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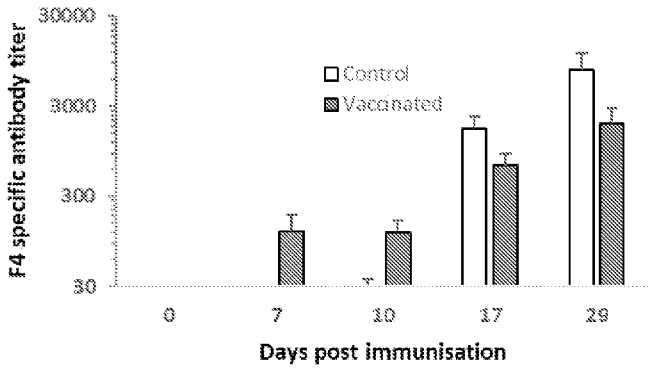

Figure 6A

(57) Abstract: The present invention relates to a subunit vaccine, especially an edible subunit vaccine, against porcine post-weaning diarrhoea, and to the production thereof in filamentous fungi, especially in Trichoderma reesei.

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A SUBUNIT VACCINE AGAINST PORCINE POST-WEANING DIARRHOEA

FIELD OF THE INVENTION

The present invention relates to a subunit vaccine, especially an edible subunit vaccine, against porcine post-weaning diarrhoea, and to the production thereof in filamentous fungi, especially in *Trichoderma reesei*.

BACKGROUND OF THE INVENTION

Demand of dietary animal protein is rising with the expanding human population. Intensive animal farming brings challenges in animal health care, e.g. post-weaning diarrhoea (PWD) is an important enteric disease that usually occurs shortly after weaning. It is characterized by watery diarrhoea, dehydration, loss of body weight and death of e.g. infected pigs. PWD is induced by enterotoxigenic *Escherichia coli* (ETEC), and it is a major cause of mortality, morbidity and deceased growth of young born piglets, and can cause severe economical losses in pig farming (Fairbrother et al., 2015).

Diet, containing e.g. acidifiers, enzymes and bacterial probiotics, can help to prevent the incidence of PWD but cannot control the outbreaks (Fairbrother et al., 2015). Indeed, PWD is one of the most common reasons for antibiotic usage in pig industry. Antibiotics and antimicrobials work against PWD; however, they are overused in meat production and their usage needs to be limited in the future due to the risks of developing multiresistant pathogenic strains and preventing outbreaks of these. In addition, multidrug resistance has transferred to human pathogens causing a severe threat to human health (Van Boeckel et al., 2015; Ye et al., 2016).

Suckling piglets are usually protected by the maternal antibodies transferred through milk but become vulnerable for *E.coli* infections post weaning (Rutter and Jones, 1973). Breeding of resistant pigs is hard and excludes the lactogenic protection during suckling period. Furthermore, passive immunotherapy remains not economically feasible.

Therefore, vaccination of the piglets is desired. Current parenteral vaccines do not elicit protective defence in piglet gut (Van den Broeck et al. 1999a). There is an oral vaccine available for use for the piglets but it is based on live-attenuated bacteria, which limits the usability (Anonymous, 2015). Thus, there is an identified need in the art for vaccines against PWD that overcome the problems associated with the current vaccines and treatment modalities against PWD.

BRIEF DESCRIPTION OF THE INVENTION

An object of the present invention is to provide a vaccine and a method of producing and using the vaccine so as to overcome the above problems associated with conventional treatment or prevention of PWD. The object of the invention are achieved by a vaccine and a method which are characterized by what is stated in the independent claims. The preferred embodiments of the invention are disclosed in the dependent claims.

Accordingly, the present invention provides a vaccine for porcine post-weaning diarrhea, comprising a filamentous fungus-derived FaeG subunit of a F4 fimbriae of enterotoxigenic Escherichia coli (ETEC).

The present invention also provides a method of producing vaccine for porcine post-weaning diarrhoea, the method comprising transforming a filamentous fungal host cell with a polynucleotide encoding FaeG, cultivating the transformed host cell under cultivation conditions suitable for expression and secretion of FaeG into conditioned culture medium, and recovering the conditioned culture medium comprising FaeG. Also provided is a vaccine composition obtainable by this method.

Further aspects provided by the present invention include a vector comprising a FaeG-encoding nucleic acid sequence, and filamentous fungal host cell genetically modified to express FaeG.

Further aspects, specific embodiments, object, details, and advantages of the invention are set forth in the following drawings, detailed description, and examples.

BRIEF DESCRIPTION OF THE DRAWINGS

In the following the invention will be described in greater detail by means of preferred embodiments with reference to the attached drawings.

Figure 1. A schematic presentation of expression constructs for FaeG production in *Trichoderma reesei*.

Figure 2. Detection of FaeG protein from small scale cultivations and from 15l fermentation samples. M1351, parent strain w/o FaeG; M1779, FaeG-producing strain transformed with pJJJ783. M1779 was used for fermentation culture and culture supernatants were collected in different time points and analyzed by total protein staining or by FaeG specific immunodetection.

Figure 3. Purification of FaeG from Trichoderma culture supernantant by immobilized metal ion affinity column chromatography.

Figure 4. Detection of FaeG protein in F4 specific double sandwich enzyme linked immunosorbent assay.

Figure 5. Inhibition of E. coli binding to villous brush borders after preincubation with FaeG protein or F4 fimbriae.

Figure 6. Oral immunization with monomeric FaeG protein evokes F4-specific immune response in pigs. Figure 6A shows that F4 specific antibodies were found in the serum of FaeG vaccinated (grey bars) piglets already 7 days post first immunization. The control group (white bars) raised a serum response only after the challenge that was done 10 days after the first immunization. Figure 6B shows that the piglets that had been immunized with FaeG (grey bars) secreted 10-1000 times (days 1-4 post challenge) less ETEC bacteria following the ETEC challenge than the unvaccinated group (white bars). n=7, error bars indicate standard error of the mean.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the prevention of porcine post-weaning diarrhea (PWD) outbreaks caused by enterotoxigenic *Escherichia coli* (ETEC) that express F4 (K88) adhesive fimbriae and produce enterotoxins that induce secretory diarrhoea.

As used herein the term "F4 fimbriae" or "F4" refer to long filamentous polymeric surface proteins of ETEC, consisting of so-called major (FaeG) and minor (FaeF, FaeH, FaeC, and probably FaeI) subunits. Three antigenic variants of the F4 fimbriae have been described, namely serotypes F4ab, F4ac, and F4ad, with F4ac being the most prevalent. All these variants are herein encompassed by the term "F4", unless otherwise indicated. The F4 fimbriae allow the microorganisms to adhere to F4-specific receptors present on brush borders of villous enterocytes and consequently to colonize the small intestine.

The present invention provides a PWD vaccine that is a subunit vaccine comprising a FaeG polypeptide of one or more F4 serotypes in any desired combination. In some embodiments, the vaccine comprises FaeG of serotype F4ac and/or serotypes, F4ab F4ad. The vaccine elicits a protective immunogenic response, i.e. provokes acquired immunity, against F4-expressing ETEC. In some embodiments, the FaeG polypeptide comprises an amino acid sequence set forth in SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NO: 5.

Accordingly, FaeG antigens that are uptaken via gut receptors, will evoke a protective mucosal immune response against ETEC in the gut. In addition,

free antigen subunits can outcompete ETEC bacteria from gut receptors immediately after administration of the vaccine thus providing also a passive protection.

In some embodiments, FaeG is produced in a fungal host, preferably in isolated cells of a filamentous fungus.

As used herein, the terms "filamentous fungus" and "filamentous fungal host" refer to any filamentous fungal cell that is suitable for producing the present subunit vaccine. Such fungal cells include, but are not limited to, cells from the genera *Acremonium, Aspergillus, Fusarium, Humicola, Mucor, Myceliophthora, Neurospora, Penicillium, Scytalidium, Thielavia, Tolypocladium,* and *Trichoderma.* Examples of particularly useful species from *Neurospora* include *N. intermedia, N. crassa, N. sitopula,* and *N. tetraspora*; examples of particularly useful species from *Aspergillus* include *A. nidulans, A. niger, A. oryzae, A. terreus*, and *A. fumegatus*; whereas examples of particularly suitable species from *Trichoderma* include *T. harzianum, T. koningii, T. longibrachiatum, T. atroviride, T. virens, T. viride,* and *T. reesei.* In some embodiments, *T. reesei* is the most preferred filamentous fungal host.

Filamentous fungal cells to be employed for producing the present vaccine may be derived from a wild-type fungal strain or from a genetically modified fungal strain, such as a protease deficient fungal strain. WO 2013/102674 discloses *Trichoderma* fungal cells having reduced or no detectable activity of at least three endogenous proteases selected from *pep1*, *pep2*, *pep3*, *pep4*, *pep5*, *pep8*, *pep11*, *pep12*, *tsp1*, *slp1*, *slp2*, *slp3*, *slp7*, *gap1*, and *gap2*, resulting in increased expression and stability of recombinantly expressed heterologous proteins. Further genetically modified *Trichoderma* fungal cells having reduced or no detectable activity of one or more endogenous proteases including *amp2, mp1, mp2, mp3, mp4, mp5, pep9, amp1,* and *sep1* are disclosed in WO 2015/004241.

In some embodiments, the filamentous fungal host cells are wild-type *Trichoderma* cells, preferably *T. reesei* cells. In some other embodiments, the filamentous fungal host cells are *Trichoderma* cells, preferably *T. reesei* cells, having reduced or no detectable activity of one or more, preferably at least three, extracellular proteases including, but not limited to, aspartic proteases, trypsin-like serine proteases, subtilisin proteases, glutamic proteases, metalloproteases, and sedolisin proteases. In a more specific embodiment, the filamentous fungal host cells are *Trichoderma* cells, preferably *T. reesei* cells, carrying deletions for one to three extracellular protease genes selected from aspartic protease genes *pep1*

and *pep4*, and trypsin-like serine protease gene *tsp1*. Alternatively or additionally, the host cells may carry a *mus53* deletion for enhanced homologous integration.

In accordance with the above, one aspect of the invention provides a method of producing the present subunit vaccine in a filamentous fungal host cell. The method comprises transforming the host cell with a polynucleotide encoding FaeG, cultivating the transformed host cell under cultivation conditions suitable for expressing FaeG, and recovering the conditioned culture medium comprising FaeG.

Transformation of a fungal host cell, i.e. incorporation of a polynucleotide encoding FaeG into the host cell, may be carried out by any suitable transformation technique available in the art. Such techniques include, but are not limited to, protoplast-mediated transformation, *Agrobacterium*-mediated transformation, electroporation, biolistic method and shock-wave-mediated transformation.

A FaeG-encoding polynucleotide may be produced by any suitable technique available in the art. Such techniques include, but are not limited to, chemical synthesis, cloning, isolation from natural sources, and DNA amplification, such as PCR amplification.

For transformation, the FaeG-encoding polynucleotide may be incorporated into an expression vector, such as a plasmid, using means and methods well known in the art. The skilled person knows which of the expression vectors available are suitable for transforming a selected fungal host cell. In some embodiments, the vector is a self-replicating vector that is maintained extra-chromosomally in the host cells after transformation. In some other embodiments, the vector is an integrating vector. In such embodiments, the entire expression cassette integrates into the genome of a fungal host transformed with the vector. To this end, the expression cassette is typically surrounded by homology flanks that enable insertion of the expression cassette to a desired locus in the host genome. Preferably, the transformation results in stable transformation.

In general, an expression vector for use in the present invention comprises a nucleic acid sequence encoding FaeG (e.g. a polynucleotide sequence set forth in SEQ ID NO: 1, or SEQ ID NO: 3) operably linked to an expression-regulating region comprising nucleic acid sequences that encode a functional promoter and a terminator sequence (including a polyadenylation sequence). Preferably, the FaeG-encoding nucleic acid molecule is also operably linked to a signal sequence resulting in the expression of FaeG as a secreted fusion protein. In some embodiments, the signal sequence comprises or consists of an amino acid

sequence set forth in SEQ ID NO: 7, or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 6.

As used herein, the term “promoter” refers to a nucleic acid sequence which is recognized by the particular filamentous fungus for expression purposes. It is operably linked to a nucleic acid sequence encoding the signal sequence and FaeG. Such linkage comprises positioning of the promoter with respect to the initiation codon of the nucleic acid sequence encoding the signal sequence of the expression vector. The promoter sequence contains transcription and translation control sequences which mediate the expression of the signal sequence and FaeG polypeptide as a fusion polypeptide. Examples include the promoter from *A. niger* glucoamylase and alpha-glycosidase, *A. nidulans* trpC, and *Trichoderma* cellulases such as cellobiohydrolase (cbh), preferably *T. reesei* cbhI. Further suitable promoters are readily available in the art. In some embodiments, the promoter is an inducible promoter. In some other embodiments, the promoter is a constitutively active promoter.

As used herein, the term “terminator” refers to a nucleic acid sequence which is recognized by the expression host to terminate transcription. It is operably linked to the 3' end of the nucleic acid encoding the FaeG to be expressed. Examples include the terminator from *A. niger* glucoamylase or alpha-glycosidase, *A. nidulans* trpC, and *Trichoderma* cellulases such as cellobiohydrolase (cbh), preferably *T. reesei* cbhI. Further suitable terminator sequences are readily available in the art. The terminator sequence comprises a "polyadenylation sequence", a nucleic acid sequence which when transcribed is recognized by the expression host to add polyadenosine residues to transcribed mRNA.

As used herein, the term "signal sequence" refers to an amino acid sequence which when operably linked to the amino-terminus of a FaeG polypeptide permits the secretion of the FaeG polypeptide from the host filamentous fungus. A signal sequence can be operably linked to the FaeG polypeptide by joining a nucleic acid sequence encoding the signal sequence to a nucleic acid sequence encoding the FaeG polypeptide in a proper reading frame to permit translation of the signal sequence and the FaeG polypeptide as a fusion polypeptide. In some embodiments, the signal sequence is a fungal signal sequence of a fungal gene encoding a protein selected from the group consisting of cellulase, cellobiohydrolase, beta-galactosidase, xylanase, pectinase, esterase, protease, amylase, chitinase, chitosanase, polygalacturonase and hydrophobin. More specific examples of suitable fungal signal sequences include *A. niger* glucoamylase and

Trichoderma cellulases, such as cellobiohydrolase (cbh), preferably *T. reesei* cbhI. However, any signal sequence capable of permitting secretion of the FaeG polypeptide may be employed in the present invention. In some embodiments, the expression-regulating region and the signal sequence are from the same source.

In some embodiments, carrier protein is linked to the FaeG polypeptide through a protein-processing site. In some embodiments, the protein-processing site is a Kex2 cleavage site having an amino acid sequence set forth in SEQ ID NO: 11 or encoded by a nucleic acid sequence set forth in SEQ ID NO: 10. Accordingly, later in the secretion pathway, FaeG is released from the fusion by endogenous cleavage by Kex2 protease that recognizes the site NVISKR between the fusion partners.

If FaeG is not expressed as a fusion with a signal sequence, the FaeG polypeptide is not secreted. In such embodiments, FaeG may be obtained from cell lysates using means and methods well known in the art.

In some embodiments, FaeG may be produced as a fusion with a carrier protein, such as CBHI carrier. In some preferred embodiments, the CHBI carrier protein comprises or consists of an amino acid sequence set forth in SEQ ID NO: 9 or encoded by a nucleic acid sequence set forth in SEQ ID NO: 8.

It is well known that deletion, addition or substitution of one or a few amino acids does not necessarily change the properties of a protein. Therefore the invention also encompasses functionally equivalent variants and fragments of the given amino acid sequences and nucleic acid sequences encoding said amino acid sequences. As used herein, the terms “functionally equivalent variant” refers to a sequence having minor changes in the amino acid or nucleic acid sequence as compared to a given sequence but it still functions in substantially the same manner as the given polypeptide or polynucleotide. Functionally equivalent nucleotide sequence variants include variants arising from the degeneration of the genetic code and from silent mutations. Nucleotide substitutions, deletions and additions are also included. Functionally equivalent amino acid sequence variants include variants arising from amino acid substitutions with similar amino acids well known in the art. Amino acid deletions and additions are also included. As used herein, the term “functionally equivalent variant” is interchangeable with the term “conservative sequence variant”. Such variants may occur naturally e.g. as an allelic variant within the same strain, species or genus, or it may be generated by mutagenesis or other gene modification.

Accordingly, the polypeptides and polynucleotides disclosed herein

include those, which have at least 80% sequence identity, or at least 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the given sequence provided that functional equivalency is retained. In other words, functionally equivalent sequence variants refer to FaeG polypeptides capable of eliciting a protective immunogenic response against F4-expressing ETEC, and to polynucleotides encoding said FaeG polypeptides.

As used herein, the percent identity between two sequences is equivalent to the percent homology between the two sequences. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using standard methods known in the art.

After transformation, viable transformants may be identified by screening for the expression and secretion of the FaeG polypeptide. Alternatively, an expressible selection characteristic may be used to isolate transformants by employing an expression vector that encodes for a selection marker. Non-limiting examples of suitable selection characteristics include resistance to various antibiotics (e.g. hygromycin) and sequences encoding genes which confer a nutritional (e.g. acetamidase) or morphological marker in the expression host.

After transformation, the host cells are cultivated in a suitable culture medium under conditions allowing the expression of the FaeG polypeptide using methods well known in the art. Suitable conditions are readily understood by a skilled person and include variables such as pH, temperature, and duration of the cultivation. Moreover, culture media suitable for cultivating filamentous fungal cells are available in the art and can be easily adjusted depending on different variables such as the fungal strain in question and specifics of the polynucleotide used for transforming the host cells (e.g. use of an inducible promoter, a selection marker, etc.) as is well known to skilled persons. Cultivation may be carried out, for example, as shake flask cultivation, continuous fermentation, batch fermentation, fed-batch fermentation, or solid state fermentation in any desired scale.

As used herein, the term “conditioned culture medium” refers to a culture or fermentation supernatant obtained by cultivating filamentous fungal host cells in a culture medium suitable for said cultivation and allowing the

expression of FaeG. The conditioned medium comprises products secreted by the host cells during their cultivation. The conditioned medium can be recovered, i.e. separated from the host cells, by conventional techniques such as centrifugation.

In some embodiments, the method of producing the present subunit vaccine may comprise, after recovering the conditioned medium comprising FaeG, one or more additional steps of isolating and/or purifying FaeG. Said isolating and/or purifying may be carried out using any appropriate technique available in the art and well known to a skilled person. Non-limiting examples of suitable isolation techniques include extraction, filtration, centrifugation, evaporation, and precipitation, while non-limiting examples of suitable purification techniques include extraction, techniques based on differential solubility, electrophoretic techniques such as gel electrophoresis and preparative isoelectric focusing, and chromatography, such as ion exchange chromatography, affinity chromatography, and size exclusion chromatography.

In some alternative embodiments, the conditioned culture medium is recovered and used as such as a subunit vaccine. In other words, no isolation and/or purification of FaeG is carried out. The vaccine so prepared comprises not only FeaG but also a cocktail of other extracellular proteins, such as various cellulolytic enzymes and proteases (including, but not limited to, cellulases such as cellobiohydrolases, endoglucanases and beta-glucosidases, and hemicellulases such as endoxylanases and endomannanases) secreted by the host cells. Thus, the composition of the present vaccine may be adjusted by way of selecting the fungal host to be employed. Proteins and enzymes secreted by *Trichoderma reesei* are reviewed e.g. by Saloheimo and Pakula, 2012. Cellulolytic enzymes and proteases comprised in the present vaccine have a probiotic effect in the porcine gut, and aid the immature digestion system to adapt to solid food during the weaning period of suckling piglets. In those embodiments, wherein FeaG is isolated and/or purified from the conditioned culture medium, desired cellulolytic enzymes and proteases may be added into the final vaccine formulation in order to achieve a corresponding probiotic effect.

Accordingly, the present vaccine acts through three different modes of action, namely i) active protection by activating pigs own immune defence against ETEC, ii) passive protection by outcompeting pathogenic ETEC from gut receptors, and iii) probiotic protection by enhanced digestion of solid food and faster gut transit time.

Further advantages associated with those embodiments, wherein the

conditioned culture medium is used as the present vaccine composition without isolating and/or purifying FaeG include cost-effectiveness and simple manufacturing as no further production steps are required. However, if desired, the recovered conditioned culture medium may be dried prior to being used as a vaccine for easy formulation and to facilitate storage, for example. Accordingly, in some embodiments, the method of producing the present subunit vaccine may comprise, after recovering the conditioned medium comprising FaeG, one or more additional drying steps. Suitable drying methods include, but are not limited to, vacuum drying, freeze-drying (lyophilization), and spray-drying.

In some embodiments, the present vaccine composition may com-prise an effective amount of one or more adjuvants. One of the main features of the adjuvant substances in general is to broaden or enhance the immune response induced by the vaccine antigens. The term “effective amount of adjuvant” includes an amount of adjuvant which is capable of stimulating the immune response against an administered antigen, i.e. an amount that increases the immune response of an administered antigen composition. Suitable effective increases include those by more than 5%, preferably by more than 25%, and in particular by more than 50%, as compared to the same antigen composition with no adjuvant. Adjuvants suitable for use in vaccine formulations are known to those skilled in the art. In some embodiments, sugar moieties on fungal cell surface or on secreted fungal enzymes may serve as vaccine adjuvants.

The present vaccine composition may be provided in various forms including, but not limited to, aqueous solutions and dry powders, e.g. as lyophilized, vacuum dried, or spray dried formulations. Preferably, said aqueous solutions and dry powders may be provided in a form suitable to be administered by oral delivery, i.e. as an edible vaccine. Thus, the present vaccine may be formulated as a feed or drink product or simply added to the food or drink supply for piglets.

In accordance with the above, the present invention also provides a method of preventing and/or reducing the risk of PWD in a piglet in need thereof, wherein the method comprises administering an efficient amount of a vaccine composition of the present invention to the piglet. In some embodiments, the method induces a protective immunogenic response against ETEC or PWD. In some further embodiments, the method provides passive protection against ETEC or PWD by occupying gut F4 receptors. In some still further embodiments, the method provides probiotic protection against ETEC or PWD by enhanced digestion of solid food and faster gut transfer time of the food. In some even further embodiments,

the method provides protection against ETEC or PWD through one or more, preferably all, mechanisms of action set forth above.

As used herein, the term "piglet" refers to a young porcine to be administered with a vaccine of the invention. Unless otherwise indicated, the term "piglet" encompasses post-weaning piglets and suckling piglets. As used herein, the term “post-weaning piglet” refers to a piglet weaned from the sow, preferably aged from about 10 to about 28 days old.

As used herein, the term “effective amount” refers to an amount, which is able to elicit an immune response that protects the vaccine recipient against the ETEC or reduces the risk of PWD either by eliciting neutralizing antibodies or a cell-mediated response, or both. The term also encompasses an amount, which is able to provide passive protection against the ETEC by occupying gut F4 receptors.

As is readily understood by those skilled in the art, the effective amount to be used may vary according to a number of factors including, but not limited to, initial weight of the piglets, growth phase of the piglets, age of the piglets, hygienic status of the animal facilities, stress after weaning, feed and supplements used, health of the piglets and accompanying diseases or treatment. The effective amount may be administered to the animal, preferably by feeding, as a single dosage or may be given according to a regimen, whereby it is effective. Accordingly, in some embodiments, the piglets may receive the vaccine of the invention for substantially the whole of their growing period, or for only a part of their growing period, for example the early part and/or the suckling period.

As is readily understood by those skilled in the art, the present vaccine may be used in combination with any other appropriate treatment modality, such as traditional preventive antimicrobial biocides including zinc oxide.

It will be obvious to a person skilled in the art that, as technology advances, the inventive concept can be implemented in various ways. The invention and its embodiments are not limited to the examples described below but may vary within the scope of the claims.

EXAMPLES

Example 1. Construction of vectors for FaeG expression in *Trichoderma reesei* (Figure 1)

DNA sequences encoding FaeG carrying modifications for N-terminal deletion and donor strand complementation (Van Molle et al., 2009) were adapted for *Trichoderma reesei* codon usage and generated synthetically omitting

restriction sites for *Bsa*I, *Bsm*BI, *Btg*ZI, *Pac*I, *Not*I and *Pme*I endonucleases. The FaeG sequences were surrounded by matching flanking regions that enabled insertion to pJJJ395 expression vector with Golden Gate reaction (Engler, Kandzia and Marillonnet, 2008) by using *Bsa*I. The pJJJ395 plasmid carries a gene construct for high level protein expression in *T. reesei*. It has promoter, secretion signal sequence and gene terminator from cellobiohydrolase I (cbh) and antibiotic resistance marker for hygromycin. The expression cassette is surrounded by homology flanks that enable insertion to *chbI* locus in *T. reesei* genome. In the created expression vectors pJJJ783 (serotype F4ac) and pJJJ784 (serotype F4ad) the FaeG protein is translated as fusion with CBHI carrier protein for high secretion. Later in the secretion pathway, FaeG is released from the fusion by endogenous cleavage by Kex2 protease that recognizes the site NVISKR (SEQ ID NO: 11) between the fusion partners. Relevant sequences of the exemplary constructs are shown in Table 1 below.

Table 1.

	DNA sequence	Amino acid sequence
FaeG ac NTD (pJJJ783)	TGGATGACCGGCCACCACCACCACCACCACCGCC AGAAGTGGGAGTGGAAGGTCGGCACCGGCCTCA ACGGCTTCGGCAACGTCCTCAACGACCTGACCAA CGGTGGCACCAAGCTGACCATCACCGTCACCGGC AACAAGCCCATCCTCCTGGGCCGCACCAAGGAAG CCTTCGCCACCCCCGTCACCGGCGGCGTCGACGG CATCCCCCACATTGCCTTCACCGACTACGAGGGCG CCTCGGTCGTCCTCCGCAACCCCGACGGCGAGAC TAACAAGAAGGGCCTCGCCTACTTCGTCCTGCCCA TGAAGAACGCCGAGGGCACCAAGGTCGGCTCGG TCAAGGTCCAGGCCTCGTACGCCGGCGTCCTGGG CCGCGGCGGCGTCACCTCGGCCGACGGCGAGCTC CTGAGCCTCTTCGCCGACGGCCTGAGCTCCATCTT CTACGGCGGCCTCCCCCGCGGCTCGGAGCTGTCC GCCGGCTCGGCTGCCGCCGAGCGCACCAAGCTCT TCGGCAGCCTGTCCCGCAACGACATCCTCGGCCA GATCCAGCGCGTCAACGCCCAGATCACCTCCCTG GTCGACGTCGCCGGCAGCTACCGCGAGAACATGG AGTACACCGACGGCACCGTCGTCAGCGCCGCCTA CGCCCTGGGCATTGCCAACGGCCAGACCATCGAG GCCACCTTCAACCAGGCCGTCACCACCTCCACCCA GTGGAGCGCCCCTCTCAACGTCGCCATCACCTACT ACGACAACAAGCAGATGACCGGCGACTTCCAGGG	WMTGHHHHHHRQKWEWKVGTGL NGFGNVLNDLTNGGTKLTITVTGNK PILLGRTKEAFATPVTGGVDGIPHIAF TDYEGASVVLRNPDGETNKKGLAYF VLPMKNAEGTKVGSVKVQASYAGVL GRGGVTSADGELLSLFADGLSSIFYG GLPRGSELSAGSAAAERTKLFGSLSRN DILGQIQRVNAQITSLVDVAGSYREN MEYTDGTVVSAAYALGIANGQTIEAT FNQAVTTSTQWSAPLNVAITYYDNK QMTGDFQGSVDIGGSITA (SEQ ID NO: 2)

	CTCCGTCGACATCGGCGGCAGCATCACCGCCTGA (SEQ ID NO: 1)	
FaeG ad NTD (pJJJ784)	TGGATGACCGGCCACCACCACCACCACCACCGCC AGAAGTGGGAGTGGAAGGTCGGCACCGGCCTCA ACGGCTTCGGCAACGTCCTCAACGACCTGACCAA CGGTGGCACCAAGCTGACCATCACCGTCACCGGC AACAAGCCCATCCTCCTGGGCCGCACCAAGGAAG CCTTCGCCACCCCCGTCACCTCGGGCGTCGACGGC ATCCCCCACATTGCCTTCACCGACTACGAGGGCGC CTCGGTCGAGCTCCGCAACCCCGACGGCGAGACT GAGAAGGGCCTCGCCTACTTCGTCCTGCCCATGA AGAACGCCGAGGGCACCAAGGTCGGCAGCGTCA AGGTCCAGGCCTCGTACGCCGGCGCCCTGGGCCG CGGCGGCGTCACCAGCGCCGACGGCGAGCTCAT GAGCCTGTTCGCCGAGGGCTCCCACGCCATCTTCT ACGGCGGCCTCCCCACCAACGTCAAGAACAGCGA GCTGAAGGGCGGCTCGGCTGCCGCCGCCCGCACC GAGCTCTTCGGCAGCCTGTCCAAGAACGACATCC TCGGCCAGATCCAGCGCGTCAACGCCCAGATCAC CAGCCTGGTCAACGTCCCCGGCTCCTTCAACGAG AACATGGCCTACACCGACGGCTCGGTCGTCAGCG TCGCCTACGCCCTCGGCATTGCCAACGGCCAGAC CATCGAGGCCACCTTCAACCAGGCCGTCACCACCA GCACCCAGTGGTCCGCCCCTCTCAACGTCGCCATC ACCTACTACGACAACAAGCAGATGACCGGCGACT TCCAGGGCAGCGTCGACATCGGCGGCTCCATCAC CGCCTGA (SEQ ID NO: 3)	WMTGHHHHHHRQKWEWKVGTGL NGFGNVLNDLTNGGTKLTITVTGNK PILLGRTKEAFATPVTSGVDGIPHIAFT DYEGASVELRNPDGETEKGLAYFVLP MKNAEGTKVGSVKVQASYAGALGR GGVTSADGELMSLFAEGSHAIFYGGL PTNVKNSELKGGSAAAARTELFGSLS KNDILGQIQRVNAQITSLVNVPGSFN ENMAYTDGSVVSVAYALGIANGQTI EATFNQAVTTSTQWSAPLNVAITYY DNKQMTGDFQGSVDIGGSITA (SEQ ID NO: 4)
FaeG ab NTD		WMTGHHHHHHRQKWEWKVGTGL NGFGNVLNDLTNGGTKLTITVTGNK PILLGRTKEAFATPVSGGVDGIPQIAF TDYEGASVKLRNTDGETNKGLAYFVL PMKNAEGTKVGSVKVQASYAGVFG KGGVTSADGELFSLFADGLRAIFYGG LTTTVSGAALTSGSAAAARTELFGSLS RNDILGQIQRVNAQITSLVDVAGSYR EDMEYTDGTVVSAAYALGIANGQTIE ATFNQAVTTSTQWSAPLNVAITYYD NKQMTGDFQGSVDIGGSITA (SEQ ID NO: 5)
CBHI signal sequence	ATGTATCGGAAGTTGGCCGTCATCTCGGCCTTCTT GGCCACAGCTCGTGCT (SEQ ID NO: 6)	MYRKLAVISAFLATARA (SEQ ID NO: 7)

CBHI carrier	CAGTCGGCCTGCACTCTCCAATCGGAGACTCACCC GCCTCTGACATGGCAGAAATGCTCGTCTGGTGGC ACGTGCACTCAACAGACAGGCTCCGTGGTCATCG ACGCCAACTGGCGCTGGACTCACGCTACGAACAG CAGCACGAACTGCTACGATGGCAACACTTGGAGC TCGACCCTATGTCCTGACAACGAAACGTGCGCGA AGAACTGCTGTCTGGACGGTGCCGCCTACGCGTC CACGTACGGAGTTACCACGAGCGGTAACAGCCTC TCCATTGGCTTTGTCACCCAGTCTGCGCAGAAGAA CGTTGGCGCTCGCCTTTACCTTATGGCGAGCGACA CGACCTACCAGGAATTCACCCTGCTTGGCAACGA GTTCTCTTTCGATGTTGATGTTTCGCAGCTGCCGT GCGGCTTGAACGGAGCTCTCTACTTCGTGTCCATG GACGCGGATGGTGGCGTGAGCAAGTATCCCACCA ACACCGCTGGCGCCAAGTACGGCACGGGGTACTG TGACAGCCAGTGTCCCCGCGATCTGAAGTTCATCA ATGGCCAGGCCAACGTTGAGGGCTGGGAGCCGT CATCCAACAACGCGAACACGGGCATTGGAGGACA CGGAAGCTGCTGCTCTGAGATGGATATCTGGGAG GCCAACTCCATCTCCGAGGCTCTTACCCCCCACCC TTGCACGACTGTCGGCCAGGAGATCTGCGAGGGT GATGGGTGCGGCGGAACTTACTCCGATAACAGAT ATGGCGGCACTTGCGATCCCGATGGCTGCGACTG GAACCCATACCGCCTGGGCAACACCAGCTTCTAC GGCCCTGGCTCAAGCTTTACCCTCGATACCACCAA GAAATTGACCGTTGTCACCCAGTTCGAAACGTCG GGTGCCATCAACCGATACTATGTCCAGAATGGCG TCACTTTCCAGCAGCCCAACGCCGAGCTTGGTAGT TACTCTGGCAACGAGCTCAACGATGATTACTGCAC AGCTGAGGAGGCAGAATTCGGCGGATCCTCTTTC TCAGACAAGGGCGGCCTGACTCAGTTCAAGAAGG CTACCTCTGGCGGCATGGTTCTGGTCATGAGTCTG TGGGATGATTACTACGCCAACATGCTGTGGCTGG ACTCCACCTACCCGACAAACGAAACGTCCTCCACA CCCGGTGCCGTGCGCGGAAGCTGCTCCACCAGCT CCGGTGTCCCTGCTCAGGTCGAATCTCAGTCTCCC AACGCCAAGGTCACCTTCTCCAACATCAAGTTCGG ACCCATTGGCAGCACCGGCAACCCTAGCGGCGGC AACCCTCCCGGCGGAAACCCGCCTGGCACCACCA CCACCCGCCGCCCAGCCACTACCACTGGAAGCTCT GGCGGCGGCGGCGGC (SEQ ID NO: 8)	QSACTLQSETHPPLTWQKCSSGGTC TQQTGSVVIDANWRWTHATNSSTN CYDGNTWSSTLCPDNETCAKNCCLD GAAYASTYGVTTSGNSLSIGFVTQSA QKNVGARLYLMASDTTYQEFTLLGN EFSFDVDVSQLPCGLNGALYFVSMD ADGGVSKYPTNTAGAKYGTGYCDSQ CPRDLKFINGQANVEGWEPSSNNAN TGIGGHGSCCSEMDIWEANSISEALT PHPCTTVGQEICEGDGCGGTYSDNR YGGTCDPDGCDWNPYRLGNTSFYG PGSSFTLDTTKKLTVVTQFETSGAINR YYVQNGVTFQQPNAELGSYSGNELN DDYCTAEEAEFGGSSFSDKGGLTQFK KATSGGMVLVMSLWDDYYANMLW LDSTYPTNETSSTPGAVRGSCSTSSGV PAQVESQSPNAKVTFSNIKFGPIGST GNPSGGNPPGGNPPGTTTTRRPATT TGSSGGGGG (SEQ ID NO: 9)
Kex2 cleavage	AACGTCATCAGCAAGCGA (SEQ ID NO: 10)	NVISKR (SEQ ID NO: 11)

Example 2. *Trichoderma* transformation and protein expression analysis of FaeG constructs

Protoplasts were prepared from *T. reesei* strain M1351 carrying deletions for tree extracellular protease genes, *pep1*, *tsp1* and *pep4*. The strain carries also a *mus53* deletion for enhanced homologous integration (Steiger et al., 2011. The FaeG expression constructs pJJJ783 and pJJJ784 were transformed as described previously (Penttilä et al. 1987). Transformants were selected with hygromycin and purified through single spore cultures. The pure cultures were sporulated on potato dextrose agar plates to be stored as spore suspensions at -80°C in the presence of 20% glycerol (pJJJ783 was used to create *T. reesei* strain M1779 and pJJJ784 was used to create M1780). The expression constructs aimed integration to CBHI locus to replace the endogenous cellobiohydrolase I gene. The locus was confirmed with primer pairs (GCTGTTCCTACAGCTCTTTC (SEQ ID NO: 12) & AGCCGCACGGCAGC (SEQ ID NO: 13)) and (GGTTGACTTACTCCAGATCG (SEQ ID NO: 14) & AGTCGTTTACCCAGAATGC (SEQ ID NO: 15)) for 5' and 3' integration, respectively. To confirm dislocation of the endogenous chbI gene primer pair (CAACTCAGATCCTCCAGGAGAC (SEQ ID NO: 16) & GCTCGTTGCCAGAGTAACTAC (SEQ ID NO: 17)) was used.

Trichoderma strains transformed with pJJJ783 and pJJJ784 expression constructs were cultivated on 24-well plates in 24-well plates in 4% (w/v) lactose, 2% spent grain extract, 110mM KH_2PO_4, 38mM Na2SO4, 100mM PIPPS, 2.4mM Mg_2SO_4, 4.1mM $CaCl_2$, *Trichoderma* trace elements (Penttilä et al., 1987) and 38 mM di-ammonium hydrogen citrate, pH 4.5 for 4 days on a humidity controlled rotary shaker (800 rpm) at 28°C. Culture supernatants were collected by centrifugation and analysed on SDS-PAGE gels (Figure 2). The gels were detected either for total protein (Gel Code Blue Stain, Pierce) or after blotting to nitrocellulose filters with immunodetection. Shortly, the membranes were blocked with 5% non-fat milk in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.4) and then probed with anti-FaeG rabbit serum (1:1000 in TBS) and followed by goat anti rabbit IRDye 680RD (1:30000 in TBS, Li-cor) and imaged with Odyssey infrared imaging system (Li-cor).

Example 3. Production of FaeG in bioreactor with strain M1779 (Figure 2)

For primary inoculum 6x10E7 spores were added to 300ml of 3% (w/v) lactose, 1.5% spent grain extract, 110mM KH_2PO_4, 38mM Na_2SO_4, 2.4mM Mg_2SO_4, 4.1mM $CaCl_2$ and Trichoderma trace elements [11], pH 5.0. The inoculum was

cultured in 2 litre erlenmayer flask for four days at 28°C in a rotary shaker (200 rpm). For secondary inoculum, 4x 300ml cultures (same media as above) were started by adding 20ml of the primary culture and grow for three days.

The fermentation (fed-batch process) was done in New Brunswick BioFlo 510 fermentor in 15 litre volume. The culture media contained 6.25% (w/v) lactose, 1% spent grain extract, 3% spent grain, 110mM KH_2PO_4, 38mM Na_2SO_4, 2.4mM Mg_2SO_4, 4.1mM $CaCl_2$ and *Trichoderma* trace elements and the pH control was set at 4.5 ± 0.2, and feed was done with lactose solution. Three days after the inoculation the culture was sampled daily and harvested 167 hours after inoculation. Culture supernatants were collected by centrifugation and analysed on SDS-PAGE gels. The gels were detected (Figure 2) either for total protein (Gel Code Blue Stain, Pierce) or after blotting to nitrocellulose filters with immunodetection. Shortly, the membranes were blocked with 5% non-fat milk in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.4) and then probed with anti-FaeG rabbit serum (1:1000 in TBS) and followed by goat anti rabbit IRDye 680RD (1:30000 in TBS, Li-cor) and imaged with Odyssey infrared imaging system (Li-cor).

Example 4. Purification of FaeG (pJJJ783) protein (Figure 3)

The FaeG protein containing 6xHistidine tag was captured from culture supernatant by immobilized metal ion affinity column chromatography. The culture supernatant was adjusted to match conductivity and pH of binding buffer (20mM Na-phosphate, 500mM NaCl, pH 7.4). The adjusted supernantant was filtered (0.20µm) and loaded to the column (25x column volume). The column was washed with five column volumes of binding buffer. FaeG protein was eluted from the column with 250mM imidazole in binding buffer (3x column volume). After the purification buffer of the FaeG containing fractions was exchanged to PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4) with gel filtration (EconoPac, 10DG, BioRad). Quantification was done with SDS PAGE analysis followed by image densitometry.

Example 5. In vitro activity testing of FaeG (pJJJ783) protein with F4 double sandwich ELISA (Figure 4)

96-well plates (Nunc Maxisorb) were coated with anti-F4-ac monoclonal antibody (IMM01) 1µg/ml, 100 µg/ well, diluted in PBS buffer for two hours at 37°C. To block unspecific binding plates were incubated over night with PBS+Tween 80 (0.2%) 300µl/well at 4°C. Then the plates were washed three times with PBS including 0.2% Tween 20. FaeG samples were diluted in PBS+0.2%

Tween 20 and 3% BSA to 10 000; 1000; 100; 10; 1; 0.1 ng/ml and incubated 1h at 37°C. After 3x washes, F4 positive rabbit serum (1/1500 dilution with PBS) was added and incubated 1h at 37°C. Detection was done after 3x washes with HRP-conjugated goat anti-rabbit IgG H+L (100 µl/well, 1/1000 diluted in reagent diluent) and incubated for 1h at 37°C. After 3x washes ABTS solution was added (50 µl/well) and the plates were incubated for 1h at 37°C. The absorbance at 405 nm was measured after 15, 30 and 60 min with a spectrophotometer.

Example 6. *In vitro* villous adhesion testing (Figure 5)

To assess the capacity of the monomeric FaeG protein to adhere to the brush border of F4 receptor positive small intestine villous enterocytes, an in vitro villus adhesion assay was performed. Villi were isolated as described by Van den Broeck et al. 1999b. Briefly, a segment (15 cm) from the mid jejunum was excised. This segment was washed twice with cold PBS and once with cold Krebs-Henseleit buffer (0.12 M NaCl, 0.014 M KCl, 0.001 M KH_2PO_4, 0.025 M $NaHCO_3$, pH 7.4) containing 1% (v/v) formaldehyde. Subsequently, the villi were scraped off with glass slides and washed 4x in Krebs-Henseleit buffer. For inhibition assay the villi were exchanged to PBS buffer containing 1% D-mannose. Protein samples were added to 50 µg/ml and 20µg/ml concentration and incubated 1h at room temperature on a rotary shaker. F4ac positive fimbriae from GIS26 ETEC strain were used as positive control and PBS as negative control. After 1 h incubation, 4*10^8 bact/ml F4ac positive ETEC bacteria (strain GIS26) were added to the mixture and again incubated at room temperature for 1h. Then, the inhibition of bacteria binding to brush borders was evaluated quantitatively by counting the number of bacteria adhering along a 50µm villous brush border at 20 randomly selected places using phase-contrast microscopy at a magnification of 600x.

Example 7. *In vivo* animal testing

To evaluate whether the monomeric FaeG protein could raise F4 specific immune response in pigs following experiment was performed. Fourteen F4 reseptor positive and F4-seronegative conventionally bred piglets from three different litters were used. The piglets were weaned and randomized to two groups and housed in isolation units where they received water and standard piglet food. One week post weaning the piglets (n=7) received intragastric dose of 7mg of purified FaeG protein in PBS buffer supplemented with 50µg of cholera toxin as adjuvant for three consecutive days. A week after the first immunization piglets were boosted for one day with similar dose. Untreated control group (n=7)

received PBS buffer alone. Three days after the boost immunization the piglets were challenged intragastrically with 1010 F4+ ETEC bacteria. To support the ETEC colonization, the stomach fluid was neutralized with isotonic bicarbonate solution prior the challenge.

To monitor the development of F4 specific antibodies in blood serum, piglet were sampled for blood on 0, 7, 10, 17 and 29 days post first immunization. To determine the excretion of F4+ ETEC, faecal samples were taken daily from challenge until 6 days post challenge. The presence of F4-specific antibodies in blood serum was determined with F4 ELISA. The serum samples were screened on microtiter plates coated with F4 fimbriae and after washing step probed for bound F4 antibodies with a labelled anti-swine secondary antibody. To analyze excretion following ETEC challenge, F4+ *E. coli* were enumerated in diluted faecal samples by dot blotting visualized by a F4-specific antibody.

The results are shown in Figure 6, which demonstrates that oral immunization with monomeric FaeG protein evokes F4-specific immune response in pigs. Similar results are obtained with lyophilized *T. reesei* culture supernatant containing unpurified FaeG).

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Claims

1. A vaccine for porcine post-weaning diarrhea, comprising a filamentous fungus-derived FaeG subunit of a F4 fimbriae of enterotoxigenic *Escherichia coli* (ETEC).

2. The vaccine according to claim 1, wherein the FaeG comprises an amino acid sequence set forth in SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 5, or a functionally equivalent variant thereof having at least 80% amino acid sequence identity therewith.

3. The vaccine according to claim 1 or 2, further comprising at least one enzyme selected from the group consisting of cellulases such as cellobiohydrolases, endoglucanases and beta-glucosidases, and hemicellulases such as endoxylanases and endomannanases.

4. The vaccine according to any one of claims 1 to 3, further comprising an adjuvant.

5. The vaccine according to claim 4, wherein the adjuvant is a sugar moiety derived from a fungal cell surface or from a fungal enzyme selected from the group consisting of cellulases such as cellobiohydrolases, endoglucanases and beta-glucosidases, and hemicellulases such as endoxylanases and endomannanases.

6. The vaccine according to any one claims 1 to 5, wherein the filamentous fungus is selected from the group consisting of cells from the genera *Acremonium, Aspergillus, Fusarium, Humicola, Mucor, Myceliophthora, Neurospora, Penicillium, Scytalidium, Thielavia, Tolypocladium*, and *Trichoderma*, preferably *Trichoderma* cell, more preferably *Trichoderma reesei* cell.

7. The vaccine according to any one of claims 1 to 6, wherein the vaccine is in the form a conditioned culture medium.

8. The vaccine according to claim 7, wherein the vaccine is in a dried form.

9. A method of producing vaccine for porcine post-weaning diarrhoea, the method comprising

a) transforming a filamentous fungal host cell with a polynucleotide encoding FaeG,

b) cultivating the transformed host cell under cultivation conditions suitable for expression and secretion of FaeG into conditioned culture medium, and

c) recovering the conditioned culture medium comprising FaeG.

10. The method according to claim 9, wherein the method does not comprise purification of FaeG.

11. The method according to claim 9 or 10, further comprising drying of the conditioned medium.

12. The method according to any one claims 9 to 11, wherein the filamentous fungal host cell is selected from the group consisting of cells from the genera *Acremonium, Aspergillus, Fusarium, Humicola, Mucor, Myceliophthora, Neurospora, Penicillium, Scytalidium, Thielavia, Tolypocladium,* and *Trichoderma,* preferably *Trichoderma* cell, more preferably *Trichoderma reesei* cell.

13. The method according to claim 12, wherein the host cell is deficient in one or more protease-encoding genes selected from the group consisting of *pep1, pep2, pep3, pep4, pep5, pep8, pep11, pep12, tsp1, slp1, slp2, slp3, slp7, gap1, gap2, amp2, mp1, mp2, mp3, mp4, mp5, pep9, amp1,* and *sep1,* preferably deficient in one to three protease-encoding genes selected form the group consisting of *pep1, pep4,* and *tsp1*.

14. The method according to any one of claims 9 to 13, wherein the polynucleotide encoding FaeG comprises a nucleic acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3, or a functionally equivalent variant thereof having at least 80% amino acid sequence identity therewith.

15. The method according to any one of claims 9 to 13, wherein the FaeG comprises an amino acid sequence set forth in SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 5, or a functionally equivalent variant thereof having at least 80% amino acid sequence identity therewith.

16. A vaccine composition obtainable by the method according to any one of claims 9 to 15.

17. A vector comprising a FaeG-encoding nucleic acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO:3, or a functionally equivalent variant thereof having at least 80% amino acid sequence identity therewith.

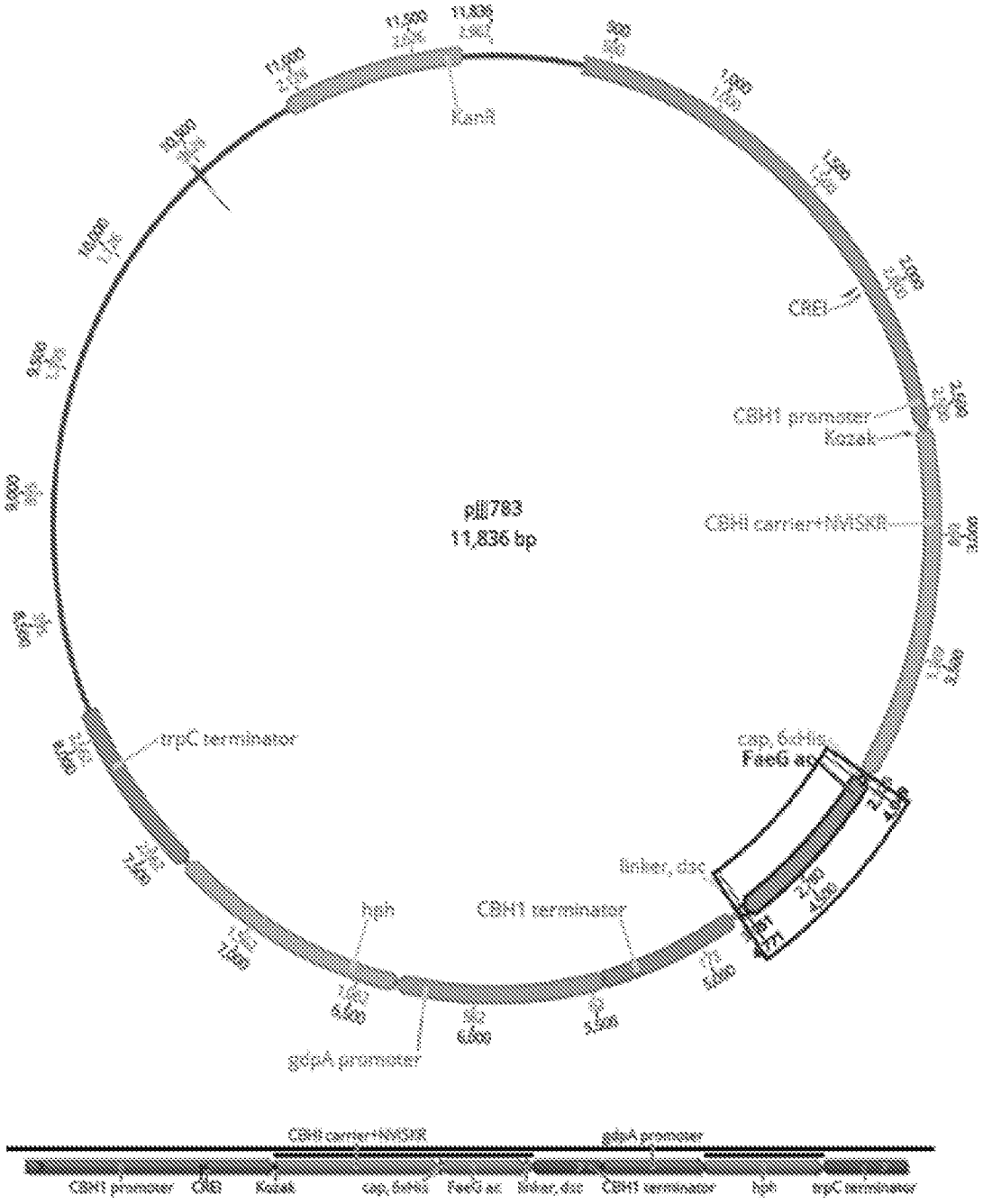
18. A filamentous fungal host cell genetically modified to express FaeG.

19. The host cell according to claim 18, wherein the FaeG comprises an amino acid sequence set forth in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 5 or a functionally equivalent variant thereof having at least 80% amino acid sequence identity therewith, or is encoded by a polynucleotide comprising a nucleic acid sequence set forth in SEQ ID NO: 1, SEQ ID NO: 3 or a functionally equivalent variant thereof having at least 80% amino acid sequence identity therewith.

20. The host cell according to claim 18 or 19, wherein the host cell comprises the vector according to claim 16.

21. The host cell according to any one of claims 18 to 20, wherein the filamentous fungus host cell is selected from the group consisting of cells from the genera *Acremonium, Aspergillus, Fusarium, Humicola, Mucor, Myceliophthora, Neurospora, Penicillium, Scytalidium, Thielavia, Tolypocladium,* and *Trichoderma,* preferably *Trichoderma* cell, more preferably *Trichoderma reesei* cell.

22. The host cell according to any one of claims 18 to 21, wherein the host cell is deficient in one or more protease-encoding genes selected from the group consisting of *pep1*, *pep2*, *pep3*, *pep4*, *pep5*, *pep8*, *pep11*, *pep12*, *tsp1*, *slp1*, *slp2*, *slp3*, *slp7*, *gap1*, *gap2*, *amp2*, *mp1*, *mp2*, *mp3*, *mp4*, *mp5*, *pep9*, *amp1*, and *sep1*, preferably deficient in one to three protease-encoding genes selected form the group consisting of *pep1*, *pep4*, and *tsp1*.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CBH1 promoter
Kozak
CBH carrier+NVSKR
trpC terminator
linker, dac
hph
CBH1 terminator
gpdA promoter
CBH carrier+NVSKR
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Figure 1

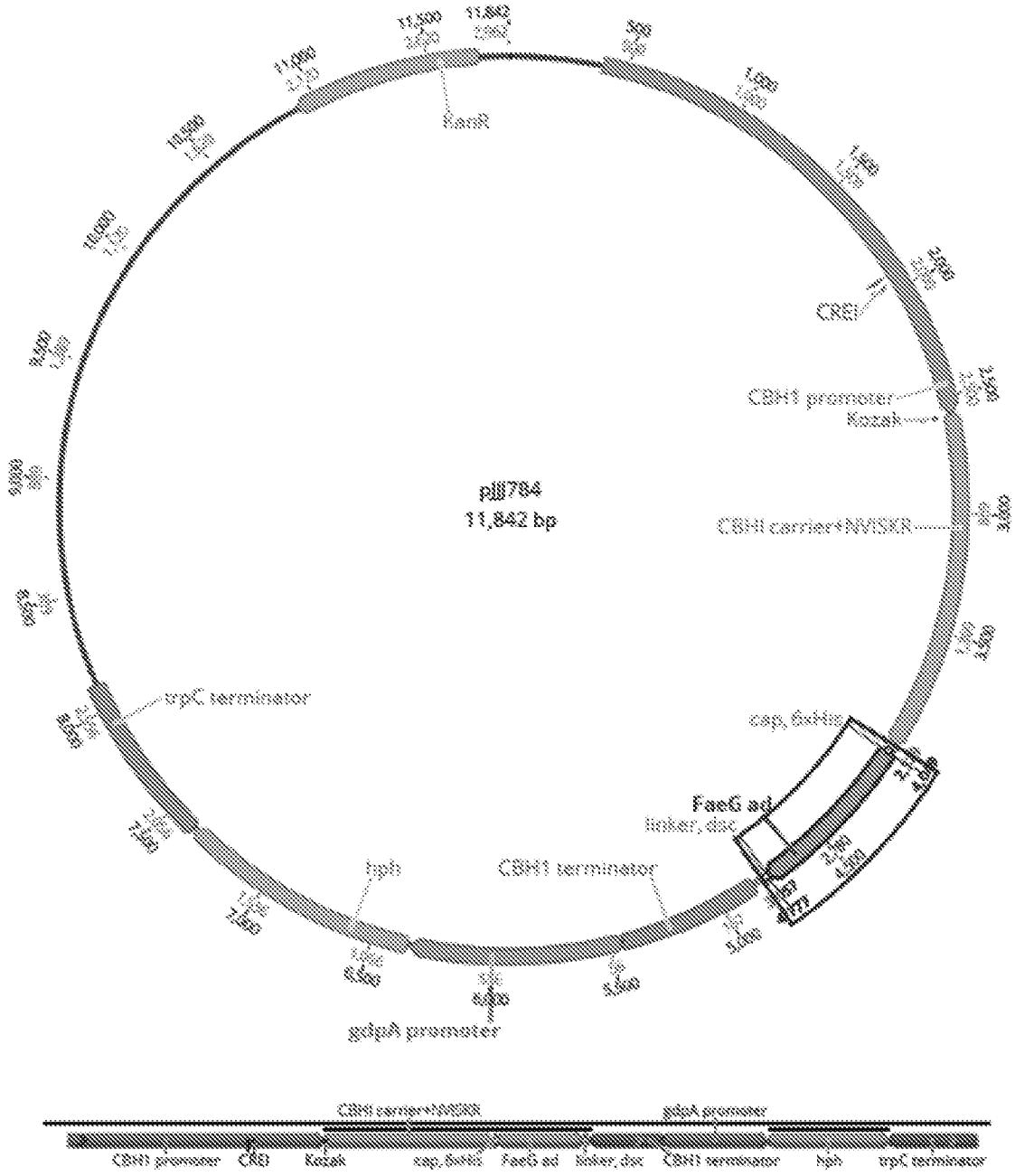
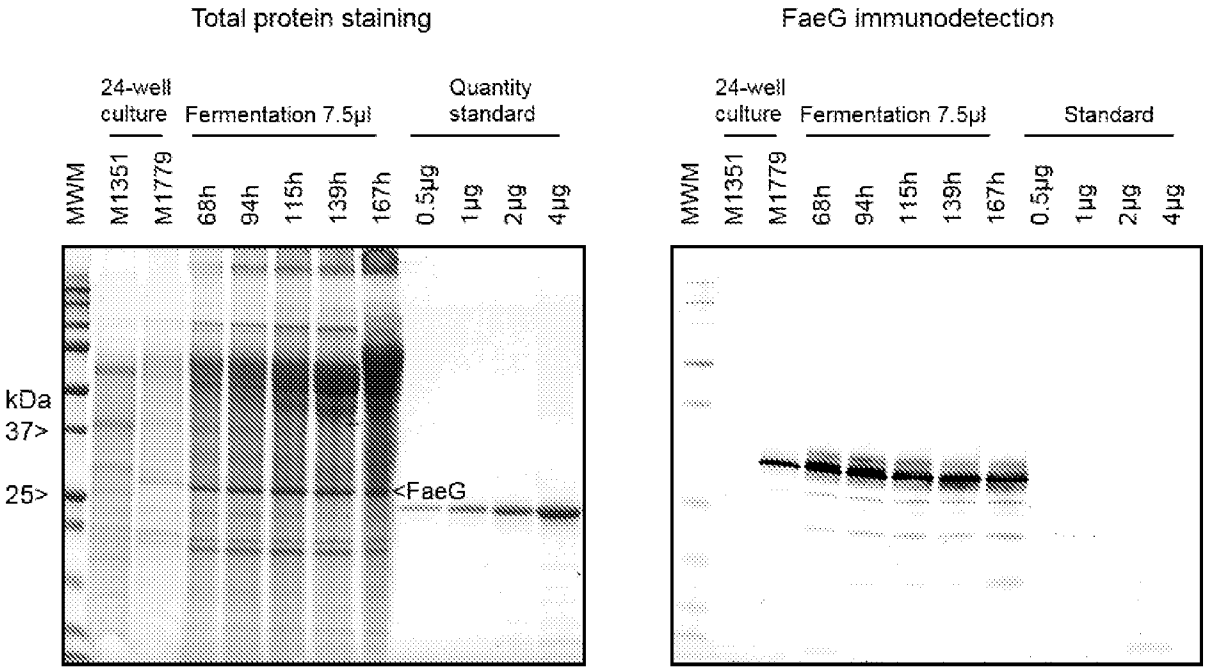


Figure 1 continued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FaeG immunodetection
24-well culture
Fermentation 7.5µl
Quantity standard
Standard
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M1351
M1779
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167h
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1µg
2µg
4µg
kDa
37>
25>
<FaeG

Figure 2

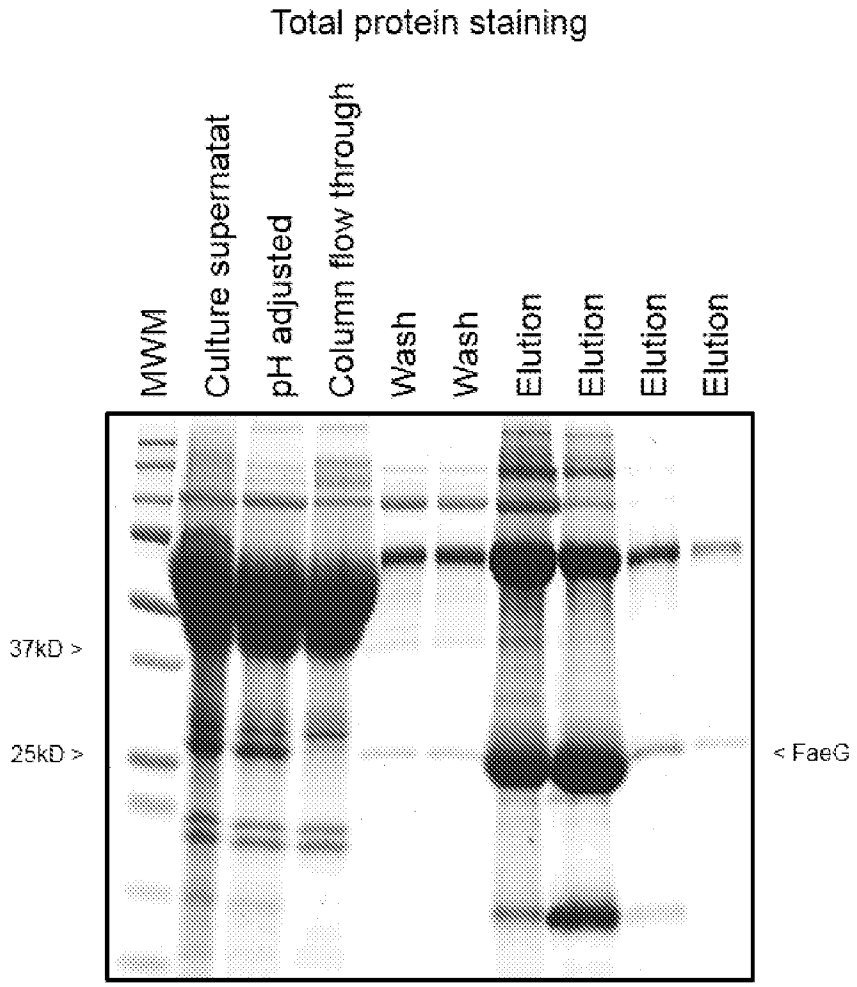
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Figure 3

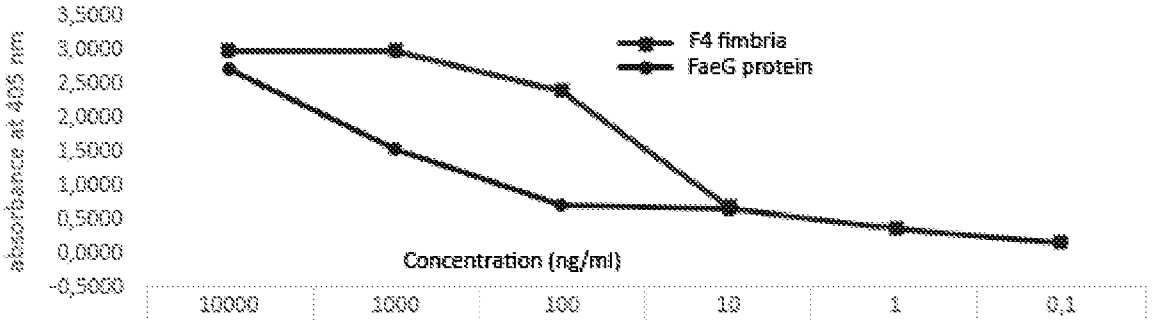

Figure 4

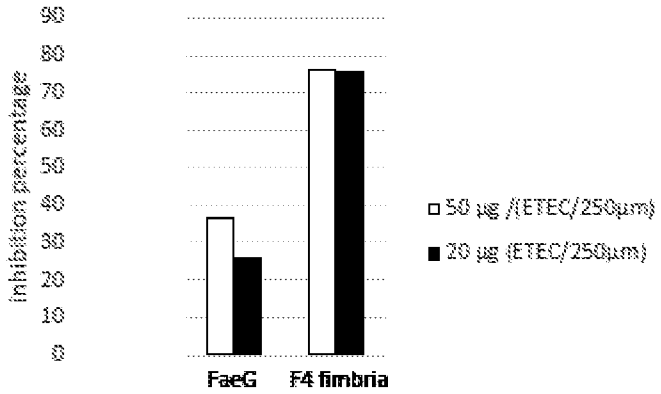

Figure 5

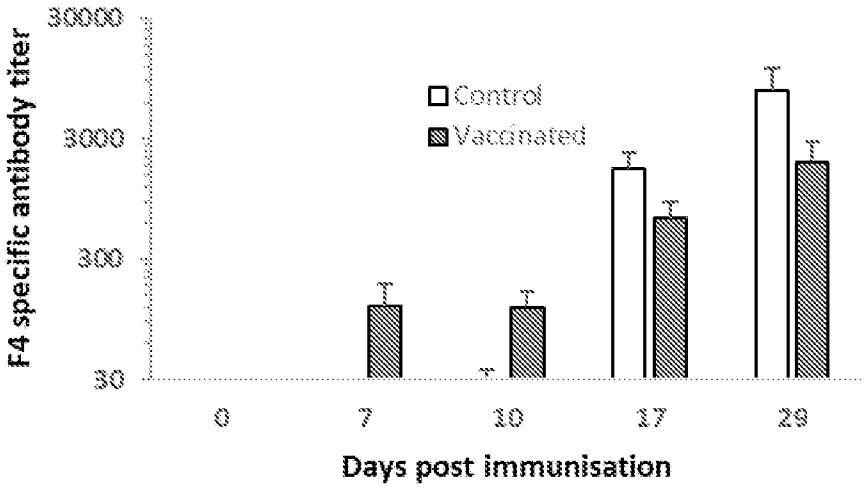

Figure 6A

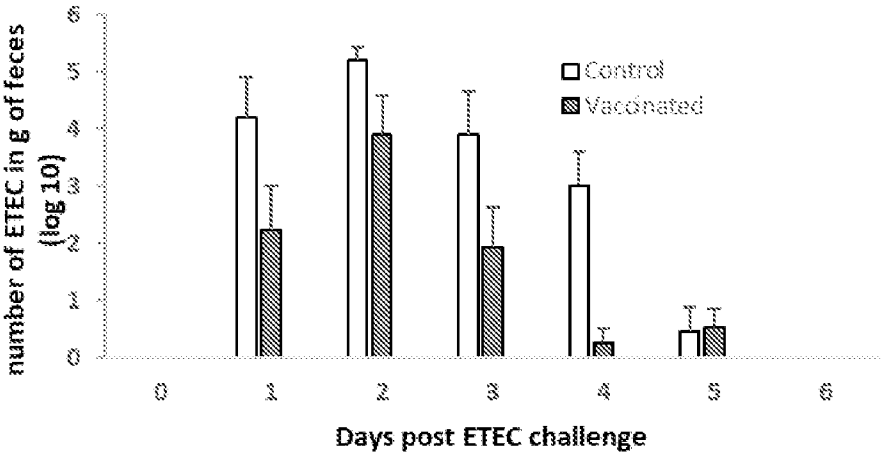

Figure 6B

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2019/050207

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/108 C07K16/12
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHUJIE LIU ET AL: "Immune responses elicited in mice with recombinant Lactococcus lactis expressing F4 fimbrial adhesin FaeG by oral immunization", VETERINARY RESEARCH COMMUNICATIONS ; AN INTERNATIONAL JOURNAL PUBLISHING TOPICAL REVIEWS AND RESEARCH ARTICLES ON ALL ASPECTS OF THE VETERINARY SCIENCES, KLUWER ACADEMIC PUBLISHERS, DO, vol. 34, no. 6, 9 June 2010 (2010-06-09), pages 491-502, XP019832139, ISSN: 1573-7446 abstract ----- -/--	1-22

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
15 May 2019	27/05/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fleitmann, J

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JUSSI J JOENSUU ET AL: "Glycosylated F4 (K88) Fimbrial Adhesin FaeG Expressed in Barley Endosperm Induces ETEC-neutralizing Antibodies in Mice", TRANSGENIC RESEARCH, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NE, vol. 15, no. 3, 1 June 2006 (2006-06-01), pages 359-373, XP019409555, ISSN: 1573-9368, DOI: 10.1007/S11248-006-0010-7 abstract --- VERDONCK F ET AL: "Oral immunization of piglets with recombinant F4 fimbrial adhesin FaeG monomers induces a mucosal and systemic F4-specific immune response", VACCINE, ELSEVIER, AMSTERDAM, NL, vol. 22, no. 31-32, 22 October 2004 (2004-10-22), pages 4291-4299, XP002523295, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2004.04.016 abstract --- Wim Van Den Broeck ET AL: "Receptor-Dependent Immune Responses in Pigs after Oral Immunization with F4 Fimbriae", Infection and Immunity, 1 February 1999 (1999-02-01), pages 520-526, XP055587380, United States Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC96350/pdf/ii000520.pdf cited in the application abstract --- IGOR KOLOTILIN ET AL: "Production of a Subunit Vaccine Candidate against Porcine Post-Weaning Diarrhea in High-Biomass Transplastomic Tobacco", PLOS ONE, vol. 7, no. 8, 3 August 2012 (2012-08-03), page e42405, XP055587390, DOI: 10.1371/journal.pone.0042405 abstract ---	1-22 1-22 1-22

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