0.5 volumes with basic culture medium and dispensed as small hemispherical droplets in 24-well plates. After the polymerization of 37°C of Matrigel, $600 \, \mu l$ basic culture medium was added per well and incubated at 37°C. Spherical ileum organoids were treated with ice-cold DMEM/F12 medium firstly, and then incubated in Tryple Express dissociation medium (Gibco) for 10 min at 37°C. Single cell suspensions were finally seeded at a concentration of 78,125 cells/cm^2 in culture medium with 20% FBS. After 3-5 days incubation, cells reached 90% confluence, and are ready for the WST-1 cytotoxicity assay. Each isolate was anaerobically grown at 37°C in YCFAwo (YCFA medium without addition the short fatty acids), and harvested when the optical density ($OD_{600 \, nm}$) reached 0.8-1.0. Bacteria culture was next centrifuged at 3000 g for 10 mins, the supernatant filtered by 0.45 μm filter. One hundred microliter of 10% diluted supernatants (v/v, bacteria supernatant/ basal culture medium) per well were added in each 96 well-plate. Organoids with stimulations were cultured at 37°C (5% CO2) for 1 hour before adding Cell Proliferation Reagent WST-1 (10 μl /well). $OD_{460/655 \, nm}$ value was measured immediately after adding the WST-1 reagent. Cells continued growing for two extra hours at 37°C (5% CO2), and $OD_{460/655 \, nm}$ value was measured every 30 min during this 2h.

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Microbial inhibition i*n vitro* assay

The microbial inhibition assay was conducted against the following bacteria originated from pig: *C.perfringens* type B, *S.enterica*, monophasic *S. Typhimurium* , *E.coli* K88, *S.suis* S10 (highly virulent strain) and *S.suis* T15 (avirulent strain). For the inhibition test, *C. perfringens* was cultured under anaerobic conditions in BHI agar plates at 37ºC overnight (O/N). *S. enterica*, monophasic *S. Typhimurium* and *E. coli* K88 were cultured in BHI agar plates O/N at 37ºC, under both aerobic and anaerobic conditions. For *S. suis* S10 and *S. suis* T15, chocolate agar plates were used under aerobic conditions at 37°C, 5% CO2 O/N. The microbial inhibition assay was done using swabs fully soaked in suspensions of the corresponding pathogenic bacteria (density of 0.5 McFarland, in PBS 1X) and plated homogenously within 9 cm^2 plates. Next, a 10 µl droplet of individually isolated bacteria from warthog fecal microbiota (density of 1.0 McFarland, in PBS 1X), was softly added over the monolayer. *In vitro* growth of pathogenic bacteria was compared with control plates. Isolates showing specific inhibition results were subjected to a second assay to confirm their inhibition capacity.

Immunostimulatory ability of different bacteria on gut-associated lymphoid tissue cells

The gut-associated lymphoid tissue (GALT) cells were isolated as previously described[32]. Briefly, the muscularis external was first removed with scissors and once mucus was removed with continuous shaking, the mucosa and submucosa were treated four times at 37℃ for 10 min in Ca^{2+} and Mg^{2+}-free HBSS (1% Pen/Strep and 5 mM EDTA) with continuous shaking to remove the epithelial cells. Finally, tissues were incubated a 37℃ for 45 min with 30 µg/ml DNase and 5 mg/ml collagenase D in RPMI with continuous shaking, vortexed for 30s, and filtered with 70 µm filter. The filtered liquid was centrifuged 400 g for 10 min at 4℃ to collect the GALT cells. GALT cells seeded in 4*10^6 cells in each well (24-well plate) were stimulated for 24 h with fresh cultured bacteria, at a density of 100:1 (bacteria: cell ratio). Plates were centrifuged at 3000g for 20 min and supernatants were recovered to detect the presence of different cytokines by ELISA: Interleukin (IL)6 (Catalog # DIY0727S-003, Kingfisher Biotech), IL10 (Catalog # DIY0744S-003, Kingfisher Biotech), IL17 (Catalog # DIY0730S-003, Kingfisher Biotech), IFNα (Catalog # DIY0724S-003, Kingfisher Biotech), interferon

(IFN)γ (Catalog # DIY0725S-003, Kingfisher Biotech), tumor necrosis-factor (TNF)α (Catalog # ES24RB, Invitrogen), transforming growth factor (TGF)β (Catalog # 162501/A, Invitrogen), IL8 (Catalog # AR0658-005, Kingfisher Biotech) and IL18 (Catalog # BMS672, Invitrogen).

Inoculation of bacteria from warthog feces in pigs

Fifteen bacterial strains isolated from warthog feces were selected for *in vivo* inoculation: WAE1, WAN5, WAE21, WAE28, WSA48, WSN45, WSN48, WAN31, WAE29, WAE30, WAE46, WAE49, WAN26, WAN32 and WAN43.

Fresh bacteria were cultured as above described and 10^9 CFU (colony forming unit) of each isolate in a total volume of 10 ml of PBS was inoculated into the esophagus of each piglet (21 days after birth) with the help of a 10 cm-long catheter. The procedure of inoculation of bacteria from warthog (BFW) was done for 5 consecutive days using freshly prepared material, using a total of 10 pigs (#11-20, BFW group). An additional control group with 10 pigs was inoculated with PBS (#1-10, PBS group). Rectal temperature was recorded, and pigs were weighted at 0, 8, 15, 22 and 28 dpi (days post inoculation).

African swine fever virus experimental infection with the attenuated E75CV1 ASFV strain

At 28 dpi, 7 out of ten animals per group were randomly selected for intramuscular challenge with 1,000 HAU of E75CV1, an attenuated ASFV strain, following previously described protocols[33]. Temperature and clinical signs were recorded daily. Serum and nasal swabs were taken at 0, 4, 7, 13 and 21 dpc (days post challenge). Specific antibody responses against ASFV were tested by ELISA[33] with serum, nasal swabs and feces. For IgG ELISA, the dilution of sera was as before 1:1000; for IgA ELISA for ASFV, the dilution of sera was 1:100, dilution of nasal swabs and feces was 1:10. Serum and nasal swabs were used to quantify ASFV by qPCR[33]. PBMCs were extracted at 21 dpc to monitor specific T‑cell responses by ELISPOT[33] as previously described. Sera from 0 dpc (28 dpi) were also used for checking the total IgA with the dilution 1:10 000. Total sera IgA was measured as recommended by the manufacturer (Bethyl).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS Inc, Chicago, USA). Differences in animal weight and daily weight gain were analyzed by one-way ANOVA. The differences observed were considered as highly different ($P < 0.005***$), significantly different ($P < 0.01**$), statistically different ($P < 0.05*$) and trending statistically differences ($P < 0.10$).

The standard curve for total sera IgA was generated with 4-Parameter Logistic Regress curve fit using one online software (https://elisaanalysis.com/). The formula is $y= d+(a-d)/(1+(x/c)^b)$; a= 1.221; b= -1.163; c= 483.419; d= 0.051.

Results

Phylogenetic analysis of 16S rDNA gene from bacteria isolated from warthog feces

We isolated 148 phenotypically different bacterial colonies from warthog (118) and pig (30) feces. The genomic DNA of all of the isolates was extracted and the 16S rRNA was amplified. Further analysis performed on isolates from same genus, allowed identifying identical ERIC-PCR profiles (data not shown) for 13 isolates from warthog, therefore only 135 of the 148 colonies available were used for further analysis. These 135 isolates were classified in 24 different genera: 19 for the warthog feces bacteria and 14 for pig microbiota (Table 1). The most abundant genera isolated from both animal species was *Bacillus,* accounting for 43 (40.9 %) and 10 (33.3 %) of the bacterial isolates from warthog and pig feces, respectively. Together with *Bacillus,* another 7 genera were identified in feces of both animal species, i.e. *Streptococcus*, *Clostridium*, *Acinetobacter*, *Escherichia*, *Eubacterium/Clostridium*, *Lysinibacillus*, *Painebacillus* and *Staphylococcus*. Ten genera were only isolated from warthog feces belonging to *Arthrobacter*, *Actinobacillus*, *Bhargavaea*, *Enterococcus*, *Lactobacillus*, *Pasteurella/Haemophilus*, *Rothia*, *Paeniclostridium*, *Terribacillusk* and *Turicibacter*. On the other hand, five genera, *Jeotqalicoccus*, *Psychrobacillus*, *Sharpea/Bacterium*, *Sporosarcina* and *Ornithinibacillus* were exclusively found in pig feces.

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Table 1. Summary of taxa predicted by 16s DNA of isolations from pig and warthog feces

Predicted genus	Warthog feces	Percentage (%)	Pig feces	Percentage (%)
Acinetobacter	1	0.95	1	3.33
Arthrobacter	1	0.95	0	0.00
Bacillus	43	40.95	10	33.33
Bhargavaea	1	0.95	0	0.00
Clostridium	10	9.52	2	6.67
Enterococcus	4	3.81	0	0.00
Escherichia	8	7.62	3	10.00
Lactobacillus	1	0.95	0	0.00
Eubacterium/Clostridium	1	0.95	2	6.67
Lysinibacillus	1	0.95	1	3.33
Paeniclostridium	1	0.95	0	0.00
Painebacillus	1	0.95	1	3.33
Pasteurella/Haemophilus	1	0.95	0	0.00
Rothia	1	0.95	0	0.00
Staphylococcus	9	8.57	2	6.67
Streptococcus	12	11.43	3	10.00
Terribacillus	6	5.71	0	0.00
Turicibacter	1	0.95	0	0.00
Actinobacillus	2	1.90	0	0.00
Jeotqalicoccus	0	0	1	3.33
Ornithinibacillus	0	0	1	3.33
Psychrobacillus	0	0	1	3.33
Sharpea/Bacterium	0	0	1	3.33
Sporosarcina	0	0	1	3.33
Total	105		30	

The 16s rRNA gene of these isolates was used to build a phylogenetic tree in order to understand the genetic relationship among the isolates (Figure 1). To identify potential specific bacteria from ASF-resistant warthog microbiota, we excluded all the bacteria belonging to a cluster shared with isolates from pig microbiota. Mostly, we selected those bacteria coming from warthog that conform a unique cluster in the tree. But, when multiple bacteria from warthog microbiota clustered together, only one or
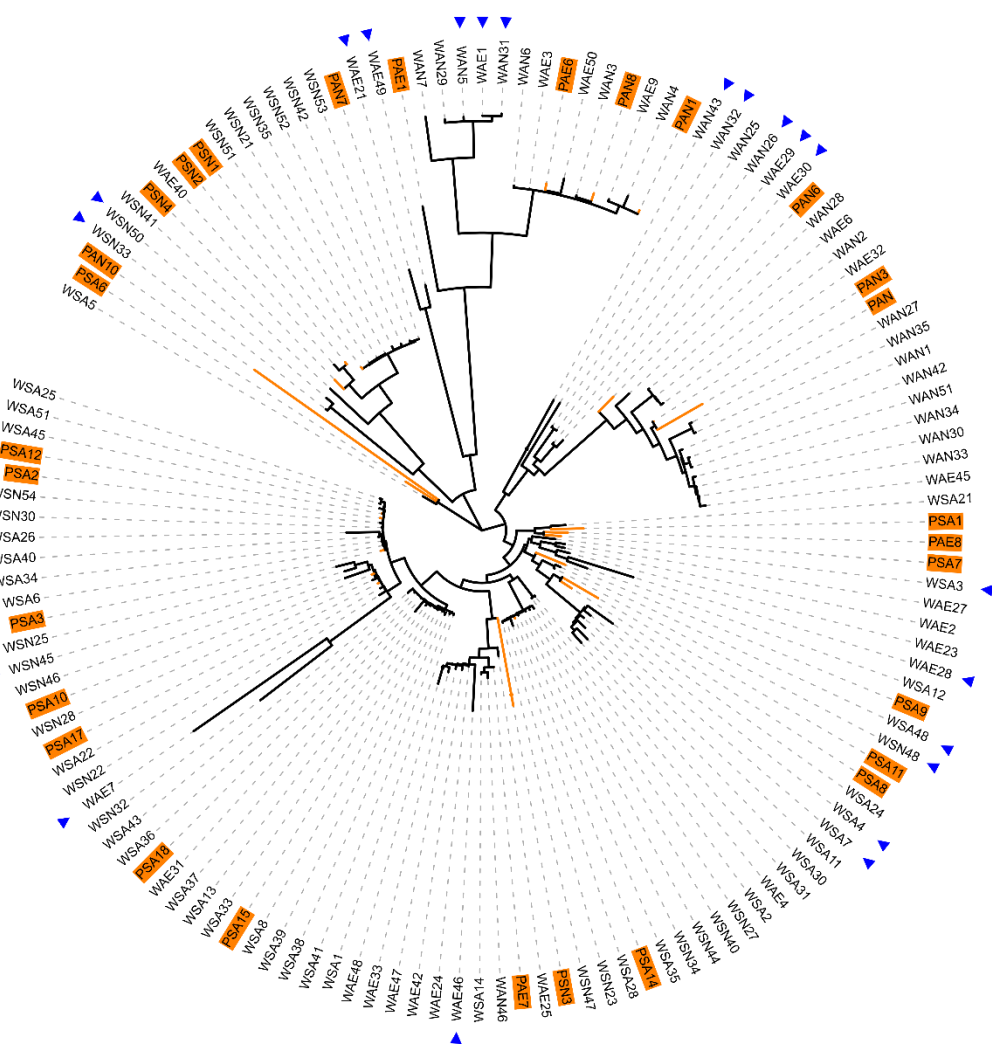
Figure 1. Phylogenetic relationship among isolates from warthog and pig feces based on the 16S rRNA gene sequences. The phylogenetic tree was constructed using the Maximum Likelihood method and Kimura 2-parameter model with a discrete Gamma distribution at 1,000 bootstrap. Circles in branches represent bootstrap values with sizes proportional to the bootstrap value (only values higher than 0.5 are shown). The analyses were conducted in MEGA X. The isolates isolated from pig feces are labeled in orange, while the selected isolates from warthog' microbiota are indicated with a blue triangle.

two were picked for further analysis. Following this strategy, we selected 21 strains from 10 different genera, where six strains were classified as *Bacillus* (WAE28, WAE7, WSA48, WSA3, WSN48, WSN45), three where from *Enterococcus* genus (WAE29, WAE30, WAN26), two from *Clostridium* genus (WSN33, WSN50), three from *Actinobacillus* genus (WAE1,WAN5 and WAN31), one from *Staphylococcus* genus (WAE46), two from *Terribacillus* genus (WSA7, WSA4), one strain belonged to *Arthrobacter* genus (WAE21), one to *Rothia* genus (WAE49), one to *Lactobacillus/Bacterium* genus (WAN32) and one strain was classified as *Turicibacter* genus (WAN43).

In vitro characterization of bacteria isolated from warthog feces

With the exception of five bacterial strains (WAE7, WSA3, WSA4, WSA7 and WAE49), which failed to grow in YCFAwo using strict anaerobic condition, all selected bacteria were capable to grow in this medium. Aiming to evaluate their *in vitro* viability, intestinal organoid cells were cultured in the absence or presence of supernatants from isolated bacteria, individually grown in anaerobic conditions.

Regarding the supernatant from individual bacteria isolated from warthog fecal microbiota, WAE1, WAE29, WAE30, WAN26, WAN5, WSN50 and WSA48 did not show any toxicity effect on ileum cells, while WAE21, WAE28, WAN31, WAN43, WAN32, WSN48 and WSN45 showed beneficial growing effects on ileum cells (Figure 2a). WAE28 showed stimulatory capabilities on ileum organoid cells similar to that detected for pig feces. WSN45 was the only bacteria capable to benefit also the growth of colon organoid cells (Figure 2b). Unfortunately, WAE46 and WSN33 could not be tested in the WST assay, because of a contamination problem of organoid later happened.
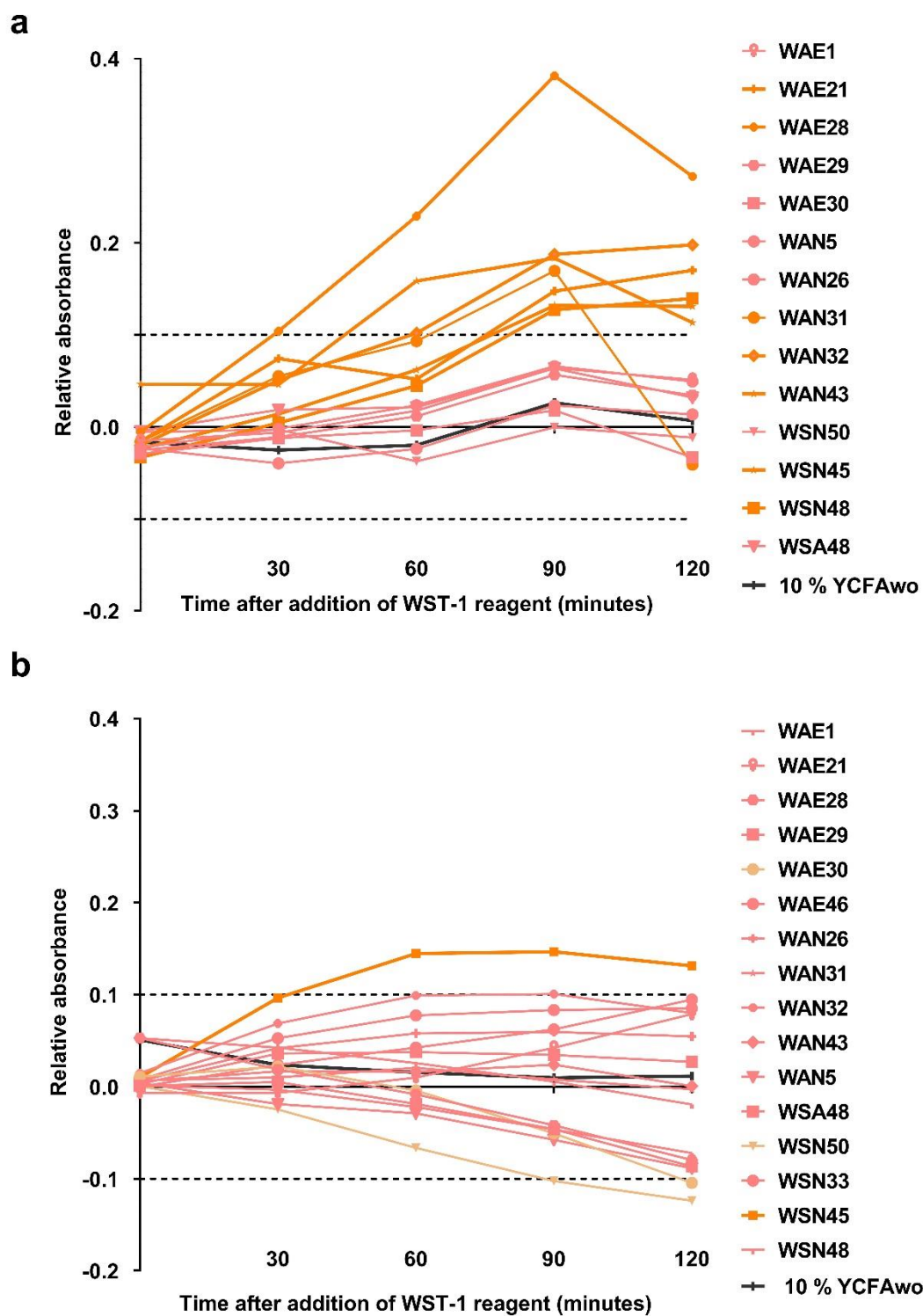
Figure 2. Growth activity of ileum (a) and colon (b) organoid cells measured by WST-1 cytotoxicity assay. Brown lines correspond to those isolates positively affecting the *in vitro* growth of organoid cells. Pink lines correspond to those isolates nontoxic to *in vitro* growth of organoid cells.

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To determine the microbicidal capacity of the 21 selected bacteria, we evaluated the growth in agar plates pre-seeded with each one of the following pathogenic bacteria: *C. perfringens* (Type B), *E. coli* K88, *S. enterica*, monophasic *S. Typhimurium*, *S. suis* S10 and *S. suis* T15 (as a control for S10). Since the strain WAN32 had very slow growth on BHI agar medium, it was not included in this assay. The inhibition effect on each pathogen was tested for each isolate (Table 2). Seven out of the 20 bacterial isolates inhibited *C. perfringens* Type B strain (Figure 3), with three of them showing high inhibitory capacity (WAE1, WAN5 and WAN31) (Figure 3). Interestingly, WAN31 inhibited pathogenic *C. perfringens* only, while two other members from *Actinobacillus* (WAE1 and WAN5) inhibited both *C. perfringens* and pathogenic *Salmonella*. We found two additional strains inhibiting only *C. perfringens* with low efficacy (WAE30 and WAE46), while two other strains (WAE7 and WAN26) showed intermediate inhibitory capacity and WAN26 also inhibited the growth of pathogenic *S. suis in vitro*. The ability to inhibit type B pathogenic *C. perfringens* was not limited to one genus, but five additional ones: *Clostridium*, *Enterococcus*, *Staphylococcus*, *Actinobacillus* and *Bacillus* (Table 2). Besides from WAN26, four additional bacteria inhibited pathogenic *S. suis*, which were WSA48 strain (*Bacillus* genus), that interestingly only inhibited the highly virulent *S. suis* S10 strain, and WAE49 (*Rothia*), WSA3 (*Bacillus*) and WSA4 strain (*Terribacillus*), that were three of the bacteria that did not survive in YCFAwo medium in strict anaerobic chamber.

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Table 2: Microbicidal capability and cytotoxicity to intestine organoid cells of 21 warthog isolations

GenusPredicted by 16S rRNA sequencing	Isolate name	*S. suis S10*	*S. suis T15*	*C. perfringens*	*S. enterica*	*monophasic S. typhimurium*	*E. coli K88*
Actinobacillus	WAE1	/	/	Y ++++	Y	Y	/
Arthrobacter	WAE21	/	/	/	/	/	/
Bacillus	WAE28	/	/	/	/	/	/
Enterococcus	WAE29	/	/	/	/	/	/
Enterococcus	WAE30	/	/	Y	/	/	/
Staphylococcus	WAE46	/	/	Y	/	/	/
Enterococcus	WAN26	Y+	Y	Y++	/	/	/
Actinobacillus	WAN31	/	/	Y++++	/	/	/
Lactobacillus	WAN32	/	/	/	/	/	/
Turicibacter	WAN43	/	/	/	/	/	/
Actinobacillus	WAN5	/	/	Y++++	Y	Y	/
Bacillus	WSA48	Y	/	/	/	/	/
Bacillus	WSN45	/	/	/	/	/	/
Bacillus	WSN48	/	/	/	/	/	/
Clostridium	WSN33	/	/	/	/	/	/
Paeniclostridium	WSN50	/	/	/	/	/	/
Rothia	WAE49	Y+	Y+	/	/	/	/
Bacillus	WAE7	/	/	Y++	/	/	/
Bacillus	WSA3	Y+	Y	/	/	/	/
Terribacillus	WSA4	Y+	Y+	/	/	/	/
Terribacillus /Salinibacillus	WSA7	/	/	/	/	/	/

Based on the radiometer of the inhibition zone, the effect of inhibition was quantified as follows: "Y++++" represents very strong inhibition, >0.5 cm inhibition halo. "Y+++" represents strong inhibition. "Y++" represents mild inhibition, (0.1-0.3 cm). "Y+" represents light inhibition, corresponding with a thin inhibition halo around the microbiota drop. "Y" represents only inhibition where the microbiota drop drops. "/" represent no inhibition halo was found. "NT": Not tested.
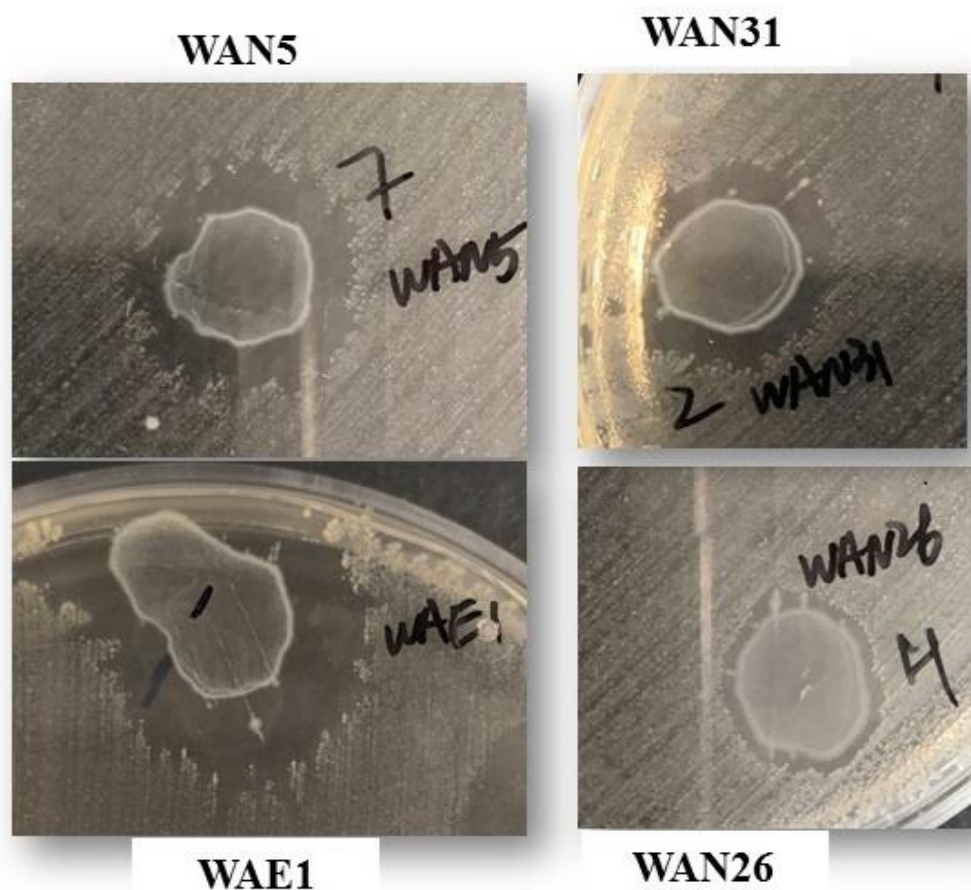
Figure 3, Warthog microbiota bacteria isolates inhibit the *in vitro* growth of *C. perfringens*. Representative illustration showing the inhibition "halo" observed after *C.perfringens* co-cultivation with strong inhibitory isolates WAE1, WAN5 and WAN31 or with WAN26, warthog bacteria isolate that shows moderate inhibition effect.

To evaluate the immunostimulatory capacity of the selected bacteria, pig cells isolated from GALTs were stimulated for 24h with live bacteria and a panel of cytokine production was measured. Thus, from all the bacteria tested, only WAE49 was capable to specifically stimulate the induction of IFNγ, when incubated with cell preparations from different lymphoid tissues *in vitro* (Table 3). Conversely, no IL6, IL8, IL10, IL17, IL18, IFNα, TNFα and TGFβ, was found in the supernatants of cells after stimulation with WAE49 and no other bacteria induced any significant stimulation of the cytokines measured.

Table 3. Cytokines induction from GALT cells after stimulation with the indicated warthog fecal bacteria (grouped by genus)

Genus	Isolate	IL6	IL10	IL17	IFNα	IFNγ	TNFα	TGFβ	IL8	IL18
Actinobacillus	WAE1, WAN5	0.163	0.217	0.142	0.139	0.252	0.096	1.928	0.239	0.279
Arthribacter	WAE21	0.387	0.433	0.368	0.348	0.453	0.105	0.913	0.233	0.198
Bacillus	WAE28, WSA48, WSN45, WSN48	0.2	0.212	0.174	0.164	0.282	0.093	1.12	0.118	0.256
Actinobacillus	WAN31	0.118	0.172	0.141	0.093	0.184	0.093	1.22	0.208	0.194
Enterococcus	WAE29, WAE30, WAN26	0.106	0.109	0.108	0.067	0.154	0.089	0.785	0.205	0.147
Rothia	WAE49	0.216	0.262	0.214	0.193	1.276	0.09	0.83	0.133	0.176
Staphylococcus	WAE46	0.102	0.11	0.092	0.049	0.178	0.088	0.749	0.282	0.079
	negative control for stimulation	0.081	0.072	0.06	0.055	0.124	0.09	0.89	0.216	0.07

OD $_{450\,nm}$ reads were showed in the table. The positive OD value of cytokines are highlight as red.

Study II—Microbicidal and immunostimulatory capabilities of BFW

Inoculation of a cocktail of 15 bacteria isolated from warthog's feces improves the mucosal immunity induced after experimental ASFV infection.

From the 21 warthog *in vitro* characterized bacteria, 15 were finally selected for further *in vivo* testing due to different characteristics. Seven bacteria were selected due to their beneficial effects on the ileum organoid (WAE21, WAE28, WAN31, WAN32, WAN43, WSN45 and WSN48; Table 2) and WAE29 was selected because of their lack of toxicity not only for ileum but also for colon organoids. Similarly, seven additional bacteria were selected according to their capability to inhibit pathogenic bacteria and to grow in YCFAwo medium in strict anaerobic conditions (WAE1, WAE30, WAE46, WAN26, WAN31, WAN5 and WSA48). Moreover. The isolate WAE49, was selected not only due to its ability to inhibit the growth of *S. suis in vitro,* but mainly for its unique immunostimulatory capabilities on GALT cells.

In total, 10 three-week-old piglets were inoculated with a cocktail of 15 selected isolates from warthog fecal microbiota. Inoculation of BFW showed no negative effects on pig productivity, furthermore, bacteria inoculation showed a tendency to improve piglets' average weight gaining compared with control animals (Figure 4a). This increase tendency was more evident during the first two weeks after inoculation though it was not statistically significant, and less apparent from week 3, coinciding with low detectable fever in half of the transplanted pigs, compared with only one animal from control group (Figure 4b). No other clinical signs were recorded after experimental inoculation.
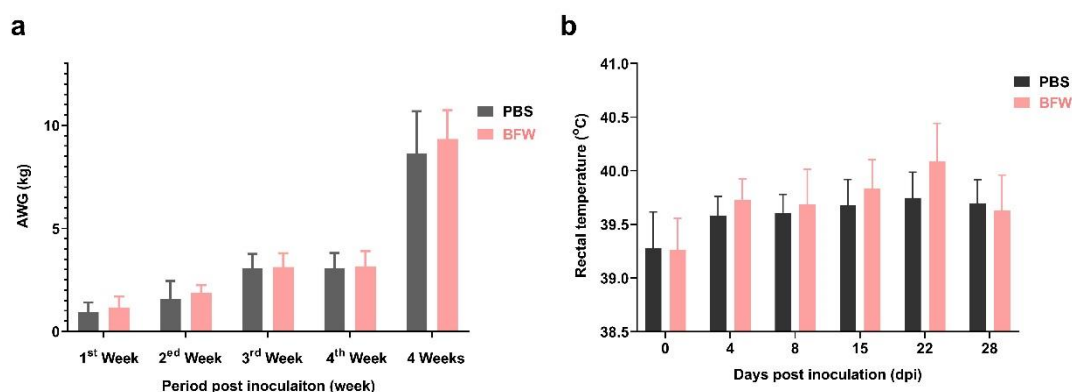
Figure 4. Intragastric inoculation of warthog feces bacteria is safe for domestic pigs. Average weight gain (AWG) of pigs inoculated with warthog and non-inoculated pigs, measured weekly and in the 4 weeks-period after inoculation (a) and, average rectal temperature kinetics of these same groups of animals taken at different days after inoculation (b). Means and standard deviation is represented for each time point and group.

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Twenty-eight days after inoculation, seven pigs from each group were challenged with 1,000 HAU of E75CV1, an attenuated strain of ASFV, aiming to mimic the protection studies recently performed after fecal warthog transplantation (Zhang *et al.* 2020). Conversely to previous experiments using the same viral strain and dose, none of the challenged pigs showed fever, neither viremia nor viral shedding at any time post-infection disabling any useful comparison between the experiments. Despite the lack of fever and viral detection, all pigs independently of the group they belonged, showed ASFV-specific T-cells in their blood by 21 dpc (days post challenge), detectable by IFNγ ELISPOT (Figure 5a) and most of them, also showed detectable ASFV-specific IgG in sera by ELISA (Figure 5b), with no significant differences between groups. Importantly, statistically significant differences were found in ASFV-specific IgA measured in blood when the groups were compared (Figure 5c), with pigs #15 and #19 from the inoculated group showing detectable IgA responses since 14 dpc. No pigs from PBS group showed detectable IgA in sera before 21 dpc. Confirming the enhancement of mucosal immunity, the same animals (#15, #19) showed much higher IgA titers in nasal swabs (Figure 5d). However, specific IgA for ASFV was not detected in feces. To more deeply characterize the effects of warthog bacteria in mucosal immunity, total IgA in blood was measured at 28 dpi as observed in figure 6, the total IgA was higher for pigs inoculated with BFW than for PBS control animals.
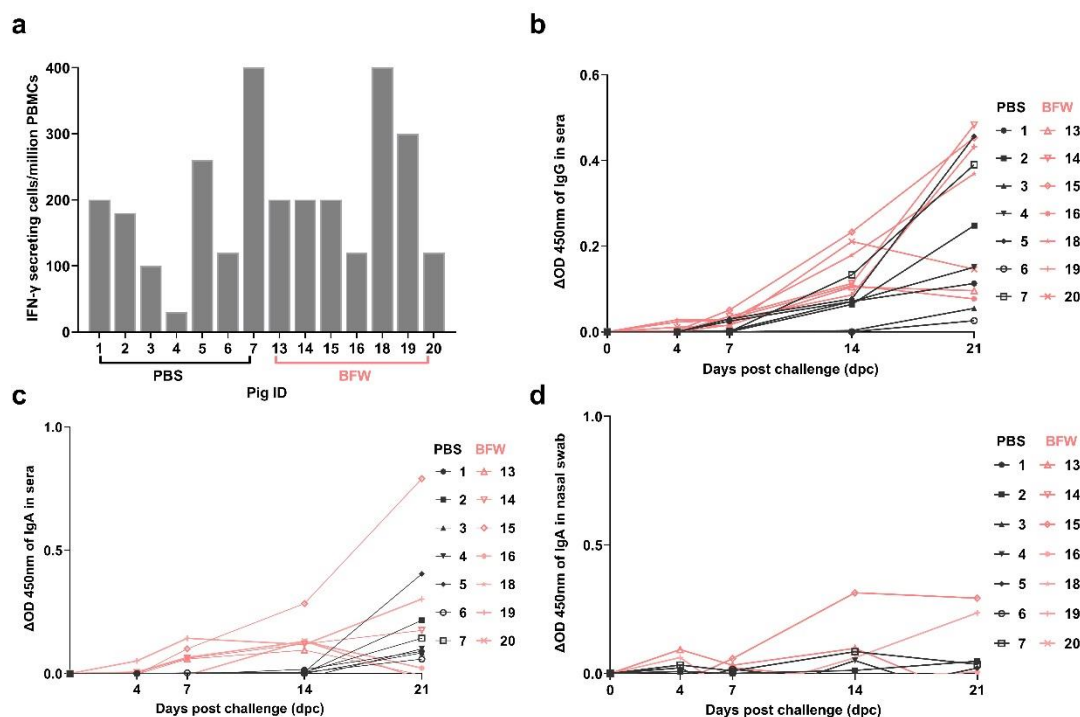
Figure 5. Intragastric inoculation of warthog feces bacteria seems to improve the humoral response induced against ASFV and accelerates the mucosal responses induced against ASFV. Quantification of ASFV-specific T-cells at 21 days after E75CV1-challenge, in the blood of pigs pre-inoculated (BFW) or non-pre-inoculated (PBS) with warthog feces bacteria, measured by IFNγ-ELISPOT (a); and, kinetics of ASFV-specific IgG found after E75CV1-challenge in both groups of pigs. Kinetics of ASFV-specific IgA induction found at different days after E75CV1-challenge, in the blood (c) or nasal swab (d) of pigs pre-inoculated (BFW) or non-pre-inoculated (PBS) with warthog feces bacteria, measured by an ASFV-specific ELISA. The maximum number of positive spots quantifiable in our ELISPOT-assay is 500 spots/million PBMCs. Values are given as ΔOD, corresponding to the OD value at a time point minus the OD at the time of challenge (day 0), for each animal.
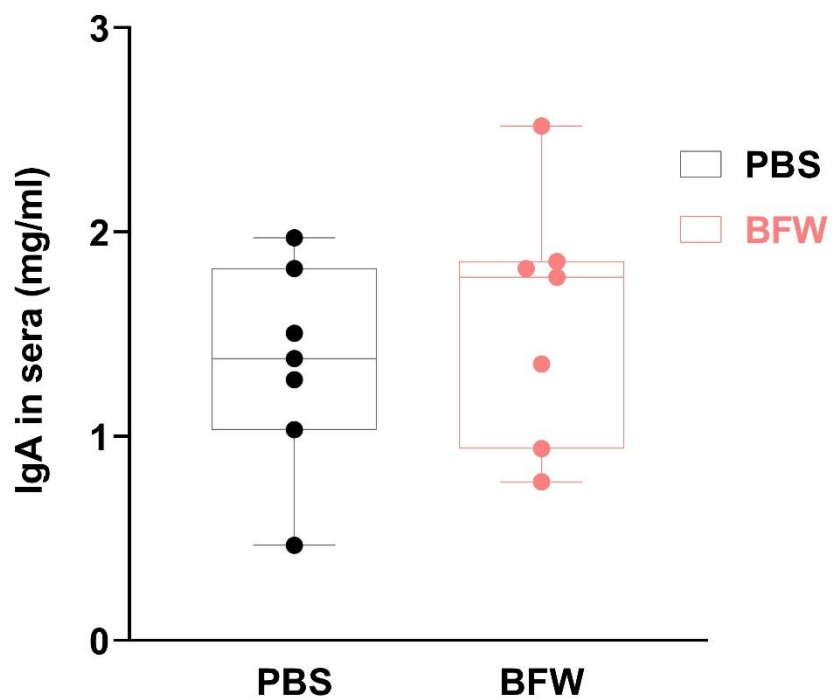
Figure 6. Intragastric inoculation of warthog feces bacteria improves general mucosal immunity. Comparison of total sIgA in the blood of non-inoculated (PBS) and inoculated (BFW) pigs at 28 days post-inoculation detected by ELISA.

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Discussion

The intestinal tract harbors a variety of resident bacteria, 90% of which are obligate anaerobes, while less than 5% of fecal bacteria are aerobes and facultative anaerobic bacteria[34]. When the intestinal content is shed to the environment, bacteria exposed to the environment atmosphere show different tolerance to the oxygen[35] and obligate anaerobic bacteria may die within minutes of exposure[36]. Thanks to the development of microbiology, more and more bacteria have been isolated from human[36] and animal (including pigs) fecal content[13], soil and environment[37]. Still, the isolates' outcome is limited by several factors, i.e. presence of oxygen[35], specific needs for nutrition included in the growth media[38], pH conditions and incubation temperatures[37]. Confirming these results, here we were able to isolate 135 colonies from 19 genera from warthog feces, accounting for 36% of the genera predicted from the warthog fecal microbiota (19 out of 44 genera[9]). Despite using aerobic and non-strict anaerobic conditions, many bacteria were lost, including those highly sensitive to the oxygen and those impossible to culture in the conditions used. Thus, some bacteria predicted to conform the core gut population were not obtained through this procedure, such as those belonging to *Bacteroides*, *Butyrivibrio*, *Lactobacillus*, *Prevotella* and *Ruminococcus* genera[39].

Warthog bacteria were isolated for two times from two different fecal pools, while only one attempt was done from pig feces (as control for the assay), thus explaining, at least partially, the reduced number of colonies obtained from pig feces (30 isolates belong to 14 different genera). Thus 21 isolates from warthog feces were selected based on the phylogenetic evolutionary analysis. In spite of these limitations, and taking into account that fungus, viruses and other microbiota components different to bacteria would be absent from our analysis, we continued with our search of warthog microbiota (bacterial) components with cytotoxicity effect, microbicidal and/or immuno-stimulatory capabilities with potential as future probiotics. It is well established that the commensal flora residing in the animal's gut, is essential for compromising the host intestinal barrier integrity, maintaining the animal intestine health, particularly regulating the immune homeostasis[40,41]. Intestinal organoid is a mininature organ derived from intestinal crypts, serving as an reliable and accurate *ex vivo* model for investigate the host intestine-bacteria interaction[42]. Except for isolates WAE30 and WSN50, which showed low toxicity effect on colon organoid cells,

supernatant from others isolates showed beneficial or non-toxic effect on both ileum and colon organoid cells. Further work revealed the presence of several bacteria isolates from warthog feces, as capable of inhibiting *C. perfringens*, virulent *S. suis* or both pathogens concomitantly; six of them included in our *in vivo* experiment. These bacteria (WSA3, WAN26, WSA48) deserve further investigation since they might act as potential probiotics against *S. suis* disease, inhibiting the pathogenic strain without interfering the growth of commensal non-pathogenic strain *S. suis* T15.

Probiotics might exhort their action using multiple mechanisms. Besides acting as antimicrobial components, promoting intestinal health and or immune maturation. In this regard, it is worthy to mention WSN45 and WAE49. WSN45 the only bacteria assayed capable to promote the *in vitro* growth of ileum and colon organoids, is identified as *B. mojavensis* or *B. tequilensis*. Interestingly, a *B. tequilensis* strain isolated from goat milk, showed the ability to produce levan, an immunostimulatory moiety, which is able to induce the anti-inflammatory cytokine IL4 at HT-29 cells[43] and exert strong activity to induce production of IL12 p40 and TNFα by macrophage cell lines *in vitro*[44]. *B. mojavensis* is used as dietary additive in fish food, showing a beneficial effect by efficiently fighting yersiniosis in fish, a bacterial infection[45]. Together with its microbicidal properties, WSN45 might be an excellent candidate for further characterization. In the other hand, WAE49, a bacteria belonging to the *Rothia* genus, is capable to stimulate GALT cells *in vitro* to stimulate the production of IFNγ, a Th1 cytokine involved in multiple mechanisms including the enhancement of CD8 T-cell responses, a matter that could be essential to fight ASFV[46,47]. Treatment with a high dose of *Lactobacillus rhamnosus* GG ($2*10^9$ CFU) before rotavirus (RV) infection in RV-infected mice significantly decreased viral shedding of RV in feces, increased the secretion of intestinal mucosa secretory IgA (sIgA), production of production of serum IFNγ, IgA[48]. PBMCs stimulated with lactic acid bacteria showed an increased concentration of TNFα, IFNγ, IL10 and TGFβ[49].

Inoculation of 15 bacterial colonies, which were selected due to their ability to promote intestinal health, *in vitro* inhibit pathogenic bacteria and/or act as immunostimulators, translated in an immediate growth benefit on weaned piglets. Bacteria inoculation resulted in higher average weight gain, statistically significant only during the first 15 days after transplantation, consistent with improved food conversion[50]. Lack of effect alter after inoculation might correlate with the occurrence

of a peak of fever two weeks after bacteria cocktail treatment, described in occasions FMT[51]. Interestingly, fecal transplantation of warthog feces improved the average weight of the animals during the second half of the treatment (Zhang *et al.*, 2020). Differences that might be explained by the different composition of the inoculum, infinitely more complex for the fecal transplant material. Besides this, oral inoculation of the warthog bacteria was evident with an increase in the total IgA present in the blood of the animals by day 28 dpi. The enhancement in the mucosal immunity observed, also affected to the specific mucosal immune response induced against ASFV challenge, recently described also after fecal transplantation of warthog feces[9].

We really believe that these results open new avenues to study these and other warthog microbiota components as future candidates as alternative probiotics for (and not limited to) the swine industry.

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Chapter 5

Study III

Full-length sequencing of isolated bacteria from warthog fecal microbiota: unravelling their potential microbicidal mechanisms of action.

(Manuscript in preparation)

Study III—Genome analysis unravels potential microbicidal mechanisms

Abstract

Gut microbiota play crucial roles in many aspects of living eukaryotes, including body homeostasis, immunity and health. The use of probiotics based on beneficial microbiota components, has an ancient history in human and veterinary medicine and their potential use as antibiotic alternatives is gaining new strength. Previous work in our laboratory allowed demonstrating the astonishing properties of warthog feces microbiota. If in previous work we concentrated our effort in some immunostimulatory microbiota components that contributed to partially protect against African swine fever virus experimental challenges with attenuated strains, here concentrate our efforts to extend our knowledge about microbicidal properties of bacteria isolated from warthog feces. To do so, we first characterized the capabilities of 135 isolated warthog feces bacteria to *in vitro* inhibit a panel of pathogenic bacteria, including *Clostridium perfringens* (type B), S*treptococcus suis* S10, *Escherichia coli* K88*, Salmonella enterica*, *Salmonella enterica* serovar *Typhimurium* monophasic variant (antigenic formula 4,12:i:-), and a nonpathogenic S*treptococcus suis* T15; and second, to fully sequence the genome of the 14 most promising microbicidal warthog microbiota components. Full-length sequencing of these bacteria, allowed the in silico identification of different mechanisms for pathogen inhibition worthy to be further explored if willing to use some of these microbiota components as alternative to antibiotics in the future.

Key words: warthog, gut microbiome, microbicidal, probiotics, genome, in silico prediction

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Introduction

Microorganisms residing in the digestive tract contribute not only to food digestion, but also play important roles in maintaining the intestinal homeostasis and protection against pathogens by using different mechanisms, immune and non-immune related. Thus, gut commensal flora protects the host from pathogenic bacteria infection through competition for the nutrients and surface area[1]. Basic research has allowed the commercialization of some probiotics, i.e. live microorganisms which, when administered in adequate amounts, confer a health benefit on the host[2]. Lactic acid bacteria (LAB), within genera such as *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Bacillus* are probably well known probiotics, but other genera, such as *Clostridium*, *Escherichia* are being also reported as potential probiotics, because of the antimicrobial ability or potential health-promoting benefits[3,4].

There are multiple ways through which probiotics provide health benefits to the host. Being a probiotic, it is necessary that a) surviving in harsh environment, such as acid and bile juice inside the gut, b) adhesion to the intestinal mucosa, which is important for colonization and possible immune stimulation, c) producing metabolites such as antimicrobial components, organic acids, thus inhibiting gut pathogenic bacteria or modulating immune response, d) safe for the host[5,6]. Biofilms are the aggregates of micro-organisms that are embedded in a self-produced polymeric matrix in a sessile state[7]. Biofilm formation facilitates the colonization and maintain of bacteria in the surface. On one hand, probiotic biofilms can stimulate longer stability of probiotics in the host mucosa that prohibit colonization of pathogenic bacteria[8–10]. Certain bacteria can produce a large number of bactericidal molecules which act towards other related or unrelated members of intestinal commensal and enteric pathogens. Though some bacteriocins have a wider spectrum of activity, most of them are usually active against taxonomically closely related bacteria[4]. In general, genes encoding bacteriocins reside either in genome, plasmids or other mobile genetic elements, are inducible and require secretion and extracellular accumulation of peptides for induction[11]. Some bacteria can also exert beneficial effects on the intestinal epithelial cells (IEC) and immune cells by producing metabolites, such as short chain fatty acids (SCFA), organic acid and essential vitamins[12–14].

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Recent work performed in our laboratory allowed the isolation and identification of a total of 73 spore-forming (with heat shock) and 62 (non-heat shock) bacteria from warthog feces in both aerobic and non-strict anaerobic conditions[15]. *In vitro* and *in vivo* studies performed with a proportion of them, allowed characterizing individual isolates that promoted the growth of ileum and colon organoids, the induction of IFNγ by GALT cells grown *in vitro* and/or the inhibition of pathogenic bacteria *in vitro*[15]. Here, we extended the latter studies to all the bacteria collection obtained from warthog feces, studying the ability to inhibit the growth of the following pathogenic bacteria strains: *Clostridium perfringens* (*C.perfringens*) (type B), S*treptococcus suis* (*S.suis*) S10, *Escherichia coli* (*E.coli*) K88*, Salmonella enterica* (*S.enterica*), *Salmonella enterica* serovar *Typhimurium* monophasic variant (monophasic *S.Typhimurium*) (antigenic formula 4,12:i:-), and a nonpathogenic S.*suis* T15. Based on the inhibition effect on bacteria tested above, 14 individual isolates were selected for full-length sequencing and deep *in silico* characterization of their genome. Furthermore, inhibition effect of bacteria-free culture supernatants on pathogenic bacteria, immune stimulatory effect on gut associated lymphoid tissues and biofilm formatting ability were also tested. The findings obtained and their future potential will be here discussed.

Material and methods

Fecal sampling, processing, bacteria isolation and identification

The whole procedure has been recently described[15]. Warthog and pig feces were used as controls for our assays. Individual colonies isolated in aerobic and anerobic conditions are named according to their origin with a W (from warthog), followed by AE or AN (from aerobic or anaerobic, respectively) or by SAE or SAN, for heat-shock treated under aerobic or anaerobic conditions, respectively.

Pathogenic microbial inhibition assay

The microbial inhibition assay was conducted with the following pathogenic bacteria from pig: *C. perfringens* (type B, isolated from piglets showing diarrhea), *S. enterica*, monophasic *S. typhimurium* (antigenic formula 4,12:i:-), *E. coli* K88, *S. suis* S10 (highly virulent strain) and also the *S. suis* T15 avirulent strain as a control.

To perform the inhibition test, each of these bacteria was cultured following the recommendations. *C. perfringans* was cultured under anaerobic conditions in BHI agar

plates at 37ºC overnight (O/N). *S. enterica*, monophasic *S. Typhimurium* and *E.coli* K88 were cultured in BHI agar plates O/N at 37ºC, under both aerobic and anaerobic conditions. For *S. suis* S10 and *S. suis* T15, chocolate agar plates were used under aerobic conditions at 37°C, 5% CO2 O/N.

The microbial inhibition assay was done using swabs fully soaked in pathogenic bacteria suspensions (density of 0.5 McFarland in PBS 1X) and plated homogenously within 9 cm^2 plates. Next, a 10 µl droplet of individually isolated bacteria from warthog fecal microbiota (density of 1.0 McFarland, in PBS 1X), was softly added over the monolayer. Assays with those isolated showing specific inhibition results were repeated twice independently.

Selection and further characterization of a group of bacteria

A group of bacteria were selected for later genome analysis, mainly based on the inhibition effect on the tested bacteria obtained above, together with the involvement in the *in vivo* bacteria inoculation experiment[15]. Which are WAE30, WAN26, WAN45, WAE46, WAE47, WAN28, WAE1, WAN5, WAN31, WSA25, WSA26, WSA48, WSN32 and WSN46. Thus, microbial inhibition effect of bacteria-free culture supernatant, biofilm formatting ability, immunostimulatory effect of these selected bacteria were tested further, also the full genome were sequenced and analyzed.

Pathogenic microbial inhibition assay using supernatants of warthog bacteria.

The inhibition effect of bacteria-free culture supernatant from these selected on *C. perfringens, S. enterica*, monophasic *S. typhimurium* and *E. coli* were also tested. Briefly, isolates culture in BHI broth was harvested when the bacteria reach logarithmic phase, cultures were centrifuged at 4000 g for 10 min, then the supernatant was filter-sterilized using a 0.2 µm membrane syringe filter.

Pathogenic bacteria were adjusted to 0.5 MacFarland in PBS 1X, soft BHI agar (7 g/L) was prepared. One ml of adjusted bacteria was added to the 100 ml of soft BHI agar (39 to 41ºC). Twenty ml BHI agar + pathogenic resuspension was poured into Petri dish (9 cm). 100 µl of filtered SN is introduced into the well, in the hole (6-8 mm diameter) created aseptically by punching with a sterile tip. Antimicrobial inhibition test was conducted with total bacteria resuspension as positive control and BHI broth as negative control. The plates were incubated at the aerobic and/or anaerobic condition based on the characteristic of the pathogen tested.

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Biofilm assay

Biofilm assays were performed in 96-well cell culture plates under both aerobic and anaerobic conditions following a previously published protocol with some modifications[16]. Selected isolates above from warthogs were grown in BHI broth until $OD_{600nm} = 0.3$, and then a 1:100 dilution was made for each individual culture. For each isolate, 100 µl of diluted culture was added in each well in quadruplicates. Plates were incubated at 37ºC in either aerobic or anaerobic conditions under static conditions for 24h, 48 h and 7 h, quantified at OD_{600nm} and next, plates were emptied and rinsed with tap water. Wells were stained with 100 µl 0.1 % (w/v) crystal violet (Merck) for 2 mins at room temperature. After extensive washing, plates were dried at 37ºC. Then 100 µl of 70 % ethanol and quantified at $OD_{590\ nm}$.

Immunostimulatory ability on gut-associated lymphoid tissue cells

The whole procedure has been recently described[15] . The immunostimulatory effect of WSA25, WSA26, WAN28, WAN45 and WSN46 were tested.

Genome analysis

Assembly and annotation

The genomes of 14 bacterial species were sequenced using Illumina MiSeq platform, performing a 2×150 bp paired-end sequencing. Additionally, 16S rRNA gene was sequenced using Sanger for all bacterial species (except for WAN45). The quality control of the reads was assessed using FastQC v0.11.8[17]. Because no reads were flagged as poor quality, all reads were *de novo* assembled into contigs and scaffolds using SPAdes genome assembler v3.14.0[18] with the flags --isolate, recommended for high-coverage data for viral isolate organisms, --cov-cutoff auto and including the full-length sequence of the 16S gene obtained through Sanger sequencing with the --trusted-contig option to increase assembly quality. The quality of genome assemblies was assessed using QUAST v5.0.2[18]. Finally, the 14 assemblies were annotated using Prokka v.1.14.6[19], specifying the Gram characteristics of the species previously determined *in vitro* (--gram), a minimum contig length of 200 bp (--mincontiglen) and without including tRNA (--notrna).

Taxonomic assignment

Taxonomic assignment of the 14 isolates with sequenced 16S rRNA gene was

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performed using RDP classifier tool v2.11[20] with the RDP 16S rRNA training set 16 and default parameter options. Additionally, BLCA[21] was also performed under the default parameters. Four isolates (WSA25, WSN32, WAN45 and WAE46) yielded an unclassified status, and in these cases BLCA was run using the 16S rRNA sequence annotated by Prokka instead of the Sanger sequence.

In silico identification of antimicrobial resistance genes, pathogenic and virulence factors

All 14 assemblies were screened for known resistance genes and both pathogenic and virulence factors. ResFinder database v3.2[22] was interrogated with a 70% of identity threshold and a minimum length threshold of 60%. PathogenFinder v1.1[23] was used under the default parameters. We performed a Blastp locally against Victors database[24] with all the annotated proteins. We kept hits with a minimum percentage of identity of 70% and significant *P value* ($p < 0.001$).

The annotated genomes were further analyzed using the Rast tool kit integrated in the Pathosystems Resource Integration Center (PATRIC)[25]. The genomes were visualized with the genome browser option, available in the PATRIC resource. Furthermore, these genomes were interrogated for AMR genes, using the specialty gene service of PATRIC V.3.6.5. This specialty genes filtered data type, including reference sequences of the Comprehensive Antibiotic Resistance Database (CARD)[26] and the Antibiotic Resistance Genes Database (ARDB)[27], which are integrated in PATRIC (http://patricbrc.org). The pathways and subsystems were also explored to obtain information regarding functional characterization of these bacteria.

Results

In vitro characterization of bacteria isolated from warthog feces

The inhibition effect on a panel of pig pathogenic bacteria were tested with 104 isolates from warthog feces and 37 pig isolates (Supplementary Table 1). Interestingly none of the bacteria isolated from pigs showed inhibition capability, while in total 55 isolates from warthogs showed to inhibit several pathogens (Table 1). From this selection, we found 22 warthog isolates capable to inhibit *C. perfringens* type B strain, 6 of them with high efficacy (Table 2). Interestingly, two of these isolates, belonged to *Acinetobacter* and *Streptococcus*, two genera not previously described in our former study as capable to inhibit *C. perfringens* (Zhang *et al.* 2020b). Remarkably, only

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supernatants from WSA25 and WSA26 (*Bacillus*) were capable to inhibit *C. perfringens* type B (marked with asterisk in Table 1); also when GALT cells were stimulated with WSA25 and WSA26, a detectable increase in interleukin (IL)18 secretion was found in the supernatant (supplementary Table 2).

Table 1. Summary of microbicidal effect of isolates from warthog feces on a panel of bacteria

Isolates	*S. suis* S10	*S. suis* T15	*C. perfringens*	*E. coli* k88	*S. enterica*	Monophasic *S.Typhimurium*
WAE2	Y	/	/	/	/	/
WAE24	Y	Y	/	/	/	/
WAE31	Y+	/	/	/	/	/
WAE32	/	/	Y+++	/	/	/
WAE42	Y+	/	/	/	/	/
WAE45	/	Y+	/	/	/	/
WAE47	/	/	Y++++	/	Y	Y
WAE48	/	/	/	/	Y	Y
WAE6	/	/	Y++	/	/	/
WAN22	/	/	Y	/	/	/
WAN27	/	/	Y	/	/	/
WAN28	Y+	Y	Y+	/	/	/
WAN29	/	/	Y+++	/	/	/
WAN33	/	/	Y+++	/	/	/
WAN41	Y	Y	Y	/	/	/
WAN45	/	/	Y++	Y+	Y+	Y+
WAN50	Y+	Y	/	/	/	/
WAN51	Y+	Y+	/	/	/	/
WSA1	Y	Y	/	/	/	/
WSA5	/	/	/	Y+	/	/
WSA8	Y++	/	Y	/	/	/
WSA12	/	/	/	/	Y	Y
WSA13	Y+	Y+	/	/	/	/
WSA21	Y+	Y++	/	/	/	/
WSA23	Y+	Y+	/	/	/	/
WSA24	Y	Y	/	/	/	/
WSA25*	Y+++	Y+++	Y	/	/	/
WSA26*	/	Y+	Y	Y+	/	/
WSA30	Y+	Y	/	/	/	/
WSA33	Y+/	Y+/	/	/	/	/
WSA34	Y+	Y+	/	/	/	/
WSA35	/	/	Y++	/	/	/
WSA36	Y++	Y++	/	/	/	/
WSA37	Y+	Y+	/	/	/	/
WSA38	Y	Y	/	/	/	/
WSA39	Y+	Y	/	/	/	/
WSA40	/	Y	/	/	/	/
WSA41	Y	Y	/	/	/	/
WSA43	Y++	Y++	/	/	/	/
WSA45	/	Y	/	Y+	/	/

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WSA46	/	/	Y++	/	/	/
WSA50	Y	Y	/	/	/	/
WSA51	/	/	Y++	/	/	/
WSN22	/	/	/	Y	Y	Y
WSN32	/	/	Y+++	Y+	Y+	Y+
WSN34	Y	Y	Y++	/	/	/
WSN52	/	Y+	/	/	/	/
WSN53	/	/	Y++++	/	/	/
WSN54	/	/	Y++	/	/	/
WSN37	/	/	Y+	/	/	/
WSN41	/	/	Y	/	/	/
WSN42	Y	Y	/	/	/	/
WSN46	/	/	/	Y+	Y+	Y+
WSN49	Y+	/	/	/	/	/

Based on the radiometer of the inhibition zone, the effect of inhibition was recorded in 4 level: "++++" represents very strong inhibition, more than 0.5 cm. "+++" represents strong inhibition., "++" represents mild inhibition, a clear inhibition zone (0.1-0.3 cm) was observed around the bacteria resuspension drop, "+" represents light inhibition, only a thin inhibition circle around the drop. "y" represents only where the bacteria resuspension drop dropped, there is no growth of pathogen bacteria. "*" represent that the bacteria free-culture-supernatant from this bacteria can inhibit the growth of *C. perfringens*.

Table 2. Summary of microbicidal inhibition result for isolates from warthog feces.

	S. suis S10	*S. suis* T15	*C. perfringens*	*E. coli* k88	*S. enterica*	**Monophasic S.Typhimurium**
Y++++	0	0	2	0	0	0
Y+++	1	1	4	0	0	0
Y++	3	2	7	0	0	0
Y+	14	9	1	6	3	3
Y	9	13	7	1	4	4
Total	27	25	22	7	7	7

Based on the radiometer of the inhibition zone, the effect of inhibition was recorded in 4 level: "Y++++" represents very strong inhibition, more than 0.5 cm. "Y+++" represents strong inhibition. "Y++" represents mild inhibition, a clear inhibition zone (0.1-0.3 cm) was observed around the bacteria resuspension drop. "Y+" represents light inhibition, only a thin inhibition circle around the drop. "Y" represents only where the bacteria resuspension drop dropped, there is no growth of pathogen bacteria.

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Moreover, 27 bacterial isolates inhibited *S. suis,* most of them indistinguishably inhibiting the two strains evaluated i.e. *S. suis S10* and *S. suis T15*, more importantly six of these isolates (WAE2, WAE31, WAE42, WSA8, WSA50 and WSN49) were capable to exclusively inhibit the virulent S10 strain (Table 1). In summary, six different genera were able to inhibit *S. suis*, i.e. *Terribacillus*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Balillus* and *Lysinibacillus*. Additionally, 7 isolates inhibited the growth of the two pathogenic *Salmonella* strains tested *in vitro* (Table 1 and 2). Finally, 7 bacteria inhibited the growth of *E. coli* K88 *in vitro* (Table 1 and 2)*,* a non-observable phenomenon for any of the 21 bacteria tested before (Zhang *et al.* 2020b, in preparation). Interestingly, one isolate WAN45 (*Enterococcus*) and four other isolates belong to bacillus genus (WSN22, WSN32, WSN46), showed inhibitory activity against both *E. coli K88* and *Salmonella*. Moreover, two other isolates (WSN32 and WAN45), strongly inhibited pathogenic *C. perfringens* (Table 1). None of the isolated bacteria was capable to inhibit all the pathogenic strains used in this study.

Selection of bacteria and further characterization

Fourteen bacteria with *in vitro* microbicidal potential, were selected to sequence their full genome based on different characteristics observed, described below. Half of them were selected due to the *in vitro* microbicidal potential against *C. perfringens* (WAE1, WAE30, WAE46, WAN5, WAN26, WAN31 and WSA48) and the other half were selected due to their ability to concomitantly inhibit more than one pathogen (WAN45, WSA25, WSA26, WSN32, WSN46. WAE47 and WAN28).

Biofilm formation

We also evaluated the biofilm formation ability in both aerobic and anaerobic conditions in BHI broth medium. We found that this ability varied among the 14 selected strains (Figure 1). The isolate WAE47 was able to form biofilm in both aerobic and anaerobic condition at both 24h and 72 h, followed by WAE46 and WSA25 which formed less biofilm under anaerobic condition than in aerobic. Conversely, isolate WSA48 formed more biofilm under anaerobic condition than growing in aerobic condition. Isolate WAN26 showed increasing biofilm formation ability from 24h to 72h in both conditions, while the isolate WAN5, only showed biofilm under anaerobic condition. The rest of the isolates, showed very low biofilm capabicity in either aerobic or anaerobic condition**.**
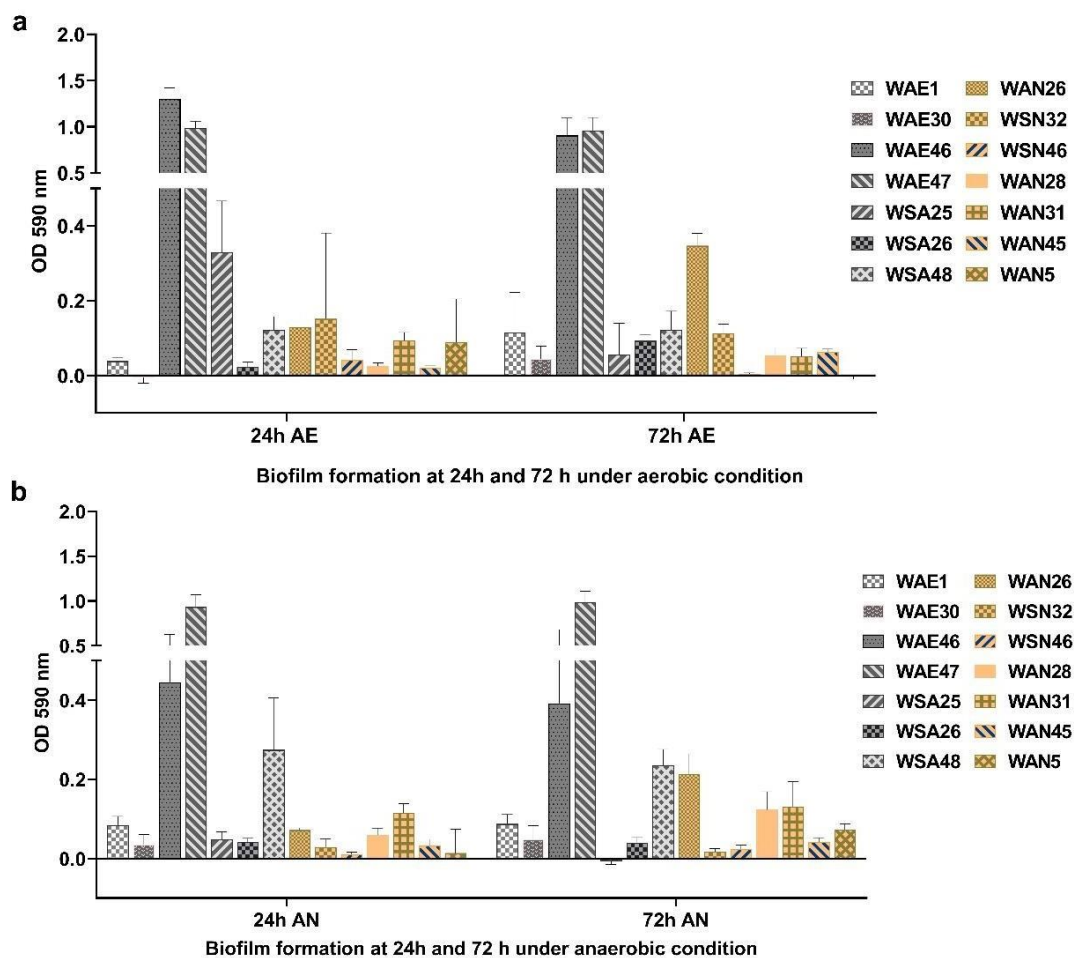
Figure 1. Biofilm formation measured in 14 selected isolates. Biofilm formation ability grown for 24 h and 72 h at aerobic condition (a) at anaerobic condition (b). Grey colors represent bacteria grown under aerobic condition, while brown bars correspond to anaerobic growth condition.

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Full-length sequencing of warthog feces bacteria with microbicidal properties

Shot-gun sequencing was performed over 14 bacterial isolates from warthog feces. The read sequence length was 150 pb and the mean quality (Phred score) 36. No reads were flagged as poor quality in any of the 14 genomic files (Supplementary Table 3). Genome assembly sizes of the 14 bacteria ranged from 2.3 Mb to 4.3 Mb and the GC percentage content ranged from 32% to 36%. After annotation with Prokka, 2114 to 5250 predicted protein-coding genes were found. The complete information related with genome assembly for 14 isolates is included in Supplementary Table 4.

RDP and BLCA classifiers were used to assess the taxonomy classification, yielding consistent results. The bacterial strains were classified to genus level with a cutoff of 100% for 12 isolates except for WAE1 and WAN5 from *Actinobacillus*, while at species level 7 out of 14, the cutoff was less than 100 % for *Actinobacillus* (WAE1 WAN5, WAN31), *Staphylococcus* (WAE47) and *Bacillus* (WSA25, WSA26 and WSN46). The detailed information is available in Supplementary Table 5.

The pathways' analysis on the annotated genomes revealed that two isolates, WAE30 and WAN45, belonging to *Enterococcus* genus, encode *enterocin A* gene, a well-known bacteriocin[28] (table 3). Three additional strains: WAE1, WAN5 and WAN31, encode *colicin V* related genes, another well-known bacteriocin[4,29] (Table 3). Interestingly, *in silico s*tudies found that these three strains would have the ability to produce succinate, as they have genes encoding phosphoenolpyruvate carboxylase (EC 4.1.1.31)[30], malate dehydrogenase (EC 1.1.1.37)[30], fumarate dehydratase (EC 4.2.1.2)[30], and fumarate reductase complexes in their genome (Table 4). The strain WSA48 may be also capable of synthesizing succinate, since it encodes several genes of this pathway (Table 4).

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Table 3. Bacteriocin encoding gene for 5 isolates based on genomic analysis

	Superclass	Class	Subclass	Subsystem Name	Role ID	Role Name	Active	Product
WAE30	PROTEIN PROCESSING	Protein Fate (folding, modification, targeting, degradation)	Protein processing and modification	Peptide methionine sulfoxide reductase	Peptide-methionine_(S)-S-oxide_reductase_MsrA_(EC_1.8.4.11)	Peptide-methionine (S)-S-oxide reductase MsrA (EC 1.8.4.11)	active	Enterocin A Immunity domain / Peptide-methionine (S)-S-oxide reductase MsrA (EC 1.8.4.11)
WAN45								
WAE1	METABOLISM	Secondary Metabolism	Ribosomally-synthesized post-translationally modified peptides (RiPPs)	Colicin V and Bacteriocin Production Cluster	Colicin_V_production_protein	Colicin V production protein	active	Colicin V production protein
WAN5								
WAN31								

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Table 4. Encoded genes related with succinate in 14 isolates based on genomic analysis

		Active	Product
WAE1		active	Fumarate reductase flavoprotein subunit (EC 1.3.5.4)
		active	Fumarate hydratase class I, alpha region (EC 4.2.1.2); L(+)-tartrate dehydratase alpha subunit (EC 4.2.1.32)
		likely	Malate dehydrogenase (EC 1.1.1.37)
		active	Phosphoenolpyruvate carboxylase (EC 4.1.1.31)
WAE46		active	Malate dehydrogenase (EC 1.1.1.37)
		active	TCA Cycle Fumarate hydratase class II (EC 4.2.1.2)
WAE47		active	Malate dehydrogenase (EC 1.1.1.37)
WAN26		active	Phosphoenolpyruvate carboxylase (EC 4.1.1.31)
WSA48		active	Fumarate hydratase class I, aerobic (EC 4.2.1.2)
		active	Phosphoenolpyruvate carboxylase (EC 4.1.1.31)
WSN32		active	Malate dehydrogenase (EC 1.1.1.37)
WSN46		active	Malate dehydrogenase (EC 1.1.1.37)
WAN5		active	Fumarate hydratase class I, alpha region (EC 4.2.1.2); L(+)-tartrate dehydratase alpha subunit (EC 4.2.1.32)
		active	Fumarate reductase flavoprotein subunit (EC 1.3.5.4)
		active	Phosphoenolpyruvate carboxylase (EC 4.1.1.31)
		likely	Malate dehydrogenase (EC 1.1.1.37)
WAN31		likely	Malate dehydrogenase (EC 1.1.1.37)
		active	Fumarate hydratase class I, beta region (EC 4.2.1.2); L(+)-tartrate dehydratase beta subunit (EC 4.2.1.32)
		active	Fumarate reductase iron-sulfur protein (EC 1.3.5.4)
		active	Phosphoenolpyruvate carboxylase (EC 4.1.1.31)

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Besides this individual characterization, we compared the particular features encoded in the different isolates by grouping them according to their *in vitro* characteristics. First, we compared the genome-encoded pathways regarding to their ability to inhibit *C. perfringens* growth. Thus, WAN31, WAE1 and WAN5, being strong inhibitors ('high' group), while WAE30 or WAE46 showed intermediate or low inhibitory capabilities ('low' group). When these two groups were compared, 120 pathways were found to be shared among 'high' and 'low' groups, twelve pathways were exclusively found in the 'low' group, and only 3 appeared to be exclusive for the 'high' group. Interestingly, these three pathways were lipid-related, i.e. 'biosynthesis of unsaturated fatty acids', 'C21-steroid hormone metabolism' and 'linoleic acid metabolism' (Figure 2). Interestingly, two of these three core pathways were also found in WSA25 and WSA26 (C21-steroid hormone metabolism and linoleic acid metabolism), bacteria capable to secret anti-clostridium active components. A deeper analysis allowed identifying the specific virulence factor, *galU* in the genome of four (WSA26, WAE1, WAN5 and WAN31) of the five bacteria (except for WSA25) included in the 'high' group (Supplementary Table 6).

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A: Biosynthesis of unsaturated fatty acids;	
B: Caffeine metabolism; Sesquiterpenoid biosynthesis; Fluorobenzoate degradation;	
C: C21-Steroid hormone metabolism; Linoleic acid metabolism;	
D: Isoflavonoid biosynthesis; Diterpenoid biosynthesis; Brassinosteroid biosynthesis; Secondary bile acid biosynthesis; Primary bile acid biosynthesis; 2,4-Dichlorobenzoate degradation; Styrene degradation; DDT degradation; beta-Lactam resistance; Penicillin and cephalosporin biosynthesis; D-Arginine and D-ornithine metabolism; mTOR signaling pathway;	

Figure 2. Venn diagrams to compare predicted pathways of different isolates based on different inhibition effect on *C. perfringens*. In purple, the number of pathways shared by strong inhibitors: WAE1-WAN5-WAN31, in green the number of exclusive pathways present in bacteria capable to inhibit *C. perfringens* with their supernatant, WSA25-WSA26 and in yellow, the number of shared pathways for WAE30-WAE46, two groups showing moderate/light inhibition effect.

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Second, we compared the particular features of each of the isolates from warthog microbiota by grouping them according to the microbicidal effect on *S. suis* S10 and *S. suis* T15 growth. We compared all the members from *Bacillus* genus, since most of the isolates showing inhibition growth to *S. suis* were from this genus. Therefore, we compared the bacterial genomes of WSA48 (inhibiting S10), WSA25 (inhibiting S10 and T15), WSA26 (inhibiting T15). We also included WSN32 and WSN46 in the analysis since they showed no inhibition capability to the growth of any of the two *S. suis* strains tested. We found the majority of the predicted pathways (90/139) were shared by all these five *bacillus* bacteria (Supplementary Figure 1a), Interestingly, the sesquiterpenoid biosynthesis pathway was exclusively found in WSA25, while secondary bile acid biosynthesis and fluorobenzoate degradation pathways were exclusively detected in both WSA25 and WSA26 genomes. Only three predicted virulent factors (out of 11) were shared: adenylosuccinate lyase (*purB*), adenylosuccinate lyase (*purA*), *codY* (Supplementary Figure 1b).

Based on the subsystem analysis, genes related with biosynthesis of B vitamins were found in the genome of these 14 isolates (Table 5). Briefly, biosynthesis gene related with Vitamin B2, B7 and B9 were found in all the isolates, while vitamin B5 and B12 were found in none of these isolates. Genes related with biosynthesis of vitamin B1 (thiamine) and B6 (pyridoxal) were found in the genome of WAN5, WAN31 and WAE1; four isolates WSA25, WSA26, WSN32 and WSN46 are with genes related with vitamin B1, B3 (niacin), and B6. Isolate WSA48 are with vitamin B3 and B6. Furthermore, Gene related with vitamin B3 was found in WAN28, Gene related with vitamin B6 were found in the genome of WAN26, WAE46, and WAE47. We also found some genes related with biofilm formation in the genome of WSN46 and WSN32, which included genes encoding several Rap proteins (Rap A, D, G, I, K, E) (Table 6). Interestingly, genes related with sucrose-to-levan conversions (Levanase EC 3.2.1.65) (Table 7). were found in the genome of weak biofilm producer, WSN32, WSN46 and WSA26, although not exclusively since it was also present in WSA48

Except for WAE46 and WAE47, a pathway related with T-cell receptor signaling pathway was found in all the sequenced genomes (Supplementary Table 7). Furthermore, a mTOR pathway appeared in some of the isolates, i.e. WAE46, WAE47, WSA48, WSA25, WSA26, WSN32 and WSN46 (Supplementary Table 7).

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Table 5, Encoding gene related with B vitamins in the isolates based on subsystems analysis

	WAE1	WAE46	WAE47	WAN26	WAN31	WAN5	WSA25	WSA26	WSA48	WSN32	WSN46	WAN28
EC 2.2.1.7	likely	/	/	likely	likely	likely	active	active	active	active	active	/
EC 1.1.1.262	likely	/	/	/	likely	likely	/	/	active	active	active	/
EC 1.1.1.95	likely	active	active	likely	likely	likely	active	active	active	active	active	/
EC 1.2.1.12	likely	active	active	likely	likely	likely	active	active	active	active	active	/
EC 2.7.1.35	/	active	active	likely	/	/	active	active	active	active	active	/
EC 2.6.1.52	likely	/	/	likely	likely	likely	active	active	active	active	active	/
EC 4.3.3.6	/	active	active	/	/	/	active	active	active	active	active	/
EC 2.7.1.35	likely	/	/	/	likely	likely	/	/	/	/	/	/
EC 1.4.3.5	likely	/	/	/	likely	likely	/	/	/	/	/	/
EC 2.7.4.16	active	/	/	/	active	active	active	active	/	active	active	/
EC 2.6.1.54	active	active	/	/	/	/	/	/	active	/	/	/
EC 2.8.1.4	active	/	/	/	active	active	/	/	/	/	/	/
Substrate-specific component NiaX of predicted niacin ECF transporter	/	/	/	/	/	/	/	/	/	/	/	active
niacin transporter NiaP	/	/	/	/	/	/	active	active	active	active	active	/

"EC 2.2.1.7": 1-deoxy-D-xylulose 5-phosphate synthase; "EC 1.1.1.262": 4-hydroxythreonine-4-phosphate dehydrogenase; "EC 1.1.1.95": D-3-phosphoglycerate dehydrogenase; "EC 1.2.1.12": NAD-dependent glyceraldehyde-3-phosphate dehydrogenase; "EC 2.7.1.35": Novel pyridoxal kinase, thiD family ; "EC 2.6.1.52": Phosphoserine aminotransferase ; "EC 4.3.3.6": pyridoxal 5'-phosphate synthase "glutamine hydrolyzing", synthase subunit ; "EC 2.7.1.35": pyridoxal kinase; "EC 1.4.3.5": pyridoxamine 5'-phosphate oxidase ; Substrate-specific component NiaX of predicted niacin ECF transporter; "EC 2.7.4.16": thiamine-monophosphate kinase ; "EC 2.6.1.54": Transcriptional regulator of pyridoxine metabolism/pyridoxamine phosphate aminotransferase; "EC 2.8.1.4": tRNA 4-thiouridine synthase/ Rhodanese-like domain required for thiamine synthesis; "/" no encoding gene was found.

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Table 6, Encoding gene related with rap proteins in isolates WSN32 and WSN46

Superclass	Class	Subclass	Subsystem Name	Role ID	Role Name	Active
CELLULAR PROCESSES	Microbial communities	Quorum sensing and biofilm formation	Phr peptides - Rap phosphatases signaling	Response_regulator_aspartate_phosphatase_E	Response regulator aspartate phosphatase E	active
				Response_regulator_aspartate_phosphatase_K	Response regulator aspartate phosphatase K	active
				Response_regulator_aspartate_phosphatase_I	Response regulator aspartate phosphatase I	active
				Response_regulator_aspartate_phosphatase_D	Response regulator aspartate phosphatase D	active
				Response_regulator_aspartate_phosphatase_A	Response regulator aspartate phosphatase A	active
				Response_regulator_aspartate_phosphatase_D	Response regulator aspartate phosphatase D	active
				Response_regulator_aspartate_phosphatase_G	Response regulator aspartate phosphatase G	active
				Response_regulator_aspartate_phosphatase_A	Response regulator aspartate phosphatase A	active
				Response_regulator_aspartate_phosphatase_D	Response regulator aspartate phosphatase D	active
				Response_regulator_aspartate_phosphatase_G	Response regulator aspartate phosphatase G	active
				Response_regulator_aspartate_phosphatase_D	Response regulator aspartate phosphatase D	active
				Response_regulator_aspartate_phosphatase_I	Response regulator aspartate phosphatase I	active
				Response_regulator_aspartate_phosphatase_K	Response regulator aspartate phosphatase K	active

Table 7, Encoding gene related with levan in isolates WSA26, WSA48, WSN32 and WSN46

Superclass	Class	Subclass	Subsystem Name	Role ID	Role Name	Active
METABOLISM	Carbohydrates	Di- and oligosaccharides	Sucrose to levan conversions	Levanase_(EC_3.2.1.65)	Levanase (EC 3.2.1.65)	active
				Levansucrase_(EC_2.4.1.10)	Levansucrase (EC 2.4.1.10)	active
				Sucrose-6-phosphate_hydrolase_ (EC_3.2.1.26)	Sucrose-6-phosphate hydrolase (EC 3.2.1.26)	active

Discussion

It is well established that the beneficial bacteria residing in the animal's gut, the microbiota, is essential for maintaining the animal health, particularly regulating the immune homeostasis[31]. Basic research has allowed the commercialization of some probiotics of animal origin, i.e. live microorganisms which, when administered in adequate amounts, confer a health benefit on the host, including humans[2]. Lactic acid bacteria (LAB), within genera such as *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Bacillus* are probably well known probiotics, but other genera, such as *Clostridium*, *Escherichia* are being also reported as potential probiotics, because of the antimicrobial ability or potential health-promoting benefits[3,4].

Antimicrobial agents are commonly applied in pig farming industries for improving productivity[32], preventing or curing bacterial infections such as the ones produced by *S. suis*[33], *E. coli*[34], *Salmonella*[35] or *C. perfringens*[36]. Massive usage of antimicrobials during the last decades, increased the antimicrobial resistant (AMR) prevalence and caused contamination of environment, increasing also the risk to human health through zoonotic diseases[37]. Despite the beneficial role antimicrobials have exerted against pathogens, they are proven to critically affect the microbiota[38,39]. It is necessary to find other solutions, i.e. probiotics or prebiotics to replace the use of antibiotics. We found several isolates from warthog feces capable of inhibiting *C. perfringens, S. suis, Salmonella and E. coli*, while none of the isolates from pig feces demonstrated this ability. Interestingly, among the bacteria showing inhibition on the growth of *S. suis* S10, 6 of them only affected the virulent *S. suis* strain without interfering the growth of commensal non-pathogenic strain *S. suis* T15. These bacteria (WAE31, WAE42, WSA3, WSA8, WSN49, WSA48) deserve further investigation since they might act as potential probiotics against *S. suis* disease. Further more, more *S.suis* strains should be tested to confirm this inhibition. Four and four isolates from warthogs showed also mild and light inhibition of both *E. coli* K88 and *Salmonella* growth., However, since there are several reports revealing strains from pig feces capable of inhibiting the growth of *E.coli* (K88/F4) or *Salmonella*[40–43], we cannot rule out that the absence of isolates of pig with this characteristics in this study, could be biased due to the low number of bacteria we isolated from pig feces, and also it is most probably reflecting a lack of sensitivity in our assay, confirmed with the weak inhibitory

Study III—Genome analysis unravels potential microbicidal mechanisms effect of isolates from warthog feces against these two pathogens.

LAB have been shown to be capable of producing an array of active antimicrobial substances like organic acids, bacteriocins and hydrogen peroxide, among others[44]. Bacteriocins are a group of small polypeptides, which can induce similar effects as antibiotics[4]. Colicins are the most frequently studied bacteriocins produced mainly by *E. coli*[29], but also by several *Enterobacter* species[45]. Colicins can be effective against bacteria belonging to the genera *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella*, *which are* associated with the diarrhea and post-weaning diarrhea in pigs[4]. We identified *colicin V* gene in the genome of three strains (WAE1, WAN5 and WAN31) as *Actinobacillus succinogenes* inhibiting the growth of *C. perfringens* (strongly) and *Salmonella* (mild effect). Enterocins are a broad-spectrum bacteriocins produced by *Enterococci* that showed strong inhibition effects on the growth and survival of *Salmonella spp* and *E.coli* strains[28]. The gene for enterocin A, was found in the genome of two isolates capable of inhibiting some of the tested pathogens; WAN45 strain showed inhibition effects on *C. perfringens*, *Salmonella* and *E. coli* growth, while WAE30 showed slight inhibition effect on *C. perfringens* growth. Other bacteria were able to inhibit the pathogens' growth, where WSA25 (*B. velezensis*) and WSA26 (*B. mojavensis*) deserve further attention due to the fact that, when grown in liquid the supernatant of these bacteria was capable of inhibiting *C. perfringens in vitro*. Both antimicrobial and anti-fungal ability potential have been described for endophytic *B. mojavensis* and *B. velezensis*[46,47]. Linoleic acid (LA), or 9-cis, 12-cis-octadecadienoate, is a polyunsaturated omega-6 fatty acid[48]. LA is toxic for many bacteria, as the length of the lag phase was dependent on LA concentrations[49]. Its main isomers, also known as conjugated linoleic acid (CLA), have antiproliferative and anti-inflammatory effects on colonocyte[50,51]. Virulent factor galU is exclusively found in the genome of WSA26, WAE1, WAN5 and WAN31. Mutant of galU in the uropathogenic *E.coli* leads to an significantly increased cytokine response when co-incubated with J774A.1 macrophages[52]. This virulence factor is required for corneal infection and efficient systemic spread following pneumonia caused by *Pseudomonas aeruginosa*. Sesquiterpenoids, especially those with a drimane skeleton, possess variety of biological activities, including anti-inflammatory, anti-microbial, which are used widely in agriculture, medicine[53]. The function of virulence factor or predicted pathway exclusively in the isolates with inhibition effect on *C.perfringens* and *S.suis* WSA25

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and WSA26 is not clear. The mechanisms underlying microbicidal properties observed from the bacteria described int this study, were out of the scope of this research and further investigations are needed to elucidate them.

Vitamin are essential micronutrition for mammals. Gut bacteria are the producers and consumers of B vitamins for its host[54]. Vitamin B6 deficiency weakened the immune response through affecting T lymphocyte differentiation and proliferation and IFNγ expression using BALB/c mouse model[55]. Vitamin B1 depletion impairs TCA cycle activity, and Vitamin B1 is required for the maintenance of naïve B cells to induce intestinal IgA responses against oral vaccines[56]. When weaned piglets were treated with fecal microbiota from warthog or a group of isolates from warthog feces, the concentration of total IgA was increased[15,57], also ASFV specific IgA was enhanced in the bacteria inoculated pigs, compared with PBS treated pigs[15]. Here, genes related with biosynthesis vitamin B1 was found in the genome of WAE1, WAN5 and WAN31, which are included in the bacteria inoculation pool. The correalation between these three isolates and strengthened IgA should be investigated further. Some of the selected bacteria used in the cocktail for inoculation, were able to produce acetic acid, many of them could produce propionic acid and butyric acid (data not shown). Moreover, based on the genome analysis, we found at least four strains (WAE1, WAN5, WAN31 and WSA48) theoretically capable of producing succinate, a metabolite known to stabilize proinflammatory HIF1α, favoring differentiation of T lymphocytes into pro-inflammatory Th17 cells and, hence, attenuating regulatory T cell development[58].

Biofilms are the aggregates of micro-organisms that are embedded in a self-produced polymeric matrix in a sessile state[7]. Biofilm formation facilitates the colonization and maintain of bacteria in the surface. On one hand, probiotic biofilms can stimulate longer stability of probiotics in the host mucosa that prohibit colonization of pathogenic bacteria[8–10.] For the tested 14 isolates, almost all of them have the ability to form biofilm, though for several isolates the biofilm format ability is extreamingly low (WAE30, WSA25, WSA26, WSN32, WSN46, and WAN45). Two isolates from *Bacillus* WSN32, WSN46 form biofilm with great difficulty, this might be correlated with the presence of genes encoding several Rap proteins (Rap A, D, G, I, K, E) and 'Sucrose-to-levan conversions Levanase (EC 3.2.1.65)' in their genome, with Rap K recently described as capable of inhibiting the biofilm formation[59]. On other hand, pathogenic bacteria also take advantage of biofilm, transport antimicrobial gene inside

the biofilm resulting in persistence infection. Levan, produced by *B. tequilensis* showed a great capacity to inhibit pathogenic biofilm formation[60]. This capability of levan could be a new antibiofilm strategy in inhibiting bacterial pathogenic biofilms[61]. Levan is also with the immunostimulatory moiety, induced the anti-inflammatory cytokine IL4 at HT-29 cells[62], and exerted strong activity to induce production of IL12 p40 and TNFα by macrophage cell lines *in vitro*[63]. Interestingly, another isolate from *Bacillus* WSN45 (*B. tequilensis or B. mojavensis*), with very low biofilm format ability (data not shown)*,* is the only isolate which showed beneficial effect on both ileum and colon organoid[15]. It is very interesting to investigate antibiofilm ability on pathogenic biofilms of low biofilm formatting isolates.

The bacteria described throughout this study, together with some bacterial isolates that need further studies, represent future candidates for bacteriotherapy as probiotics against commensal pathogen infection to (and not limited to) swine industry. Before so, the mechanisms involved in the microbicidal activity exhorted by warthog fecal bacteria deserve better characterization.

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a

b

Supplementary Figure 1. Comparison of predicted Pathway and predicted virulence factors based on inhibition effect on *S.suis*.

Supplementary Table 1, summary of isolates from both pig and warthog feces

Warthog isolates				Pig isolates			
WSN4	WSA1	WAN1	WAE1	PSN1	PSA1	PAN1	PAE1
WSN5	WSA2	WAN2	WAE2	PSN2	PSA2	PAN2	PAE2
WSN6	WSA3	WAN3	WAE3	PSN3	PSA3	PAN3	PAE4
WSN21	WSA4	WAN4	WAE4	PSN4	PSA4	PAN5	PAE5
WSN22	WSA5	WAN5	WAE5		PSA5	PAN6	PAE6
WSN23	WSA6	WAN6	WAE6		PSA6	PAN7	PAE7
WSN25	WSA7	WAN7	WAE7		PSA7	PAN8	PAE8
WSN27	WSA8	WAN25	WAE8		PSA8	PAN10	
WSN28	WSA9	WAN26	WAE9		PSA9		
WSN3	WSA11	WAN27	WAE21		PSA10		
WSN30	WSA12	WAN28	WAE22		PSA11		
WSN31	WSA13	WAN29	WAE23		PSA12		
WSN32	WSA14	WAN30	WAE24		PSA13		
WSN33	WSA21	WAN31	WAE25		PSA14		
WSN34	WSA22	WAN32	WAE27		PSA15		
WSN35	WSA23	WAN33	WAE28		PSA16		
WSN37	WSA24	WAN34	WAE29		PSA17		
WSN39	WSA25	WAN35	WAE30		PSA18		
WSN40	WSA26	WAN39	WAE31				
WSN41	WSA27	WAN40	WAE32				
WSN42	WSA28	WAN41	WAE33				
WSN43	WSA29	WAN42	WAE39				
WSN44	WSA30	WAN43	WAE40				
WSN45	WSA31	WAN45	WAE41				
WSN46	WSA33	WAN46	WAE42				
WSN47	WSA34	WAN49	WAE43				
WSN48	WSA35	WAN50	WAE44				
WSN49	WSA36	WAN51	WAE45				
WSN50	WSA37		WAE46				
WSN51	WSA38		WAE47				
WSN52	WSA39		WAE48				
WSN53	WSA40		WAE49				
WSN54	WSA41		WAE50				
	WSA43		WAE51				
	WSA45						
	WSA46						
	WSA48						
	WSA49						
	WSA50						
	WSA51						

Study III--Genome unravels potential microbicidal mechanisms

Supplementary Table 2, Cytokines expression from Gut associated lymphoid tissue stimulated by selected bacteria

	IL6	IL10	IL17	IFNα	IFNγ	TNFα	TGFβ	IL8	IL18
WSA25 and WSA26	0.226	0.261	0.213	0.218	0.299	0.108	2.922	0.111	0.562
WAN28	0.09	0.097	0.085	0.062	0.171	0.086	1.262	0.227	0.088
WAN45 and WSN46	0.125	0.118	0.144	0.089	0.202	0.106	0.832	0.225	0.108

OD $_{450\,nm}$ reads were showed in the table. The positive OD value of cytokines are highlight as red

Study III--Genome unravels potential microbicidal mechanisms

Supplementary Table 3, FastQC quality control statistics

	PE1			PE2		
Bacteria	**N reads**	**GC**	**Mean quality**	**N reads**	**GC**	**Mean quality**
WAE1	5441872	46	36	5441872	46	36
WAE30	5206482	38	36	5206482	38	36
WAE46	5396574	32	36	5396574	32	36
WAE47	4669706	32	36	4669706	32	36
WAN5	4709496	46	36	4709496	46	36
WAN26	5418612	36	36	5418612	36	36
WAN28	5139068	42	36	5139068	42	36
WAN31	4847050	46	36	4847050	46	36
WAN45	4684828	36	36	4684828	36	36
WSA25	4942827	46	36	4942827	46	36
WSA26	5060002	44	36	5060002	44	36
WSA48	5506734	35	36	5506734	35	36
WSN32	4416632	46	36	4416632	46	36
WSN46	5069544	46	36	5069544	46	36

Study III--Genome unravels potential microbicidal mechanisms

Supplementary Table 4-1, Genome assemble results of each isolates.

	WAE1	WAN5	WAN31	WAE30	WAE46	WAE47	WAN26	WAN28	WAN45	WSA25	WSA26	WSA48	WSN32	WSN46
Contigs	41	41	40	64	13	42	25	82	141	46	28	66	17	10
GC Contents	45.95	45.95	45.95	38.25	32.93	32.52	36.32	42.44	36.78	46.51	43.88	35.19	49.26	45.84
Plasmids	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Contig L50	3	3	3	6	2	4	4	9	19	4	3	7	3	1
Genome Length	2297275	2297245	2297011	3256510	2720158	2996480	2379007	2297448	3082393	3874853	4083022	5389284	4094260	4304515
Contig N50	266807	266807	266807	212595	895.786	340086	241169	88028	58034	260230	529922	263669	610453	2305563
Chromosomes	0	0		0	0		0	0	0	0	0	0	0	0
Annotated Genome Features														
CDS	2288	2288	2290	3056	2609	2856	2331	2370	2685	3965	4320	5635	4420	4743
tRNA	52	52	51	46	53	49	49	80	60	76	59	69	77	77
Repeat Regions	37	37	37	3	4	4	26	32	45	3	3	3	3	28
rRNA	3	3	3	0	0	0	3	3	3	0	0	0	0	3
Partial CDS	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miscellaneous RNA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Protein Features														
Hypothetical proteins	371	372	374	1094	548	628	613	765	911	686	996	2049	897	1093
Proteins with functional assignments	1917	1916	1916	1962	2061	2228	1718	1605	1774	3279	3324	3586	3523	3650
Proteins with EC number assignments	743	743	743	661	789	800	636	557	612	1008	1042	1086	1053	1079
Proteins with GO assignments	596	596	596	539	664	676	534	471	506	842	868	908	884	906
Proteins with Pathway assignments	529	529	529	451	596	611	462	391	418	749	770	790	777	792
Proteins with PATRIC genus-specific family (Plfam)	1912	1911	1911	2834	2551	2782	2052	1646	2471	3691	697	4984	4240	4507
Proteins with PATRIC cross-genus family (PGfam)	2037	2036	2036	2896	2570	2805	2104	1732	2491	3807	4134	5018	4250	4525
Subsystem (Subsystems, Genes)														
METABOLISM	(79, 577)	(79, 577)	(79, 577)	(63, 340)	(85, 557)	(85, 554)	66, 361	64, 368	54, 295	92,,717	96,,766	85,,698	96,,772	96,,783
PROTEIN PROCESSING	(42, 218)	(42, 218)	(42, 218)	(39, 215)	(38, 203)	(38, 204)	41, 216	38, 180	39, 206	43,,221	43,,221	42,,233	43,,229	43,,231
STRESS RESPONSE, DEFENSE, VIRULENCE	(27, 94)	(27, 94)	(27, 94)	(29, 100)	(30, 111)	(30, 114)	28, 73	25, 71	28, 99	33,,130	33,,135	32,,124	34,,123	35,,123
ENERGY	(26, 162)	(26, 162)	(26, 162)	(16, 112)	(24, 171)	(25, 179)	21, 134	12, 73	15, 105	25,,215	25,,215	17,,191	27,,228	30,,253
DNA PROCESSING	(17, 79)	(17, 79)	(17, 79)	(16,83)	(19, 80)	(19, 81)	17, 77	6, 18	15, 68	16,,79	18,,99	16,,84	18,,90	18,,103
MEMBRANE TRANSPORT	(15, 72)	(15, 72)	(15, 72)	(11,30)	(7, 32)	(8, 36)	3, 7	6, 18	10, 25	16,,74	16,,77	15,,85	16,,92	16,,91
RNA PROCESSING	(14, 72)	(14, 72)	(14, 72)	(12,47)	(13, 48)	(13, 49)	12, 46	12, 45	12, 48	13,,52	13,,52	13,,57	13,,56	13,,53
CELLULAR PROCESSES	(10, 57)	(10, 57)	(10, 57)	(12,67)	(12, 75)	(14, 82)	14, 76	14, 66	11,,54	16,,79	29,,248	27,,191	30,,252	30,,253
CELL ENVELOPE	(6, 26)	(6, 26)	(6, 26)	(3,12)	(2, 7)	(2, 7)	4, 15	3, 12	3, 13	4,,16	4,,17	3,,13	4,,20	4,,20
MISCELLANEOUS	(5, 11)	(5, 11)	(5, 11)	(2,3)	(4, 11)	(4, 11)	1, 1	2, 2	3,,4	3,,10	4,,11	3,,5	3,,11	3,,11
REGULATION AND CELL SIGNALING	(2, 6)	(2, 6)	(2, 6)	(3,9)	(3, 10)	(4, 17)	3, 9	3, 11	3,,9	3,10	3,,10	3,,14	3,,13	3,,13
Specialty Genes														
Antibiotic Resistance_CARD	4	4	4	6	10	14	3			5	5 CARD 8/ NDARO 3	CARD 2/ NDARO1	CARD 1/ NDARO1	CARD 2/ NDARO2
Antibiotic Resistance_PATRIC	26	26	26	1	1	5	1	24	32	3	46	44	44	45
Drug target_Drug bank	36	36	36	36	40	46	24	8	6	48	69	8	34	34
Drug target_TTD	6	6	6	7	15	19	4	1	1	49	1		1	1
Transporter_TCD	20	20	20	1	6	7	2	17	19	196	299	15	113	112
Virulence Factor_PATRIC_VF	4	4	4	14	21	26	7		2	3	3	3	2	2
Virulence Factor_VFDB	7	7	7	2	12	12	2	1	3		2			
Virulence Factor_Victors	39	39	39	7			5	29	8	2	4	3	2	2

Supplementary Table 4-2, Genome assemble results of each isolates.

Antimicrobial Resistance Genes

AMR Mechanism	WAE1	WAN5	WAN31	WAE30	WAE46	WAE47	WAN26	WAN28	WAN45	WSA25	WSA26	WSA48	WSN32	WSN46
Antibiotic target in susceptible species Alr, DdEF-G, EF-Tu,gyrA,gyrB, Iso-Trna,kasA,MurA,rpoB,rpoC, S10p, S12p	dxr, folA, Dfr, folP, inhA, fabI, rho	dxr, folA, Dfr, folP, inhA, fabI, rho	dxr, folA, Dfr, folP, inhA, fabI, rho	folA, Dfr, inhA, fabI, rho	folA, Dfr, folP, inhA, fabI, rho	folA, Dfr, folP, inhA, fabI, rho	inhA, fabI, rho	folA, Dfr, folP	folA, Dfr, inhA, fabI, rho	dxr, folA, Dfr, folP, inhA, fabI, rho	dxr, folA, Dfr, folP, inhA, fabI, rho	dxr, folA, Dfr, folP, inhA, fabI,rho	dxr,, folA, Dfr, folP, inhA, fabI,rho	dxr, folA, Dfr, folP, inhA, fabI, rho
Efflux pump conferring antibiotic resistance	MacA, MacB	MacA, MacB	MacA, MacB		NorA, YkkCD	NorA, Tet(K), YkkCD				BceA, BceB, EbrA, EbrB, Lmr(B), Tet(L), YkkCD	BceA, BceB, EbrA, EbrB, Lmr(B), YkkCD	BceA, BceB, YkkCD	BceA, BceB, EbrA, EbrB, YkkCD	BceA, BceB, EbrA, EbrB, YkkCD
Gene conferring resistance via absence	gidB	gidB	gidB	gidB	gidB	gidB	gidB	gidB	gidB	gidB	gidB	gidB	gidB	gidB
Protein altering cell wall charge conferring antibiotic resistance	PgsA	PgsA	PgsA	GdpD, MprF, PgsA	GdpD, MprF, PgsA	GdpD, MprF, PgsA	GdpD, PgsA	GdpD, PgsA	GdpD, MprF, PgsA	GdpD, MprF, PgsA	GdpD, MprF, PgsA	GdpD, PgsA, VanXY-unclassified	GdpD, MprF, PgsA	GdpD, MprF, PgsA
Regulator modulating expression of antibiotic resistance genes	H-NS, OxyR	H-NS, OxyR	H-NS, OxyR	LiaF, LiaR, LiaS, VanR-unclassified, VanS-unclassified	BceR, BceS, LiaF, LiaR, LiaS	BceR, BceS, LiaF, LiaR, LiaS	LiaF, LiaR, LiaS	LiaF, LiaR, LiaS	LiaF, LiaR, LiaS	BceR, BceS, LiaF, LiaR, LiaS	BceR, BceS, LiaF, LiaR, LiaS	BceR, BceS, LiaR, LiaS, VanF/M-type, VanG-type	BceR, BceS, LiaF, LiaR, LiaS	BceR, BceS, LiaF, LiaR, LiaS
Antibiotic target modifying enzyme				RlmA(II)			RlmA(II)		RlmA(II)	Cfr, RlmA(II)	RlmA(II)	RlmA(II)	RlmA(II)	Erm(D)/Erm(K), RlmA(II)
Antibiotic inactivation enzyme				AAC(6')-Ia (and related AACs)	FosB	ANT(9)-I, BlaZ family, Lnu(A), Mph(C) family			AAC(6')-Ia (and related AACs)	BcII family, FosB	ANT(6)-I, BSU family, FosB, Vgb(B)		CatA family, FosB	CatA family, FosB
Antibiotic target protection protein				Lsa(A), Msr(c)		Msr(A)	Tet(M)		Lsa(A)	BcrC	BcrC		BcrC	BcrC
Antibiotic resistance gene cluster,cassette,or operon					TcaA, TcaB, TcaB2, TcaR	TcaB, TcaB2, TcaR								
Antibiotic target replacement protein								FabK		fabL	fabL	fabL	fabL	fabL

Supplementary Table 5, Taxonomic assignment with RDP and BLCA for 14 isolates

		Species	bootstrap cutoff (%)	Genus	bootstrap cutoff (%)	family	bootstrap cutoff (%)
WAE1		*Actinobacillus succinogenes*	72.67	*Actinobacillus*	72.67	*Pasteurellaceae*	100
WAE30		*Enterococcus mundtii*	100	*Enterococcus*	100	*Enterococcaceae*	100
WAE46	**	*Staphylococcus succinus*	100	*Staphylococcus*	100	*Staphylocoaceae*	100
WAE47		*Staphylococcus saprophyticus*	54.83	*Staphylococcus*	100	*Staphylocoaceae*	100
WAN26		*Enterococcus cecorum*	100	*Enterococcus*	100	*Enterococcaceae*	100
WAN28		*Streptococcus hyointestinalis*	100	*Streptococcus*	100	*Streptocoaceae*	100
WAN31		*Clostridium polynesiense*	98	*Clostridium*	100	*Clostridiaceae*	100
WAN5		*Actinobacillus Succinogenes*	67.67	*Actinobacillus*	68.67	*Pasteurellaceae*	100
WSA25	**	*Bacillus velezensis*	93.5	*Bacillus*	100	*Bacillaceae*	100
WSA26		*Bacillus mojavensis*	68.13	*Bacillus*	100	*Bacillaceae*	100
WSA48		*Bacillus circulans*	100	*Bacillus*	100	*Bacillaceae*	100
WSN32	**	*Bacillus licheniformis*	100	*Bacillus*	100	*Bacillaceae*	100
WSN46		*Bacillus licheniformis*	97.5	*Bacillus*	100	*Bacillaceae*	100
WAN45	**	*Enterococcus hirae*	100	*Enterococcus*	100	*Enterococaceae*	100

**Prokka 16S was used. Clustal alignment

WAE1	WAE30	WAE46	WAE47	WAN5	WAN26	WAN28	WAN31	WAN45	WSA25	WSA26	WSA48	WSN32	WSN46
accA	bopD	asd	asd	accA	clpP	ccpA	accA	bopD	clpX	bslA/yuaB	clpX	codY	codY
afuB	bopD	citB	citB	afuB	clpP	ciaR	afuB	bopD	codY	clpX	codY	purA	purA
alaS	clpP	clpX	clpX	alaS	lepA	clpP	alaS	bsh	purA	codY	fur	purB	purB
argG	clpP	lysA	femB	argG	map	cpsY	argG	clpP	purB	galU	purA	recA	recA
aroA	lepA	mgrA	mgrA	aroA	psaA	fba	aroA	clpP	RsfA	galU	purB		
atpA	map	msrA	msrA	atpA	purB	gidA	atpA	KtrB		hasC	sodA1		
dksA	perR	oppD	oppD	dksA		glnA	dksA	lepA		purA			
dnaK	purB	purL	purL	dnaK		guaA	dnaK	lmo2067		purB			
fdx-2	thyA	pyrAA	pyrAA	fdx-2		hasC	fdx-2	map		sodA1			
galU		recA	recA	galU		lepA	galU	perR					
gmhA/lpcA		SA1453	SA1453	gmhA/lpcA		leuS	gmhA/lpcA	purB					
guaA		trpB	trpB	guaA		luxS	guaA	thyA					
hemE				hemE		pepC	hemE						
hlyX				hlyX		purB	hlyX						
hupA				hupA		purH	hupA						
kdsA				kdsA		purN	kdsA						
lpdA				lpdA		sodA	lpdA						
lpxC				lpxC		SP_0121	lpxC						
metB				metB		SP_0494	metB						
metF				metF		SP_0829	metF						
mglA				mglA		SP_0856	mglA						
msbB				msbB		SP_0943	msbB						
napB				napB		SP_1278	napB						
nqrE				nqrE		SP_1396	nqrE						
nqrF				nqrF		SP_1398	nqrF						
obgE				obgE		SP_1399	obgE						
parE				parE		SP_1780	parE						
PM1466				PM1466		SP_1970	PM1466						
pnp				pnp		SP_1976	pnp						
prfC				prfC		SP_2210	prfC						
ptsI				ptsI			ptsI						
pyrF				pyrF			pyrF						
recR				recR			recR						
rfaD				rfaD			rfaD						
rfaE				rfaE			rfaE						
rpmF				rpmF			rpmF						
rpoE				rpoE			rpoE						
thrC				thrC			thrC						
trpB				trpB			trpB						
tufA				tufA			tufA						
ureC				ureC			ureC						
yegQ				YegQ			yegQ						

Supplementary Table 7, Other predicted pathways for isolates

	WAE 46	WSA 48	WAE 47	WSN 32	WSN 46	WSA 26	WSA 25	WAN 28	WAN 45	WAE 1	WAN 5	WAN 31	WAN 26	WAE 30
mTOR signaling pathway	Y	Y	Y	Y	Y	Y	Y	/	/	/	/	/	/	/
T cell receptor signaling pathway	/	Y	/	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

"/" : represents this predicted pathway was not found in this isolate.

PART III

General discussion and conclusions

Chapter 6

General discussion

From the work in this Thesis performed, two major findings can be highlighted:

(a)   On one hand, FMT from warthogs to domestic pigs, definitively demonstrates the importance of microbiota in ASF susceptibility

(b)   On the other hand, warthog fecal microbiota contains individual bacteria with astonishing probiotic, microbicidal and immunostimulatory capabilities.

As probably occurs with most Theses, our work has opened more questions than provided answers. We will focus our next pages to discuss some of the most intriguing results obtained, aiming to provide some light favoring the continuation of this exciting new line of research.

1.   Microbiota and ASFV infection. The first question we ask ourselves is why warthog FMT only worked against attenuated E75CV1, but not against virulent E75 strain. The aid provided by the warthog FMT, most probably in the form of non-specific immune stimulation, could be enough to provide help to mount an immune response against the E75CV1 slow infection, having no chances against the virulent E75, a virus that triggers a marked immunosuppression in a matter of hours, killing pigs within a week (Lacasta *et al.*, 2015). Taking into account the mucosal benefits of FMT, confirmed also here after inoculating 15-selected bacteria (Zhang *et al*., 2020b), we propose performing an in-contact challenge with E75 in the near future, resembling more to what occurs in nature. Work performed in our laboratory confirms that in-contact challenge is less aggressive than the standardized intramuscular one, and ideal for testing the protective potential of this new strategy willing to control the virus at the site of entry, the oronasal mucosa (Bosch Camós, 2019).

2.   Microbiota and Health. The characteristics of the bacteria isolated from warthog feces clearly demonstrate that their potential goes beyond the ASF fight and may have a role against other pig diseases or diseases affecting other animals or, even, to be used in human beings. At the end, probiotics used today in human health proceed from other animal species, including cows, goats or sheep (Yerlikaya, 2014). The first principle of a probiotic seems to be accomplished, since FMT from warthog to domestic pigs was not harmful to domestic pigs and, at the same time, the daily weight gain for warthog-feces transplanted pigs was higher than both PBS and pig-feces transplanted pigs. In correspondence with previous FMT works, the microbiota composition changes were evident enough to ensure the transplantation success, but not for identifying any

specific bacterial genus as responsible of the beneficial effects observed. One potential explanation for this result might come from the detection methods used, not sensitive enough to detect specific bacterial populations that exert their benefits without the need to be present in large amounts (Benjamino *et al.*, 2018). This has been specially demonstrated for bacteria with immune stimulatory capabilities that do not need to "colonize" the gut, in the strict sense of the term, to provoke the desired effect (Benjamino *et al.*, 2018; Jun *et al.*, 2018). The use of amplicon sequencing has proven to be cost-effective for the microbiome analysis, but has some vulnerabilities regarding biases through sample preparation and sequencing errors. Moreover, 16S rRNA sequencing is limited to taxonomical classification at genus level providing useful but limited information (Rausch *et al.*, 2019). We plan for the next experiment to perform metagenomics shot-gun expanding the analysis, increasing the sensibility and allowing functional characterization of the bacterial communities.

3.   Microbiota and Immunostimulation.

a.   *Rothia*. In this regard, a specific bacterium isolated from warthog feces have caught our attention: WAE49 isolate, belonging to *Rothia* genus. From the ones tested, this is the only isolate capable to stimulate GALT tissue *in vitro* a dramatic overexpression of a cytokine that plays a key role in ASF protection and against many other intracellular pathogens (Murray, 1992). IFNγ secretion by ASFV-specific lymphocytes correlates with the protection afforded by attenuated ASFV strains (King *et al.*, 2011; Lacasta *et al.*, 2015; Monteagudo *et al.*, 2017), including E75CV1, the ASFV strain used in this study (Lacasta *et al.*, 2015; Monteagudo *et al.*, 2017). Additionally, treatment with pig IFNγ can inhibit the replication of ASFV on PAMs *in vitro*, significantly reducing the ASFV viral load in blood *in vivo* (Fan *et al.*, 2020). Mainly due to its immunostimulatory properties, WAE49 was included in the cocktail of 15 warthog bacteria isolates used in our second *in vivo* experiment. Taking into account the positive effect that IFNγ provokes on IgA production (Estes, 2010), it allows hypothesizing with WAE49 playing a key role in the increased of total IgA found in the sera of inoculated pigs. The improvement in the mucosal immunity, translated in a larger number of plasma cells secreting large amounts of secretory IgA (both ASF-specific and non-specific), would promote in turn, the induction of IFNγ by T-cells using TNFα dependent mechanism (Chen *et al.*, 2020). The concomitant

overexpression of IFNγ, a key player in immune protection against ASFV (Fan *et al.*, 2020; King *et al.*, 2011) and ASFV-specific IgA, also correlate with immunoprotection (Leitão *et al.*, 2001). Unfortunately, the IFNγ ELISPOT performed did not allow distinguishing between immunization groups. Technical limitations did not allow the quantification of the ASFV-specific T-cells induced, nor to measure their potentially different avidity. Members from the *Rothia* genus are commensal bacteria common in oral and intestinal microbiomes in humans, pigs, and rodents (Kernaghan *et al.*, 2012; Mann *et al.*, 2015; Zaura *et al.*, 2009), thus providing additional advantages for their use as mucosal adjuvant (Menard *et al.*, 2007). Future work will concentrate in evaluating the use of these bacteria, specially *Rothia* isolate, as potential mucosal adjuvant of live attenuated ASFV vaccines, delivered by the intranasal route, an immunization pathway that has improved the protection results obtained after intramuscular delivery (Sánchez-Cordón *et al.*, 2017).

b. ***Bacillus*.** Two additional bacteria, WSA25 (*B. velezensis*) and WSA26 (*B. mojavensis*), excluded in the *in-vivo* experiment with 15 selected bacteria, belonging to the *Bacillus* genera, stimulated GALT to secrete IL18, albeit the activation levels achieved did not seem as high as that observed with WAE49 and IFNγ . Interestingly, certain species from *B. mojavensis* and *B. velezensis* are commonly used in agriculture, showing anti-fungal, anti-viral and antimicrobial abilities for the benefit of plant roots, improving the growth rate of plants (Jasim *et al.*, 2016; Mounia *et al.*, 2014). Additionally, *B. mojavensis* is used as dietary additive in fish food, showing a beneficial effect by efficiently fighting yersiniosis in fish, a bacterial infection (Mounia *et al.*, 2014). The IL1 family of cytokines are being used as mucosal vaccine adjuvants in experimental vaccines against influenza virus, due to their ability to enhance the protective secretory IgA and CTL immunity induced (Kayamuro *et al.*, 2010). As described for *Rothia*, using WSA25 and WSA26 as mucosal adjuvants, together with our experimental ASFV vaccines, might be an avenue worthy to explore.

4. **Warthog microbiota and Virulence factors.** Besides the immunostimulatory capabilities of the bacteria described above, the study of microbicidal potential of bacteria originated from warthog feces kept much of our initial attention to the point of selecting 14 different bacteria for full-genome sequencing attending to this criteria, and coinciding with the end of my Thesis work. One of the most striking findings was the

high number or warthog bacteria feces with proved ability to inhibit the *in vitro* growth of pathogenic bacteria, being of particular interest the anti-clostridial activity observed in bacteria from different genera.

a. ***Colicin V*:** Interestingly, three of the more efficient bacteria inhibiting isolates, WAE1,WAN5 and WAN31 (both belonging to the *Actinobacillus* genus) encode *colicin V*, a bactericidal peptide found in *Enterobacteriaceae* and lactic acid bacteria (Gérard *et al.*, 2005; McCormick *et al.*, 1999), and normally active against phylogenetically closely related bacteria to the producer (Gérard *et al.*, 2005). Future work should be performed to clarify if the anti-*Clostridium* activity showed *in vitro* is due to *colicin V* and if so, why its inhibition effect is much lower against *Salmonella* and *E. coli.* WAE1,WAN5 and WA31 also share the ability to produce succinate, an inflammatory signal that induces IL1β (Mills *et al.*, 2014; Tannahill *et al.*, 2013), a key effector molecule that, together with IL18, orchestrate the immune response against pathogenic bacteria *in vivo* (Rathinam *et al.*, 2019). Interestingly, WAE1 and WAN5 stimulated GALTs cells to secrete low, albeit detectable IL18. With these results in hand, we hypothesize that there might be a direct antimicrobial effect mediated by *colicin V* and an indirect immunostimulatory effect triggered by expressing succinate; hypothesis that will need further experimental work.

b. ***Enterocin A*:** As expected for *Enterococcus* genus, two isolates from this genus (WAN26 and WAE30), possessed a gene encoding *enterocin A*, a bactericidal protein, capable to kill different bacteria (Nes *et al.*, 2000), that might be also related with the antimicrobial capacity observed in our experiments.

5. **Intestinal bacteria and their metabolites.** Including organic acids, such as short-chain fatty acids (SCFA) and succinate, several metabolites are known to play an important role in the maintenance of intestinal epithelium physiology, and modulating the response after inflammatory/infectious stimulation (Corrêa-Oliveira *et al.*, 2016; Donohoe *et al.*, 2011; Gijs *et al.*, 2013).

a. **Bile salt hydrolase.** WAE46 (*Staphylococcus*), WAE30 (*Enterococcus*) and WAN26 (*Enterococcus*) and several other bacteria not included in the *in vivo* experiment (WAE47, WAN28, WAN45, WSA25 and WSA26) encode a gene related with bile salt hydrolase (BSH, EC 3.5.1.24). The presence of BSHs is one of the probiotic selection criteria, acting as signaling molecules which can activate multiple

receptors, facilitating the secretion of glucagon-like peptide and improving strain competitiveness within the gut (Hill *et al.*, 2006; Song *et al.*, 2019). The secondary bile acids, which exclusively are generated by gut bacteria, have varies immunomodulatory effect on innate immunity (Fiorucci *et al.*, 2018).

b.   **Organic acids.** Other bacteria metabolites, including medium chain fatty acids and SCFAs, can enhance mucosal immunity (Niederwerder *et al.*, 2020). SCFAs can directly promote T-cell differentiation into T cells producing IL17, IFNγ and/or IL10 (Tan *et al.*, 2016). High fiber feeding in mice can boost IgA production and enhance T follicular helper and mucosal germinal center responses, by increasing the production of SCFAs (Niederwerder *et al.*, 2020). In a preliminary analysis, butyric acid was detected in the supernatant of WAE21, WAE28, WAE29, and WAN43; isovaleric acid was present in the supernatant of WAN5, WAE49 and WAN43; propionic acid was detected in the supernatant WAN5, WSA48, WAE21, WAE29, WAN43, WSN45 and WSN48; furthermore, acetic acid was identified in the supernatant of all the isolates above (data not shown). SCFAs produced by the inoculated bacteria might also contribute to the IgA increase observed in our study.

c.   **Vitamin B.** Vitamins are essential micronutrients for mammals. Gut bacteria are the producers and consumers of B vitamins for its host (Yoshii *et al.*, 2019). Vitamin B6 deficiency weakened the immune response through affecting T lymphocyte differentiation and proliferation and IFN γ expression using BALB/c mouse model (Qian *et al.*, 2017). Vitamin B1 depletion impairs TCA cycle activity, and Vitamin B1 is required for the maintenance of naïve B cells to induce intestinal IgA responses against oral vaccines (Kunisawa *et al.*, 2015). Vitamin B3 can increase the CD4+ T cells recovery in HIV-infected patients with suboptimal immune responses despite sustained virologic suppression (Lebouché *et al.*, 2014).   The *in-silico* analysis demonstrated the presence of genes related with biosynthesis of vitamin B1 and B6 in the genome of WAN5, WAN31 and WAE1; four isolates, WSA25, WSA26, WSN32 and WSN46, have genes related with vitamin B1, B3, and B6, while the isolate WSA48 has genes for vitamin B3 and B6 production. Furthermore, a gene related with vitamin B3 was found in WAN28 and other genes related with vitamin B6 were found in the genome of WAN26, WAE46 and WAE47.

6. **Warthog microbiota and anti-clostridial activity**. Interestingly, the supernatant of two bacterial isolates belonging to the *Bacillus* genus (WSA25, *B. velezensis* and WSA26, *B. mojavensis*), are capable to inhibit the growth of of *C. perfringens in vitro.* Conversely, the rest of the bacteria with anti-clostridial activity required direct contact with alive bacteria. So far, no canonical bactericidal molecules have been found, albeit further *in silico* assays should be performed. Identifying soluble components with anti-clostridial properties might be of great interest for the pharmaceutical companies, overall if they demonstrate efficient against C*lostridium difficile*. Regarding the use of pathogenic bacteria in our bactericidal experiments, reference strains from our lab were used, with the exception of *C. perfringens,* isolated from piglets with serious diarrhea. The pathogenicity of this *C. perfringens* isolate was confirmed with the presence in its genome of alpha, beta, epsilon and iota toxins (data not shown) associated with different digestive pathologies in diverse animal species, including pigs, at least for the case of the toxin beta (Uzal, 2013). Toxin epsilon is related to enterotoxemia in sheep, goat and/or cattle (Uzal, 2013). The complex nature of this C*lostridium* isolate, together with the strong inhibition found with several warthog microbiota isolates, reinforce our idea of further studying their inhibitory capabilities against *C. difficile,* a human pathogen against which only experimental FMT has proven efficient.

7. **Warthog bacteria and biofilm.** Interestingly, a gene related to biofilm formation in *Staphylococcus* was found only in WAE47, not in WAE46, despite both belonging to the *Staphylococcus* genus and forming good biofilms, albeit better in aerobic than anaerobic conditions. Additionally, a gene related to 'quorum sensing and biofilm formation' pathway was also found in WAE1, WAN5, WAN31, WAN28 and WSA48. Biofilms are the aggregates of microorganisms embedded in a self-produced polymeric matrix in a sessile state (Rodney, 2002) that facilitate the colonization and maintenance of bacteria on colonized surfaces. Both probiotic and pathogens can take advantage of biofilm formation. On one hand, probiotic biofilms can stimulate longer stability of probiotics in the host mucosa that in turn, prohibit colonization of pathogenic bacteria (Costerton *et al.*, 1999; Terraf *et al.*, 2012; Walencka *et al.*, 2008). On other hand, pathogenic bacteria use biofilm to achieve persistence infection. Interestingly, WSN32 and WSN46, two bacteria that form biofilm with great difficulty,

encode in their genome of genes encoding several Rap proteins (Rap A, D, G, I, K, E), with Rap K recently described as capable of inhibiting the biofilm formation (Gabriela *et al.*, 2019).

8.   A very special case affects to the WSN45 isolate, identified as *B. mojavensis* or *B. tequilensis*, the only bacterium from warthog fecal microbiota available in our collection that proved beneficial for both ileum and colon organoids viability. Unexpectedly, WSN45 showed very low biofilm formatting ability, nor immunostimulatory capabilities *in vitro,* at least for the tested cytokines. Lack of biofilm formation in another *B. tequilensis* strain isolated from goat milk, was associated with the its ability to produce levan, which can inhibit pathogenic biofilm formation (Abid *et al.*, 2019), and this capability of levan could be considered as a new antibiofilm strategy in contrasting bacterial pathogenic biofilms (Spanò *et al.*, 2016). Unfortunately, the genome of WSN45 was not sequenced, but remarkably, genes related to sucrose-to-levan conversions (Levanase EC 3.2.1.65) were found in the genome of WSN32, WSN46, WSA26 and WSA48, independently of their potential to induce biofilm formation. This apparent controversy might be explained by the multifunctional nature of the levan molecule. Thus, levan is also an immunostimulatory moiety, able to induce antagonist cytokines such as the anti-inflammatory cytokine IL4 in HT-29 cells (Taylan *et al.*, 2019) and exert strong activity to induce production of IL12 p40 and TNFα by macrophage cell lines *in vitro* (Xu *et al.*, 2006).

Closing remarks.

Interesting microbicidal results were obtained from fecal isolates of warthog. However, we are aware that these 135 isolates are limited to aerobic or facultative anaerobic bacteria capable to grow in our basic culture media. Therefore, a very low percentage of the total commensal flora will be here represented. On the other hand, we are convinced that the effect of FMT and warthog microbiota might bring benefits, not only against ASFV, but also against other pathogens. The microbicidal properties of different isolates should be tested with optimized *in vitro* systems. Furthermore, the microbicidal and immunostimulatory capabilities of simple formulations should be investigated. Finally, safety issues including the presence of virulence factors and

transferable antibiotic resistance should be investigated. We are convinced that we have a unique collection of bacteria with outstanding properties that deserve further investigation. The genome sequence of a small part of the collection, led us with valuable information that need to be considered in future experiments. Moreover, we plan for the near future the full-genome sequencing of all the isolates that showed distinctive immunostimulatory capacity or other potential probiotic characteristics. We plan for the near future investigating specific isolates as potential mucosal **adjuvant** of live attenuated ASFV vaccines. Animals, including humans, are consuming *Lactobacillus* and other microbiota components from diverse animal species. If they provide additional advantages, why not adding wild live microbiota components in the future diets?

Chapter 7

Conclusions

Conclusions

1. Fecal microbiota transplant (FMT) from warthog to domestic pigs is not harmful and conversely, the average daily weight gain observed in transplanted pigs was statistically higher than in the control group between day 15 and 30 post-FMT.

2. FMT modifies the fecal microbiota composition of the recipient domestic pigs.

3. FMT from warthogs to domestic pigs confers partial protection against experimental intramuscular challenge with an attenuated strain of ASFV (E75CV1), while pig FMT does no afford any protection.

4. Bacteria isolated from the gut microbiota of warthogs and pigs are phylogenetically distant when characterized through analysis of 16S rRNA gene.

5. One of these warthog bacteria isolates, dramatically improves the growth of ileum and colon organoid pig cells

6. Sixty-six individual bacteria isolates from warthog microbiota but none from pig microbiota, show *in vitro* microbicidal properties against one or more pig pathogens, including against: *Clostridium perfringens* type B, *Salmonella* pathogenic strains, *E. coli* K88 and/or the virulent strain *Streptococcus suis* S10 without interfering with the low-pathogenic T15 strain.

    6.1   The complete genome analysis of 14 of these bacteria, reveals two exclusive pathways, C21-steroid hormone metabolism and linoleic acid metabolism, in bacteria showing anti-*Clostridium* activity. Other pathways found related with the production of succinate colicin V, enterocin A and other virulence factors, might contribute to their microbicidal properties.

7. Three additional bacteria isolated from warthog gut, but none from pig gut, were capable to stimulate gut associated lymphoid tissue cells to secrete IFNγ or IL-18.

8. Based on their *in-vitro* characteristics, fifteen bacteria from warthogs feces were selected for intragastrical inoculation to domestic weaned piglets and further challenge with the attenuated E75CV1 strain of ASFV. This cocktail of 15 bacteria isolated from warthogs' feces improves the mucosal immunity denoted by an increase of the total IgA concentration in sera detectable before ASFV challenge and an increase in the ASFV-specific IgA detected after ASFV challenge, both in sera and nasal swabs.

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The Road Not Taken

----------Robert Frost

Two roads diverged in a yellow wood,

And sorry I could not travel both

And be one traveler, long I stood

And looked down one as far as I could

To where it bent in the undergrowth.

Then took the other, as just as fair,

And having perhaps the better claim,

Because it was grassy and wanted wear,

Though as for that the passing there

Had worn them really about the same.

And both that morning equally lay

In leaves no step had trodden black.

Oh, I kept the first for another day!

Yet knowing how way leads on to way,

I doubted if I should ever come back.

I shall be telling this with a sigh

Somewhere ages and ages hence:

Two roads diverged in a wood, and I--

I took the one less traveled by,

And that has made all the difference.