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Vaccination with Recombinant Yeast by Producing a Protective Humoral Immune Response Against Defined Antigens

Field of the invention

5 The invention relates to recombinant yeasts of the species *Kluyveromyces lactis*, which contain the VP2 antigen of the virus of infectious bursitis (IBDV), which is encoded by a foreign gene integrated into the yeast genome to generate a humoral immune response. The invention further relates to the production of these yeasts and their use for protective vaccination against pathogens containing these antigens.

10

Prior art

Vaccines are used to prevent diseases (preventive vaccines) or to treat established diseases (immunotherapeutic vaccines). Over the last 100 years or so, preventive vaccination programmes have contributed significantly to the reduction of infectious
15 diseases. Immunotherapeutic vaccines have only been developed and used for about 20 years, mainly to combat persistent infections with viruses, bacteria or parasites or against carcinogenic diseases. The objective of vaccination is the induction of a cellular (i.e. essentially T and NK cell-mediated) and/or humoral (i.e. essentially B cell/antibody-mediated) immune response as well as an immunological
20 memory against antigenic components of pathogens or malignant (tumourigenic) cells.

Traditional vaccines contain the entire pathogen in attenuated (inactivated) or killed form, including its genetic material, nucleic acids in the form of DNA or RNA. These traditional vaccines usually require special safety precautions and/or the use of
25 laboratory animals and/or the use of cell cultures for the production; furthermore, they often require extravagant storage and transport solutions using cold chains. Moreover, they carry the risk that substances from the production (e.g. from the laboratory animal or from the cell culture) in the vaccinated individual produce side effects or that unwanted re-activations of the pathogen occur. Problems also exist in
30 diagnostics: For example, after vaccination of livestock, vaccinated animals cannot be distinguished from naturally infected animals, causing failure of the early warning system, which is based on the detection of new infections. This led to the development of so-called 'subunit' vaccines that contain only parts of the pathogen. The prerequisite for this is that 'main antigens' of the respective pathogens are

already known. Main antigens are mostly surface components of the pathogen that can be recognised by the immune system, e.g. proteins of a virus envelope or the virus capsid. Even in the absence of a complete virus particle, they can induce a humoral and/or cellular immune response and an immunological memory in the host against the virus. Since '*subunit* vaccination' lacks typical components of the pathogen, a differential diagnosis can be used to distinguish between vaccinated and naturally infected individuals; this is therefore also referred to as '*subunit* marker vaccination'. The disadvantages of many *subunit* vaccines are the elaborate production and frequently insufficient immunogenicity: While the pathogens themselves can be cultivated efficiently (with the limitations outlined above), their major antigens must be genetically engineered and arduously purified using expensive and usually inefficient processes. *Subunit* vaccines produced this way are accordingly sensitive, must frequently be refrigerated for storage and transportation and have short durability. Therefore much of the bulk vaccines are still based on the traditional principle using complete pathogens. For example, most vaccines against the widely spread infectious bursal disease (IBD) in poultry are currently mostly based on attenuated (weakened) or inactivated viruses of the IBD-causing Infectious Bursal Disease Virus (IBDV).

In an attempt to compensate for the problem of weaker immunogenicity in *subunit* vaccines, adjuvants are used in addition. Adjuvants are substances that have been empirically proven to stimulate the immune system. They amplify the immune response non-specifically and often in a poorly understood way. So far, only very few adjuvants have been approved for human use. For example, the only assistive substances approved for human use in the United States are aluminium salts, aluminium hydroxide and aluminium phosphate. However, aluminium salt formulations cause additional complications in storing the vaccine in question. Furthermore, these adjuvants do not exhibit sufficient efficacy with all antigens.

Genetically engineered foreign proteins, which include most *subunit* vaccines, can be produced in various host cells. In addition to the intestinal bacterium *Escherichia coli*, mammalian cells that can be propagated in cell cultures, plant cells and various fungi have been established as host systems. Microbial systems such as bacteria and fungi can be cultivated on a large scale in a very cost-effective manner. Yeast cells of the yeast genera *Saccharomyces*, *Pichia* and *Kluyveromyces* have been used routinely for decades for the expression of foreign proteins. The advantage yeast

cells have over bacteria is that they are eukaryotes (i.e. they resemble animal cells in many aspects), and eukaryotic proteins (i.e. proteins that are formed and/or must be functional in animal cells) can be inexpensively produced in yeast in native or near-native form (Bathurst, 1994; Gellissen & Hollenberg, 1997). Yeasts were initially only
5 used to produce the foreign proteins; then the proteins were purified from the yeast cells and used as *subunit* vaccines. Only recently has it been attempted to administer yeast itself or cell fractions of the yeasts as a vaccine.

For about 5 years, attempts have been made to use *Saccharomyces cerevisiae* ('brewer's yeast', *S. cerevisiae*) itself for vaccination: It has been shown that
10 subcutaneously applied, antigen-expressing *S. cerevisiae* cells can activate dendritic cells and generate antigen-specific T cell immune responses, especially cytotoxic T cell responses, against specific antigens. This cellular immune response proved to be protective against the administration of certain tumour cells, i.e. fewer vaccines were produced in vaccinated animals after vaccination than in control animals. This
15 method is currently also being tested in immunotherapeutic applications in tumour diseases (Stubbs et al., 2001; Lu et al., 2004).

Specialists know the following sources from the prior art, in which a yeast-based vaccination is described:

A number of US patents, e.g. 20090304741, 5830463, 10738646 and 20070166323, describe the use of *S. cerevisiae* containing at least one recombinant antigen in immunotherapy. These yeasts have been shown to effectively stimulate an immune response, particularly a cell-mediated immune response.

WO 90/15140 discloses immunisation of chickens with recombinant IBDV antigen. It was derived from *Kluyveromyces lactis*, in which the antigen was expressed. However,

WO 90/15140 discloses only *K. lactis* strains in which the IBDV antigen is expressed by means of plasmid vectors, not *K. lactis* strains, in which the antigen-expressing gene is stably integrated in the genome and can be inducibly expressed (subject of the application).

WO/2006/044923 discloses yeast (*S. cerevisiae*) which recombinantly expresses various proteins of the hepatitis C virus (HCV), and which can trigger an immune reaction, especially a T cell response against these HCV proteins and should be used as a vaccine against chronic hepatitis C.

WO/2007/092792 describes the possible use of recombinant *S. cerevisiae* against influenza virus infections using a combination of different yeast strains whose application leads to a T cell induction, i.e. to a cellular immune response.

WO/2011/032119 relates to a method for improving the efficacy of a yeast-based immunotherapy in patients. This method comprises a yeast-based agent that modulates the production or survival of CD4 + TH17 cells.

None of the available patents proves that yeast induces a protective humoral immune response against infectious diseases or tumours (subject of this application).

Like *S. cerevisiae*, the 'milk yeast' *Kluyveromyces lactis* (*K. lactis*) also has GRAS status (GRAS: *generally regarded as safe*), i.e. it is suitable for use in animals and humans (van Ooyen et al. 2006). Although morphologically very similar to the brewer's yeast *S. cerevisiae*, the evolutionary lines of the two genera developed from a common precursor more than 100 million years ago in different directions. Therefore, *K. lactis* differs fundamentally from *S. cerevisiae* in many ways. Some of these differences are of great importance for applicability in biotechnological applications. The evolution of *S. cerevisiae* delivered the specialisation of the metabolism for alcoholic fermentation and thus the loss of many genes of the

precursors. Alcoholic fermentation is atypical for most yeasts. In *S. cerevisiae* it occurs at high glucose concentrations even when oxygen is present and mitochondrial respiration would actually allow a much more efficient energy yield from the sugar conversion: The function of the mitochondria, the 'power plants' of the cell, is largely suppressed through 'glucose repression'. *K. lactis* differs significantly from *S. cerevisiae* in the regulation of mitochondrial function (Chen and Clark-Walker, 1995, Clark-Walker, 2007). In contrast to *S. cerevisiae*, *K. lactis* belongs to the so-called 'crabtree-negative' yeasts. These types of yeasts generally do not form ethanol under strictly aerobic conditions but completely degrade glucose to CO₂ through mitochondrial activity to produce ATP. This physiological property is of fundamental importance, as it leads to a significant increase in biomass yield in large scale fermentations, resulting in a significant cost reduction for the use of these yeasts in producing recombinant proteins. Furthermore, *K. lactis* studies have shown that mutations in the hexokinase-mediated insulin transduction pathway can enhance the expression of heterologous genes (Donnini et al., 2004). Reduced glucose repression, especially of respiratory genes, is a characteristic of 'crabtree negative' yeasts and could be related to the empirically observed better foreign gene expression in such yeasts.

There are also significant differences between *K. lactis* and *S. cerevisiae* in the composition of the cell wall glucans (Backhaus et al., 2011); these differences are probably due to different glycosyltransferases in the Golgi apparatus involved in the maturation of glycoproteins: Hence, glycoproteins in *S. cerevisiae* often contain mannose phosphates, and the glycoproteins in *K. lactis* mainly contain terminal N-acetylglucosamine (Raschke and Ballou, 1972). It can be assumed that these differences between *S. cerevisiae* and *K. lactis* in the glycosylation and secretion of proteins as well as in the cell wall biosynthesis have a considerable influence on the intracellular localisation, protein folding as well as stability and thus also on the immunogenicity of heterologously expressed foreign proteins (Uccelletti et al., 2004). WO/2010/054649 describes the preparation of a recombinant system of *K. lactis*. In its application examples, recombinant strains derived from strain VAK367-D4 were used for mucosal or oral vaccination against various antigens. A disadvantage of oral/mucosal vaccination, however, is that the vaccines must be used in large quantities in order to achieve protective immunisation.

Description of the figures

Fig. 1 schematically demonstrates the production of vaccine strain VAK887, which carries the foreign gene IBDV VP2, via homologous recombination in the VAK367-D4 parent strain. Through transformation of the plasmid Klp3-MCS (SEQ ID No.: 10), which contained the VP2 gene of the IBDV strain D78, the VP2 foreign gene was inserted via homologous recombination into the chromosomal LAC4 gene site, which was destroyed by insertion of the *URA3* gene. During recombination into the host genome, the *URA3* gene was replaced by the VP2 gene and the *LAC4* gene was restored; recombinant yeast strains could be obtained through selection on lactose medium without uracil. Subsequently, the expression of *LAC4* (β -galactosidase) is controlled via the *KIGAL80* promoter, and the expression of the VP2 gene is controlled via the LAC4 promoter.

Fig. 2A illustrates the expression of IBDV VP2 by strain VAK887 compared to the parent strain (VAK367) and compared to IBDV infected chicken cells through Western analysis with a VP2-specific antibody. **Fig. 2B** demonstrates the expression analysis of recombinant IBDV VP2 or mutated IBDV VP2-T2S in different VP2-expressing *K. lactis* variants. The original *K. lactis* variant VP2 (VAK887) expressed only moderate amounts of viral protein. VP2 protein expression was increased in the *K. lactis* VP2-T2S strain (VAK888) by replacing threonine at amino acid position 2 of the VP2 protein with serine. A further increase was achieved by increasing the *KIGAL4* gene dosage (VP2-T2S_GAL4 = VAK890) and/or by using a yeast codon-optimised synthetic VP2 gene (oVP2-T2S = VAK910).

Fig. 3 shows that heat inactivation of the yeasts according to the invention at 90 °C for 2 hours does not lead to a loss of the recombinant VP2-T2S protein (Fig. 3A). Equal amounts of proteins from non-inactivated yeast, inactivated yeast and yeast from a feed pellet were separated on SDS PAGE and tested in a Western blot with an anti-VP2 antibody in contrast to cell lysates from poultry cells that were or were not infected with IBDV. Fig. 3 further shows that the amount of VP2-T2S in variant VAK890 is approximately 0.7 fg of heterologous protein per yeast cell (Fig. 3B). In this case, defined amounts of purified VP2-T2S in contrast to VP2 from a defined

number of cells in the fermenter-cultivated *K. lactis* (strain VAK890) were stained in the Western blot and the result was evaluated densitometrically.

Fig. 4 describes vaccination in mice with subcutaneously applied, heat-inactivated, complete yeast cells of the *K. lactis* variant VAK890 in contrast to oral vaccination with complete yeast cells of the *K. lactis* variant VAK890. Fig. 4A illustrates the immunisation plan: Subcutaneous immunisation took place three times with a two-week break; in comparison, it was fed twice for two weeks. Two weeks (arrow) after the last yeast application, serum samples from the treated mice were tested for the presence of anti-VP2 antibody in an IBDV-specific ELISA (Fig. 4B) and in an IBDV neutralisation assay (Fig. 4C). Fig. 4D summarises that mice treated with VP2-expressing *K. lactis* (strain KI VP2-T2S_GAL4 (VAK890)) have significantly higher titres of antibody/neutralising antibodies than mice treated with wild type *K. lactis* (strain VAK367). It was also shown that subcutaneously applied *K. lactis* (strain VAK890) have significantly higher titres of antibodies/neutralising antibodies than mice fed with *K. lactis* (strain VAK890). However, mice that were orally immunised with *K. lactis* (strain 890) also exhibited an increased antibody/neutralising antibody titre compared to mice treated with *K. lactis* wild type (strain VAK367).

Fig. 5 shows oral and subcutaneous vaccination in chickens with heat-inactivated, complete yeast cells of the *K. lactis* variant VP2-T2S_GAL4 (VAK890). Oral vaccination was either administered via a short 1/1/1/1/1 regimen (1 week feeding, 1 week break, 1 week feeding, etc.) or a longer 2/2/2 regimen was used (Fig. 5A). After a 1 or 2 week break following vaccination (Fig. 7A), all treated animals were infected with IBDV (Edgar strain) at a concentration level of 100 EID₅₀ per animal (virus challenge, black bars). After oral vaccination, especially after application of the extended regimen, increased titres of virus-neutralising antibodies could be detected in several animals. Subcutaneous vaccination with recombinant *K. lactis*, on the other hand, produced high titres of virus-neutralising antibodies in all treated animals (Fig. 5B, C, IBDV-specific ELISA, IBDV neutralisation assay). None of the animals treated with recombinant *K. lactis* yeast died after infection with IBDV, regardless of which treatment regimen was used. In contrast, the mortality rate in the control group was 10-35% (Fig. 5C). Analysis of the lesions in the bursae of the treated animals showed

that about 10% of the orally treated animals showed no signs of viral infection following inoculation with IBDV after the prolonged treatment regimen was applied: A so-called 'lesion score' was used: scores 1, 2 indicate undamaged or hardly damaged bursae; scores 3, 4 indicate damaged and severely damaged bursae. In contrast, all animals in which the recombinant *K. lactis* strain VAK890 was administered subcutaneously showed complete protection against IBDV (Fig. 5C).

Fig. 6 schematically shows the structure of the vector Klp3-MCS (**SEQ ID No.:10**).

Description of the invention:

The possibility of using recombinant yeasts for subcutaneous vaccination is known to specialists from the prior art: Stubbs et al., (2001) Nat. Med. 7: 625-629; Stubbs and Wilson (2002) Curr. Opin. Mol. Ther. 4: 35-40; Wansley et al., (2008) Clin. Cancer Res. 14: 4316-4325; US 5,830,463, WO/2006/044923; WO/2007/092792 and WO/2011/032119. In the design examples of these publications, however, the work was done exclusively with the yeast *Saccharomyces cerevisiae*. 'Yeast' is a collective term for unicellular growing eukaryotic microorganisms with sometimes very different properties due to divergent evolution over hundreds of millions of years (about 100 million years for *S. cerevisiae* and *K. lactis*). When *S. cerevisiae* and *K. lactis* are used for vaccination in higher eukaryotes such as animals or humans, it can therefore be assumed that an immune response triggered by *S. cerevisiae* differs greatly from a *K. lactis*-induced immune response. This applies to both the immune response against foreign antigens expressed in the yeast and to the immune response to yeast-specific antigens. Subcutaneous immunisations with complete *S. cerevisiae* cells generated a T cell induction, i.e. a cellular immune response. A protective, humoral immune response against an antigen with recombinant *S. cerevisiae* in a simple way (i.e. through direct application of a single antigen-expressing strain) has not been demonstrated in 'prior art'.

Based on the information above, the object was supposed to provide a method by which a protective, humoral immune response against the VP2 antigen of the virus of infectious bursitis (IBDV) can be generated. Another task was to produce a *subunit* marker vaccine, which would make it possible to distinguish vaccinated individuals

from naturally infected ones. Another task was to produce a *subunit* marker vaccine that also has strong adjuvant properties and thus is highly immunogenic.

These tasks were solved by providing arecombinant yeast of the species *Kluyveromyces lactis* which carries as a foreign gene a gene that codes for a VP2 antigen of the virus of infectious bursitis (IBDV), which is integrated into the yeast genome, and which enables the expression of the VP2 antigen of the virus Infectious Bursitis (IBDV) as a foreign protein, characterised in that this *Kluyveromyces lactis* strain is selected from:

Kluyveromyces lactis DSM 25405,

Kluyveromyces lactis DSM 25406, and

Kluyveromyces lactis DSM 25407.

The starting strain VAK 367-D4 (DSM 23097) used for the production of these strains permits targeted integration of the gene for expression of the VP2 antigen of the Infectious Bursal Disease Virus (IBDV) as a foreign gene into the yeast genome.

Recombinant yeasts expressing this foreign gene can be produced rapidly (i.e. within a few weeks) with this system. The yeasts can be increased in the fermenter in large quantities (e.g. kilogram/kg range) at low cost. Through regulated expression and fermentation in the fed-batch method, cytotoxic antigens can also be expressed in this yeast system. After expression of the foreign gene, the yeast is heat-inactivated and can then be stored and transported without refrigeration as a powder. The yeast powder can be used directly (i.e. without further fractionation) either as an emulsion or as a pellet (see design examples) as *subunit* marker vaccine. The antigen formulation and the adjuvant effect necessary for the effective (i.e. protective) immunisation are ensured by two factors: (i) through the possibility of targeted genetic engineering of the expressed foreign protein, (ii) through the expression of the foreign protein in the yeast and the direct application of the yeast in oral or subcutaneous form; the yeast itself has a strong adjuvant effect. subcutaneous administration is preferred. A recombinant yeast strain was created; it expresses a specific viral antigen and can be used for subcutaneous vaccination in the invention procedure. Complete preventive protection against infection by the virus was achieved. Only very small amounts of yeast (e.g. in the milligram/mg range for subcutaneous application in poultry) were used. Only 2-3 applications were necessary to achieve this protection level.

The procedure according to the invention is suitable for use in both the human and veterinary fields. The application of the method according to the invention in the veterinary field is preferred.

The procedure according to the invention is carried out with the yeast *Kluyveromyces lactis*.

The yeast *K. lactis* belongs to the so-called 'food grade' yeasts, which have GRAS status (GRAS: generally regarded as safe). Like the brewer's yeast, which has been tried and tested as a food additive over thousands of years, *K. lactis*, which is frequently used in dairy products, is considered harmless by the food industry.

In addition to the possibility of fermentation described under 'prior art', the yeast *K. lactis* has numerous advantages over *S. cerevisiae* with regard to the expression of heterologous genes. *K. lactis* belongs to the so-called 'petite negative' yeasts, which means that the loss of mitochondrial DNA is lethal (due to the collapse of the mitochondrial membrane potential (Chen et al., 1995; Clark-Walker, 2007)).

Mitochondrial function is closely linked to Ca^{2+} -dependent signalling, the production of reactive oxygen compounds, the stress response of the cell, protein glycosylation, and cell wall integrity. Thus, the mitochondrial function significantly impacts the production of recombinant glycoproteins and the composition of the cell wall.

In yeasts and mammals, the first steps of N-glycosylation of proteins taking place in the endoplasmic reticulum are the same. However, the steps taking place within the Golgi apparatus differ. The glycosyltransferases in the Golgi apparatus are different in the various yeast species. This leads to differences in the composition of the glycoproteins in the cell wall. In *K. lactis*, the glycoproteins contain terminal N-acetylglucosamine, as opposed to mannose phosphate in *S. cerevisiae*. (Raschke and Ballou, 1972). This could have a significant impact on the stimulation of the immune system by the respective yeast species in vaccinations.

The improved secretion of recombinant proteins in *K. lactis* mutants with altered α 1,6-mannosyltransferase (KIOCH1) illustrates the link between protein glycosylation/secretion and cell wall biosynthesis (Uccelletti et al., 2004). Changes in protein glycosylation also affect the intracellular localisation of recombinant proteins that are held back on the way to secretion due to defective folding.

K. lactis is one of the few yeasts that can process lactose as a carbon and energy source. Lactose is a cheap sugar that is available in high quantities as a component of whey (e.g. as a by-product in the dairy industry). *K. lactis* can achieve similar

growth rates with lactose as with glucose. Regulation of the genes involved in lactose metabolism has been extensively studied. The strong β -galactosidase promoter (LAC4) can be used to regulate the expression of heterologous genes and to produce recombinant proteins. (van Ooyen et al., 2006, Breunig et al., 2000). Due to
5 the decreased glucose repression, the heterologous expression of genes in *K. lactis* cultures cultivated in glucose-containing medium can be induced quickly and efficiently by addition of lactose.

In accordance with the invention, the *K. lactis* strains mentioned in claim 1, which represent variants of the strain VAK367-D4, are produced via genetic engineering
10 processes that permit the targeted integration of foreign genes at the LAC4 locus of the yeast genome (**Fig. 1**). This integration requires only one step via a correspondingly constructed plasmid; selection of recombinant strains is possible without the use of antibiotic resistance genes, and foreign gene expression in the recombinant strains can be induced via the *LAC4* promoter by adding lactose to the
15 medium. Using this method, *K. lactis* cells with integrated foreign genes can be generated and characterised in a few weeks. Both aspects of this system are of great importance: On the one hand, it makes the reproducible cultivation of yeast cells possible, with each containing defined amounts of a foreign protein (**Fig. 2, 3**). Furthermore, the additional integration of genes of the *KlGal4* transactivator into the
20 yeast genome can significantly increase the expression rate of the foreign gene (Kuger et al., 1990).

In another design, the invention relates to the derivatives of the *K. lactis* strain VAK367-D4 referenced in claim 1 for use in a method of subcutaneous vaccination. A series (VAK) was generated on recombinant variants derived from the *K. lactis*
25 strain VAK367-D4. Generally, these variants inducibly express significant amounts of a foreign protein, or domains of this foreign protein, or domains of this foreign protein fused with foreign protein domains. The related foreign protein domains are used for targeted stimulation of the immune response (adjuvant) or the targeted compartmentalisation of the expressed foreign protein in the yeast cell. In addition to
30 adjuvant effects, the compartmentalisation of the expressed foreign protein is important for the optimisation of the expression or the formulation of the expression product.

In another design, the method of the invention is performed with derivatives of VAK367-D4 for use as a *subunit* marker vaccine. The use of recombinant *K. lactis*

that only express defined protein antigens (foreign proteins) as a vaccine in a differential diagnosis enables the discrimination of vaccinated against naturally infected individuals. One of these recombinant *K. lactis* strains (see design examples) has been used successfully for oral and subcutaneous vaccination. After subcutaneous administration, complete protection of the vaccinated objects was achieved.

'foreign protein' in the context of this invention refers to the VP2 antigen of the Infectious Bursal Disease Virus (IBDV), which is capable of generating a protective immune response, preferably a protective humoral immune response, in humans or in animals against a pathogen. In the most preferred design of the invention, the foreign proteins are from members of the family *Birnaviridae*, such as the IBD virus, and are capable of inducing a protective immune response, preferably a protective humoral immune response.

In one example, a *K. lactis* VAK367-D4 variant VP2 (VAK887) was generated which expresses the capsid-forming VP2 antigen of the virus of infectious bursitis (IBDV strain D78) as a foreign protein (**SEQ ID NO.: 1 and 2**). Especially preferred is a *K. lactis* VAK367-D4 variant VP2-T2S (VAK888) in which the VP2 protein was mutated at amino acid position 2 (exchange of threonine for serine) ; Jagadish et al. 1991) and which exhibits the nucleotide or amino acid sequence according to **SEQ ID NO.: 3 and 4**.

In a particularly preferred design of the invention, an optimised *K. lactis* VAK367-D4 variant, VP2T2S_GAL4, was generated in which the VP2 protein was mutated at amino acid position 2 (**SEQ ID NO.: 3 and 4**) and additionally contained at least two *KIGAL4* genes (VAK890). Particularly preferred is a *K. lactis* VAK367-D4 variant, oVP2-T2S, in which the mutated VP2 antigen is encoded by the codon-optimised nucleic acid sequence with **SEQ ID NO.: 5** or in which the recombinantly expressed mutant VP2 antigen exhibits the amino acid sequence according to **SEQ ID NO.: 6**. The optimised *K. lactis* VP2-T2S_GAL4 variant (VAK890) has the following advantages:

- The mutation further stabilised the foreign protein.
- Overexpression of the transactivator and/or through codon optimisation of the sequence led to a significant increase in VP2 expression (**Fig. 2**).

- The integration of additional *KIGAL4* genes also correlated with a higher growth rate of this *K. lactis* variant.
- This *K. lactis* variant shows a particularly high reproducibility in the high cell density fermentation and the amount of expressed VP2 protein (**Fig. 3**).

5

The *K. lactis* VP2-T2S_GAL4 variant produced according to the invention, which recombinantly expresses the mutated VP2 antigen of the IBDV as a foreign protein and contains further copies of the *KIGAL4* transactivator gene (VAK890), was recorded on 29 November 2011 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures),
10 DSMZ, Inhoffenstrasse 7B, 38124 Braunschweig, Germany, in accordance with the Budapest Treaty under the number DSM 25405.

The *K. lactis* oVP2-T2S variant produced according to the invention, which recombinantly expresses the mutated and codon-optimised VP2 antigen of the IBDV
15 as a foreign protein (VAK910), was recorded on 29 November 2011 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Inhoffenstrasse 7B, 38124 Braunschweig, Germany, in accordance with the Budapest Treaty under the number DSM 25406.

The *K. lactis* oVP2-T2S variant produced according to the invention, which
20 recombinantly expresses the mutated and codon-optimised VP2 antigen of the IBDV as a foreign protein and contains further copies of the *KIGAL4* transactivator gene (VAK911), was recorded on 29 November 2011 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Inhoffenstrasse 7B, 38124 Braunschweig, Germany, in accordance with the Budapest Treaty under the number
25 DSM 25407.

Another design regards the use of the recombinant yeasts according to the invention in a method for producing protective immunisation, in particular protective humoral immunisation.

30 Such a method comprises the following steps:

- a) Cultivation and propagation of the recombinant yeasts according to the invention,
- b) harvest and inactivation of the yeasts,

- c) application of the recombinant yeasts pursuant to a defined immunisation scheme,
- d) titre determination of the formed antibodies and/or
- e) evidence of immunisation.

5

According to the invention, the cultivation and propagation of the recombinant yeasts can be carried out by any conventionally available method. Particularly preferred are methods which lead to high cell yields at low cost. This includes fermentation methods, in particular methods of high cell density fermentation. A particularly
10 advantageous method is to carry out the fermentation using a *fed-batch* fermentation protocol.

In a preferred design, the protective humoral immunisation is achieved by administering the recombinant yeasts orally/mucosally or subcutaneously. In a
15 particularly preferred design of the invention, the recombinant yeasts are administered subcutaneously. A particularly preferred method according to the invention is the use of the *K. lactis* strains mentioned in claim 1 for subcutaneous administration.

In the method according to the invention, the recombinant yeast cells should be
20 inactivated/killed before use. Therefore, the yeast cells are dried after the cultivation and expression of the foreign genes and then inactivated. Inactivation can be carried out by any conventionally available method. Particularly suitable for use in the method according to the invention is heat inactivation (e.g. heat inactivation for 2 hours at 90 °C).

25 Oral/mucosal vaccination can either be administered via a short 1/1/1/1/1 immunisation regimen (1 week feeding, 1 week break, 1 week feeding, etc.) or a longer 2/2/2 regimen (2 weeks feeding, 2 weeks break, 2 weeks feeding, etc.). For example, a two-fold or a three-fold application at intervals of two weeks may be used for subcutaneous vaccination (**Fig. 4 and 5**)

30 All conventional methods are available to establish successful immunisation. In one design of the invention, the titre of virus-neutralising antibodies is tested to demonstrate immunisation. For example, specific ELISA tests or neutralisation assays can be carried out for this purpose. A defined number of IBD viruses in the neutralisation assay are offset with a defined amount of serum from an immunised

animal or a control animal. Subsequently, the inhibition of the infection (neutralisation) by the treated viruses in cell culture is tested. Whether an immunisation was successful can also be tested in a '*challenge*' experiment, e.g. in a 'virus challenge' experiment. To accomplish that, the treated animals are administered a dose of a pathogenic microorganism or virus that would normally cause disease in unimmunised animals. If the animals do not exhibit any signs of the disease after such a *challenge*, proof of successful immunisation is provided (**Fig. 5**). Finally, the detection of immunisation can also be provided by immunohistochemistry. After the *challenge*, the target organs of the pathogen are examined for infection or lesion (**Fig. 5**).

According to the invention, it was shown that recombinant *K. lactis* variants derived from VAK367-D4 could be used successfully for vaccination by subcutaneous administration. The strain variant VAK890, which is described in the design examples, expresses the VP2 antigen of the Infectious Bursal Disease Virus (IBDV, strain D78). The VP2 of IBDV is a viral capsid-forming protein. It is a known fact about VP2 that the induction of a humoral immune response against this antigen is sufficient to protect an infected organism from a subsequent infection by the virus in question (IBDV). Triggering an effective humoral immune response could be indexed via quantification of virus-neutralising antibodies. On the other hand, the detection of a protective immune response via a 'virus challenge experiment' and immunohistochemistry was performed after the virus *challenge*. According to the invention, recombinant *K. lactis*, or recombinant *K. lactis* starting from strain VAK367-D4, could be established in subcutaneous applications as effective, i.e. 90-100% protective vaccine (90-100% corresponds to 'gold standard' in vaccination) (**Fig. 4 and 5**). The recombinant *K. lactis*, or recombinant *K. lactis* starting from strain VAK367-D4, was thus established as '*subunit*' marker vaccination against infectious agents such as viruses. That means, a single immunogenic protein subunit of a virus was used as the antigen. Use as a '*subunit*' marker vaccine implies that using it facilitates the distinction of vaccinated from non-vaccinated, infected organisms. This is possible, for example, via the use of a differential diagnostic method that detects both antibodies against the antigen used for vaccination, as well as antibodies against another antigen of the infectious agent. Immunisation with the recombinant *K. lactis* strain VAK890 starting from the strain VAK367-D4 enables generation of antibody titres against the corresponding viral antigen. These antibodies have been

shown to have a virus neutralising effect. It can already be empirically deduced from this property and the measured high titre that this humoral immune response is sufficient to protect an organism from a subsequent infection with the relevant virus. The final proof could be provided for the IBDV. The high titre of virus-neutralising antibody produced in the chicken model correlated with complete protection of the vaccinated animals against subsequent virus infection (Fig. 5).

The use of the *K. lactis* strains stated in claim 1, which are genetically engineered variants of strain *VAK367-D4*, such as *K. lactis* VP2-T2S_GAL4 (VAK890), has the following major advantages over conventional methods:

1. For use in foreign gene expression, *K. lactis* has substantial fundamental advantages over *S. cerevisiae*, which are due to the divergent physiology of *K. lactis* over millions of years of *Scerevisae*.
2. The foreign gene is not expressed via plasmid vectors as described in WO 90/15140, but after targeted and stable integration of the foreign gene in a defined locus of the *K. lactis* genome. This enables high reproducibility of protein expression under non-selective conditions. This aspect is essential for the reproducible production of the vaccine by cultivating the yeast strain in the fermenter. The principle of the strain *VAK367-D4* and its derivatives have already been described for oral vaccination (WO 20101054649 A2). The present invention now demonstrates that the strain *VAK367-D4* and its derivatives, especially *K. lactis* VP2-T2S_GAL4, result in effective protection against viral infections when administered subcutaneously using much lower amounts of yeast.
3. The gene expression is inducible and can be further raised by increasing the concentration of the transcriptional activator Gal4 and/or through codon-optimisation of the nucleotide sequence of the foreign gene in adaptation to the yeast host. The establishment of a fed-batch fermentation protocol facilitates efficient production of cytotoxic antigens.
4. Integration of the foreign gene into *VAK367-D4* and its derivatives is a 'one-step procedure'. This means that new recombinant strains can be produced in about 3 weeks; this is particularly important for the rapid development of efficient vaccines against mutated virus variants.
5. Through subcutaneous administration of recombinant yeast of type *K. lactis*, specifically recombinant yeast of the strain *VAK367-D4* and derivatives

thereof, a protective immune response could be generated both in mice and in chicken. The procedure is very simple: A defined quantity of inactivated (heat-killed) yeast cells are injected under the skin into the inoculation recipient in a 2-3 time procedure. Two weeks after the last application, the inoculation recipient's vaccine serum is assayed to look for presence and functionality of antigen-specific antibodies. Virus neutralisation tests have shown that this immune response was predominantly, if not exclusively, based on the production of neutralising antibodies (protective humoral immune response). Thus, the immune response, inducible by *K. lactis* in subcutaneous application, is fundamentally different from the immune response inducible by *S. cerevisiae*, which mainly induces a T cell response. The possibilities of subcutaneous application of *K. lactis* are therefore fundamentally different from the possibilities of subcutaneous administration of *S. cerevisiae*: while *K. lactis* may be used as a *subunit* vaccine in antigens capable of producing a protective humoral immune response (e.g. viral antigens such as the VP2 antigen of the Infectious Bursal Disease Virus, IBDV or hemagglutinin HA antigen of the influenza virus), *S. cerevisiae* may be used as a *subunit* vaccine in antigens capable of producing a protective cellular immune response (such as the NS3 protein of the hepatitis C virus or tumour antigens such as Her-2). These differences in the form of the induced immune response are likely due to the very different characteristics of the *S. cerevisiae* and *K. lactis* cells outlined above

Combined, the present invention makes a substantial contribution to the prior art and provides numerous advantageous designs over the prior art:

- The inventors managed to produce *subunit* marker vaccines with which it is possible to distinguish vaccinated individuals from naturally infected ones.
- Furthermore, *subunit* marker vaccines can be produced which also have strong adjuvant properties and are thus highly immunogenic.
- The *subunit* marker vaccines according to the invention can be used several times.
- The *subunit* marker vaccines of the invention produce a systemic protective immune response and immunological *memory* in vaccine.

- The present invention also makes it possible to produce vaccines against cytotoxic antigens.
- The method according to the invention allows the fastest possible generation of new vaccine variants.
- 5 • The vaccination methods are particularly cost-effective.
- For the production of the vaccine according to the invention, no laboratory animals or animal/human cells cultures are necessary.
- The vaccines according to the invention are not temperature-sensitive; they can be transported and stored without cooling.
- 10 • In the method of the invention, no living recombinant cells or organisms are used.
- With the method according to the invention it is possible to limit both the quantities of vaccine used and the number of applications necessary for achieving protective immunisation to a minimum level.

15

Design examples

1. Creation of the *K. lactis* strain VAK367-D4 (*metA ura3-5 lac4::ScURA3*).

The starting strain VAK367 for the heterologous expression of foreign proteins has the following characteristics: It facilitates cultivation to a high cell density without any intracellular proteins being verifiably released. In that regard, this strain differs from many closely related *K. lactis* strains. Strain VAK367 was derived from strain CBS 2359 (Centraalbureau voor Schimmelcultures <http://www.fungalbiodiversitycentre.com>) through two rounds of mutagenesis and is auxotrophic for the amino acid methionine and the nucleobase uracil. The strain VAK367-D4 (filed on 18/11/2009 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig under accession number DSM 23097) was derived from the strain VAK367 through genetic engineering, whereby the sequence of +358 to +1181 of the LAC4 gene was replaced by the ScURA3 gene with the aid of the plasmid pD4-2. The strain VAK367-D4 now allows integration of foreign genes at the LAC4 locus without additional markers by selecting lactose growth. Using a suitable integration vector, such as Klp3-MCS (**Fig. 6**), the disruption cassette is replaced through homologous recombination so that an intact *LAC4* gene is reconstituted with loss of the *ScURA3* marker. (**Fig. 1**)

2. *Generation of an integration vector that allows the inducible expression of foreign genes.*

Vector: Klp3

Vector: Klp3-MCS (SEQ ID No.: 10)

- 5 Vector Klp3-MCS (**SEQ ID No.: 10**) (**Fig. 6**) is an *E. coli* vector based on YRp7 that can not autonomously replicate in yeasts because the ARS1 sequence has been deleted. Klp3-MCS (**SEQ ID No.: 10**) contains the *K. lactis* *LAC4* promoter and sequences that allow integration at the *LAC4* locus through homologous recombination.
- 10 A DNA segment containing the *TEF1* terminator and the *KIGAL80* promoter was inserted between the *LAC4* promoter and transcription start. After reconstitution, this allows the *LAC4* reading frame to be expressed via homologous recombination under the control of the *KIGAL80* promoter. The *KIGAL80* promoter is co-regulated via transcription factor *KIGal4* with the *LAC4* promoter (Zenke et al. 1993). This design makes it possible to follow the
- 15 induction of foreign gene expression by measuring the *LAC4*-encoded β -galactosidase. Klp3-MCS (**SEQ ID No.: 10**) facilitates insertion of the foreign gene between *LAC4* promoter and *TEF1* terminator via one of the unique interfaces in the *multiple cloning site* (MCS) (**Fig. 6**). For integration, the resulting plasmid is digested with suitable restriction enzymes, so that the expression cassette is separated from the *E. coli* vector sequences. After transformation
- 20 into *K. lactis* VAK367-D4, the expression cassette is chromosomally integrated; the resulting strains do not contain any bacterial sequences.

3. *K. lactis* variant expressing the VP2 antigen of the Infectious Bursal Disease Virus (IBDV variant D78).

Production of the recombinant yeast strain

- 25 The cDNA encoded for IBDV D78 VP2 was amplified from plasmid pD78A (Icard et al., 2008) using the following oligonucleotides:
- IBDV_AscI_fwd (5'-GGCGCGCCGATGACAAACCTGCAAGATC-3') (**SEQ ID NO.: 7**), containing an *AscI* restriction cleavage site, and
- VP2_NotI_rev (5'-ATAAGAATGCGGCCGCTCACACAGCTATCCTCCTTATG-3') (**SEQ ID NO.: 8**), containing an *NotI* restriction cleavage site, and
- 30 The following oligonucleotide pair was used to generate VP2-T2S:
- IBDV_S:T_AscI_fwd (5'-GGCGCGCCGATGTCTAACCTGCAAGATCAAACCCA-3') (**SEQ ID NO.: 9**), and VP2_NotI_rev (see above).

The amplified DNA fragments were cloned after checking and confirming the nucleotide sequences on the *Ascl* and *NotI* interfaces in the vector Klp3-MCS (SEQ ID NO.: 10) (Fig. 6). This was followed by integration into the genome (Fig. 1). Specifically, the integration plasmid was digested with the restriction enzyme *EcoRI*, and the digested fragments were then transformed into *VAK367-D4* cells. The transformed cells were plated on YEPD medium and incubated overnight at 30 °C. To find positive colonies, the transformation plate was duplicated on SM medium containing lactose as a carbon source and incubated for 2 days at 30 °C. The positive clones identified in this procedure were further investigated.

Genome integration of additional *KIGAL4* gene copies was performed with a conventional method (Kuger et al. (1990). The codon optimisation followed a *Saccharomyces cerevisiae* algorithm (mr.gene.com, Raab et al., 2010). The codon-optimised DNA fragments were synthesised directly. During the synthesis, the 5' *Ascl* and 3' *NotI* restriction sites were already incorporated (mr.gene.com, Regensburg, Germany). Subsequently, the cloning was performed in vector Klp3-MCS (SEQ ID No.: 10).

Western blot analysis.

Cell pellets were resuspended in B60 buffer (50mM HEPES-KOH pH 7.3; 60mM potassium acetate; 5mM magnesium acetate; 0.1% Triton X100; 10% glycerol; 1mM sodium fluoride; 20mM glycerol phosphate; 10mM $MgCl_2$; 1mM DTT; protease complete inhibitor [Roche]) and disrupted by vigorous mixing with glass beads. The extract was centrifuged (14,000 rpm, 20 min. at 4 °C) and the protein concentration was determined. 40 µg of the protein extract were separated with SDS-PAGE in a 12% gel. Then the proteins were transferred to a membrane. Western blot analyses were performed with rabbit α -IBDV antiserum (1:15,000, Granzow et al., 1997) and goat- α -rabbit HRP-coupled antibodies (1:3000, Santa Cruz Biotechnology, Inc.) using conventional methods.

Northern blot analysis.

5 ml of a yeast culture was cooled on ice for complete extraction of the RNA. Cell lysis was performed in Prot K buffer (100 mM Tris/HCl pH 7.9, 150 mM NaCl, 25 mM EDTA, 1% SDS) and 50 mg proteinase K (Fermentas) under vigorous shaking with glass beads. The samples were incubated for 1 h at 35 °C, and the RNA was extracted, precipitated with ethanol and resuspended in DEPC water. Northern analysis was performed as described in Engler-Blum et al., 1993, but with slight deviations. 5 µg of total RNA was separated on a 1% formaldehyde agarose gel and transferred to a nylon membrane (Amersham Hybond™ N+, GE Healthcare). The membrane was incubated at 68 °C with a DIG-labelled RNA probe, which was created by *in vitro* transcription of PCR fragments in the presence of DIG-NTPs (Roche). The blot was treated with a blocking solution and incubated with an anti-DIG alkaline phosphatase-conjugated antibody (Roche). The determination of the alkaline phosphatase activity was carried out with conventional methods.

Quantification of heterologously expressed VP2.

A modified protocol (according to Saugar et al., 2005, 2000 ODE) of a yeast culture transformed with an episomal VP2 plasmid (pADH1-P VP2-T2S) was used on selective medium (0.67% YNB, 2% glucose and

the following additions: 11 mg/l Ade; 14 mg/l Tyr; je 38 mg/l His, Trp, Arg, Met; 48 mg/l

Phe; each 58 mg/l Leu, lie, Lys, Val, Thr) cultivated overnight. After harvesting and washing the cells with distilled water, they were broken down with glass beads in lysis buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 20 mM CaCl₂, 1 mM EDTA, protease complete inhibitor [Roche], pH 8.0). The resulting protein extract was centrifuged (10,000 g for 1 h at 4 °C) and the soluble fraction was layered on a 20% (w/v) sucrose cushion in sucrose buffer (10 mM Tris pH 8.0, 150 mM NaCl, 20 mM CaCl₂; contained protease complete inhibitor [Roche]). After centrifugation at 170,000 g for 3 h at 4 °C, the pellet was dissolved in 200 µl of sucrose buffer and centrifuged for another 17 h at 114,000 g in a 20 to 53% sucrose gradient in sucrose buffer. The gradient was collected in 700 µl fractions and analysed using SDS-PAGE and Western blot. Oligomeric protein complexes of the heterologously expressed VP2

was concentrated and purified in this way. The protein could be demonstrated and the amount of protein determined via SDS PAGE and Coomassie staining in comparison to a standard protein (not shown). The purified VP2 was then used as standard in a comparative Western blot with anti-VP2 antibody. The VP2 amount of a defined number of yeast cells from different fermentations was compared (**Fig. 3**).

Yeast fermentation and heat inactivation.

All experimental fermentations were performed in a DasGip parallel bioreactor system (DasGip AG, Jülich, Germany) with four fully equipped 2L fermenters. Production scale fermentations were carried out by the company Organobalance GmbH (Berlin, Germany) or in our own laboratory in a Biostat ED Bioreactor (B. Braun Biotech, Melsungen, Germany) with 10 l workload capacity. All production processes were performed with the fed-batch process. A complex culture medium with 2% yeast extract, 1% peptone and a 20% lactose feed solution was used. The temperature of the yeast culture was maintained at 30 °C and the pO₂ was controlled to 30% saturation. The pH value was maintained at 5.0 during the fermentation by adding 2M NaOH or 2M H₃PO₄.

For *in vivo* experiments in mice and chickens, the yeasts were freeze-dried and then heat-inactivated for 2 h at 90 °C. Using this method, less than 10 cells per gram of dry cell weight were viable.

4. Subcutaneous administration in mice

For subcutaneous administration of a *K. lactis* variant expressing the VP2 antigen of the Infectious Bursal Disease Virus (IBDV variant D78) (VAK890) in mice, the dried and powdered yeast for the first application was mixed with complete Freund's adjuvant (CFA); in other applications, the yeast was mixed with incomplete Freund's adjuvant (IFA) (100 µg of yeast material per 200 µl of CFA or IFA). 200 µl of the emulsions (containing 100 µg of yeast) were injected for each immunisation/boost per individual. Thus, the amount of VP2 administered per subcutaneous immunisation of an individual mouse was approximately 18 ng (**Fig. 3**). After the initial injection (day 0), the mice received 'boosters' twice at two-week intervals (on days 14 and 28; **Fig. 4**). After another two weeks, the animals were killed under anaesthesia to draw the blood serum.

5. Subcutaneous application in chickens

For subcutaneous administration in chickens, 5 mg of the dried and powdered *K. lactis* variant expressing the VP2 antigen of the Infectious Bursal Disease Virus (IBD variant D78) were dissolved (VAK890) in 750 µl of phosphate buffer/saline (PBS) and 500 µl of sterile distilled water, and an emulsion of 1.25 ml of IFA was prepared. 500 µl of this emulsion (containing 1 mg of yeast) were injected on days 0, 14 and 28 (**Fig. 5**). Thus, the amount of VP2 administered per subcutaneous immunisation of an individual chicken was about 180 ng (**Fig. 3, 4**).

6. Virus 'challenge'

After vaccination (**Fig. 5**), vaccinated chickens were infected on day 42 through oral administration with 100% EID50 of the IBDV strain 'Edgar' and mortality rates were determined after six days. After subsequent killing of the animals under anaesthesia, the sera were recovered and the bursae of the animals were removed. They were initially fixated in 10% neutral buffered formalin for 24 hours and then embedded in paraffin.

7. Enzyme-linked immunosorbent assay (ELISA).

The IBDV-specific antibody titres in the sera of the vaccinated objects were determined via a commercial ELISA assay: IDEXX FlockChek® IBD ELISA kit (IDEXX Laboratories, Inc.). For the sera from vaccinated mice, a non-proprietary secondary antibody was used (Sigma Aldrich).

8. Neutralisation assay.

The neutralisation assay for determining the concentration of virus-neutralising antibodies was carried out according to the protocol of Schröder et al., 2000.

9. Immunohistochemistry.

4 micron thick organ sections were prepared from the paraffin-embedded bursae. After removal of the paraffin, they were stained with hematoxylin and eosin in accordance with standard procedures. The samples were examined microscopically and the 'lesion score' was determined on a scale of 1-4 (1 = normal to 10% follicular

atrophy; 2 = 10-30% follicular atrophy; 3 = 30-70% follicular atrophy; 4 => 70% atrophy).

Results

5 *Production and optimisation of the K. lactis strain expressing IBDV VP2*

Different K. lactis variants with integrated IBDV VP2 gene were produced. For the vaccination experiments, an optimised variant was used in which the VP2 protein was mutated at amino acid position 2 (exchange threonine for serine; Jagadish et al., 1991), and which contained an additional tandem integration of at least two *KIGAL4* genes (variant VP2-T2S_GAL4; strain VAK890). The mutation further stabilised the
 10 foreign protein; overexpression of the transactivator led to a significant increase in VP2 expression (**Fig. 2**). The integration of additional *KIGAL4* genes also correlated with a higher growth rate of this *K. lactis* variant. The growth conditions for the respective VP2-expressing *K. lactis* strain VAK890 were optimised so that the yeast
 15 could be fermented in high densities and with reproducible quantity of expressed VP2. After preparation, the yeast was freeze-dried and inactivated at 90 °C for 2 hours. Proof of inactivation was carried out: Each gram of inactivated yeast material contained less than 10 living yeast cells. The quantity of VP2 per yeast cell was determined: It was about 0.7 fg heterologous VP2 protein per yeast cell for the strain
 20 VAK890 (**Fig. 3**)

Subcutaneous administration in mice and chickens

The immunisations were implemented as described above; two weeks after the last application, the sera of the treated vaccinated objects were examined for presence of
 25 neutralising antibodies. An IBDV-specific ELISA was used and an IBDV neutralization assay was performed (**Fig. 4 and 5**). Moreover, a 'virus challenge' experiment was performed with the vaccinated chickens. For this task, the animals received a virus dose of 100 EID50 per animal of the highly virulent IBDV strain 'Edgar', a concentration that leads to significant bursitis in non-vaccinated poultry
 30 with a mortality rate of about 10-35% (**Fig. 5D**). After the 'virus challenge' experiment, the bursae of the vaccinated objects were examined for signs of infection and lesions in the bursae using immunohistochemistry and assessed using the so-called 'lesions score' (**Fig. 5**).

Both the experiments with mice and the experiments with chickens demonstrated that subcutaneous application of *K. lactis* strain VAK890 produced high titres of virus-neutralising antibodies in virtually all treated animals (**Fig. 4B, 4C; Fig. 5B, 5C**). It was also shown that virtually all vaccinated chickens were protected against virus challenge and showed virtually no signs of viral infection in their bursae (**Fig. 5**). All animals that were inoculated subcutaneously with *K. lactis* strain VAK890 showed a significant humoral immune response against VP2. This immune response was observed after a single boost, suggesting that two injections, which can also be carried out with incomplete Freund's adjuvant (immunisation and boost), are already sufficient to provide protection. In addition, all chickens that were inoculated with *K. lactis* strain VAK890 were protected against subsequent viral infection (**Fig. 5**).

Abbreviations

	ARS1	autonomously replicating sequence; nucleotide sequence on DNA at which replication is initiated
	Asc I	Restriction endonuclease Asc I
5	CFA	complete Freund's adjuvant
	DNA	Deoxribonucleic acid
	DEPC	diethylpyrocarbonate
	DIG-NTP	Digoxigenin nucleotide triphosphate
	DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
10	(German Collection of Microorganisms and Cell Cultures)	
	DTT	dithiothreitol
	E. coli	<i>Escherichia coli</i>
	EcoRI	Restriction endonuclease EcoR I
	EDTA	ethylenediaminetetraacetic acid
15	EID50	Egg or embryo infectious dose - the number of infectious viruses necessary to cause infection in 50% of infected eggs
	ELISA	Enzyme-linked immunosorbent assay
	GAL4	yeast-specific transcriptional activator
	GRAS	generally regarded as safe
20	HEPES	2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethane sulphonic acid
	Hpa I	Restriction endonuclease Hpa I
	HRP	horseradish peroxidase
	IBDV	Infectious Bursal Disease Virus
	CFA	incomplete Freund's adjuvant
25	<i>K. lactis</i>	<i>Kluyveromyces lactis</i>
	<i>KIGAL4</i>	<i>K. lactis</i> gene encoding the KIGal4/Lac9 protein
	<i>KIGAL80</i>	<i>K. lactis</i> gene encoding the KIGal80 protein
	<i>LAC4</i>	<i>K. lactis</i> gene encoding a β -galactosidase enzyme
	Not I	Restriction endonuclease Not I
30	ODE	Optical density unit
	PBS	phosphate buffer/saline
	PCR	Polymerase chain reaction
	RNA	Ribonucleic acid
	<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>

	<i>Sal</i> I	restriction endonuclease <i>Sal</i> I
	SDS	sodium dodecyl sulphate
	SDS-PAGE	Polyacrylamide gel electrophoresis using SDS
	TEF1	<i>Arxula adenivorans</i> gene
5		encoding translation factor EF-1 alpha
	VP2	Capsid-forming virus protein of the IBDV
	VP2-T2S	VP2 with an amino acid exchange of threonine against serine at position 2
	VAK	vaccine strain
10	YEPD	Yeast extract peptone dextrose
	YRp7	<i>S. cerevisiae</i> - <i>E.coli</i> shuttle vector, Gene bank Accession U03501 (Botstein et al., 1979)

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P A T E N T K R A V

1. Rekombinant gær af arten *Kluyveromyces lactis*, der bærer et gen, som koder for et VP2-antigen af smitsom bursitisvirus (IBDV), som fremmedgen, der er integreret i gær-genomet, og som muliggør udtrykkelsen af VP2-antigenet af smitsom bursitisvirus (IBDV) som fremmedprotein, k e n d e t e g n e t ved, at denne stamme af *Kluyveromyces lactis* er udvalgt blandt:

Kluyveromyces lactis DSM 25405;

Kluyveromyces lactis DSM 25406, og

10 *Kluyveromyces lactis* DSM 25407.

2. Rekombinant gær ifølge krav 1, k e n d e t e g n e t ved, at fremmedgenudtrykkelsen sker konstitutivt, eller at fremmedgenudtrykkelsen er inducerbar.

3. Rekombinant gær ifølge krav 1 eller 2, k e n d e t e g n e t ved, at fremmedgenudtrykkelsen kan kvantificeres indirekte via udtrykkelsen af et endogent rapportørgen.

- 15 4. Rekombinant gær ifølge et eller flere af de foregående krav til anvendelse i en fremgangsmåde til subkutan vaccinerings.

5. Rekombinant gær ifølge krav 4, k e n d e t e g n e t ved, at de rekombinante gærstammer anvendes som *subunit*-markørvacciner.

- 20 6. Rekombinant gær ifølge krav 5, k e n d e t e g n e t ved, at *subunit*-markørvaccinerne anvendes til at skelne vaccinerede individer fra naturligt inficerede individer.

7. Rekombinant gær ifølge krav 5 eller 6, k e n d e t e g n e t ved, at *subunit*-markørvaccinerne desuden udviser stærke adjuvansegenskaber.

- 25 8. Rekombinant gær ifølge krav 5 til 7, k e n d e t e g n e t ved, at *subunit*-markørvaccinerne er stærkt immunogene.

9. Rekombinant gær ifølge krav 1 til anvendelse i en fremgangsmåde til subkutan vaccinerings ved hjælp af hele gærceller af en rekombinant gær, k e n d e t e g n e t ved, at der fremkaldes en beskyttende humoral immunisering mod udtrykt fremmedprotein, og at fremgangsmåden omfatter følgende trin:

- 30 a) dyrkning og opformering af den rekombinante gær,
b) høst og inaktivering af gæren,
c) anvendelse af den rekombinante gær ifølge et fastlagt immuniseringsskema,
d) titerbestemmelse af de dannede antistoffer og/eller
e) påvisning af immunisering.

- 35 10. Rekombinant gær til anvendelse ifølge krav 9, k e n d e t e g n e t ved, at der ved hjælp af subkutan indgivelse af hele gærceller af en rekombinant gær af arten *Kluyveromyces lactis* fremkaldes en beskyttende humoral immunisering mod udtrykt fremmedprotein.

- 40 11. Oligonukleotidpar, som udviser en nukleinsyresekvens ifølge SEQ ID NR. 8 og SEQ ID NR. 9.

12. Ekspressionsvektor Klp3 eller Klp3-MCS ifølge SEQ ID NR. 10, som bærer et

fremmedgen, k e n d e t e g n e t ved, at fremmedgenet udviser nukleinsyresekvensen ifølge SEQ ID NR. 3 eller SEQ ID NR. 5, til integration i udgangsstammen VAK367-D4 af *Kluyveromyces lactis*, deponeret under DSM 23097.

13. Ekspressionsvektor ifølge krav 12, k e n d e t e g n e t ved, at fremmedgenet
- 5 koder for proteinet IBDV VP2-T2S med aminosyresekvensen ifølge SEQ ID NR. 4 eller proteinet IBDV oVP2-T2S med aminosyresekvensen ifølge SEQ ID NR. 6.

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Drawings

Figure 1

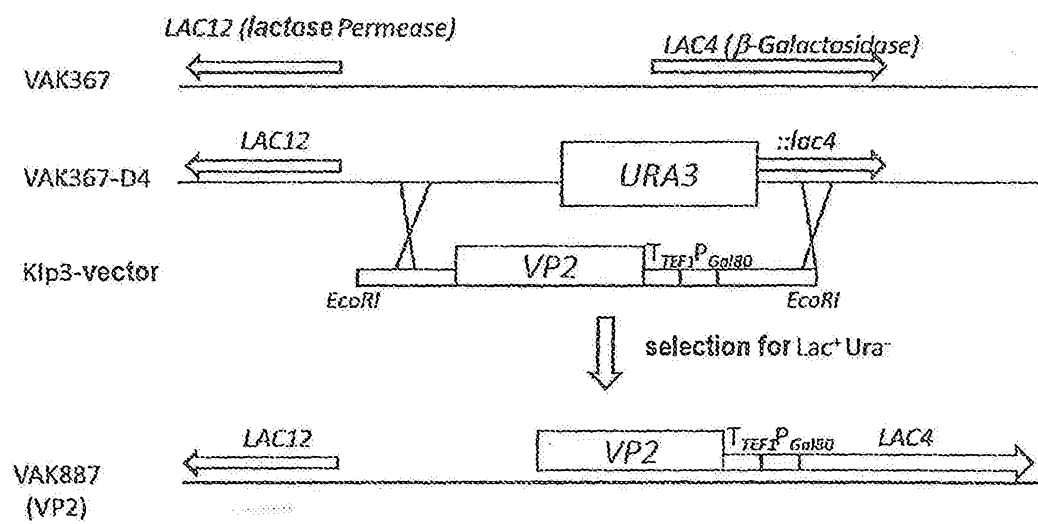


Figure 2A

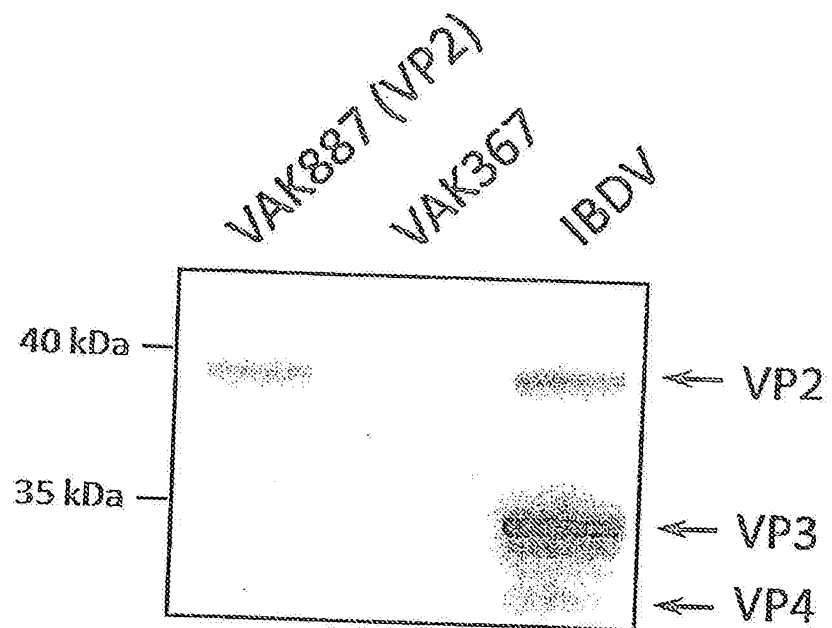
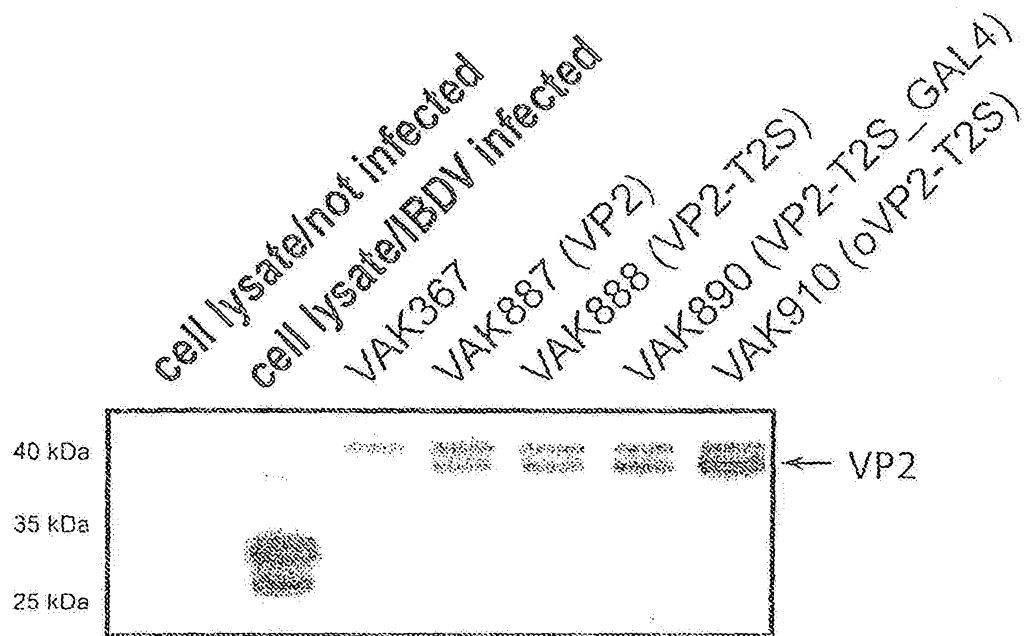
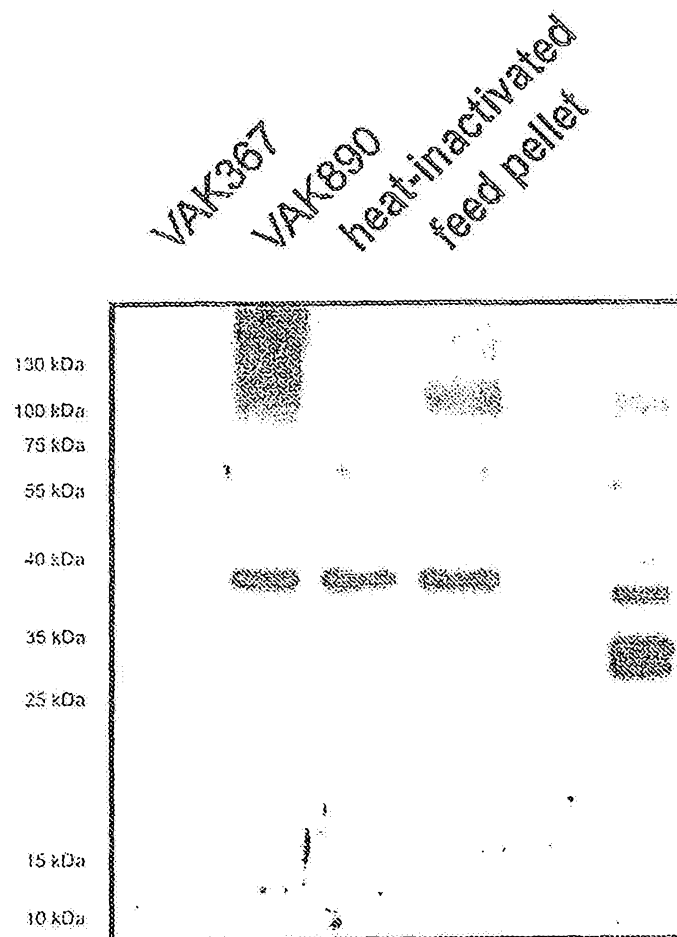


Figure 2B



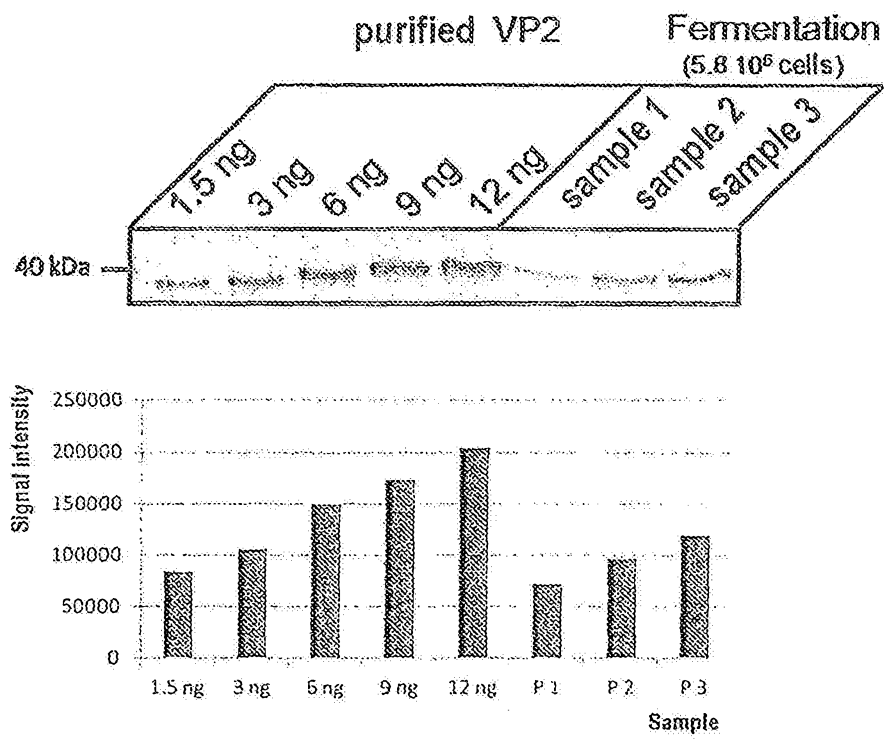
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Figure 3A



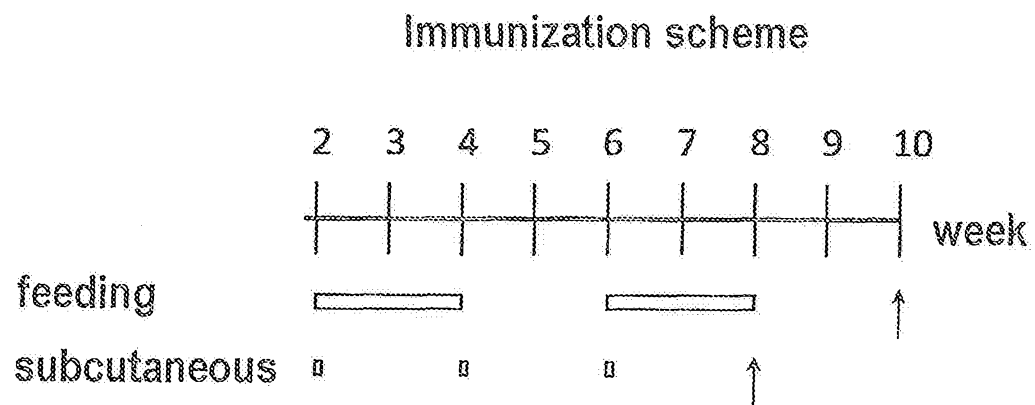
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Figure 3B



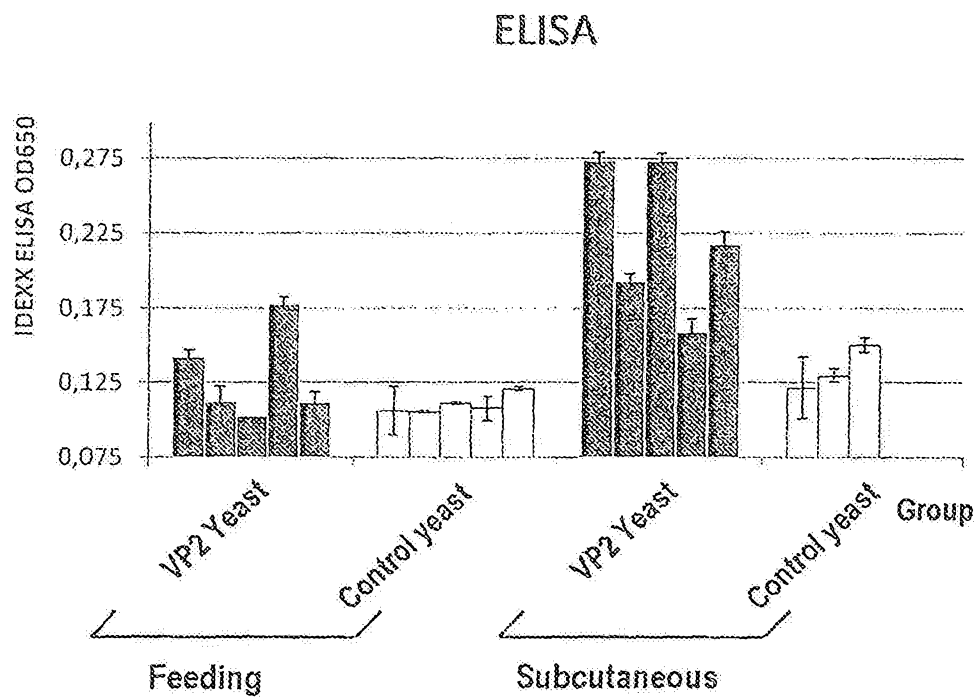
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Figure 4A



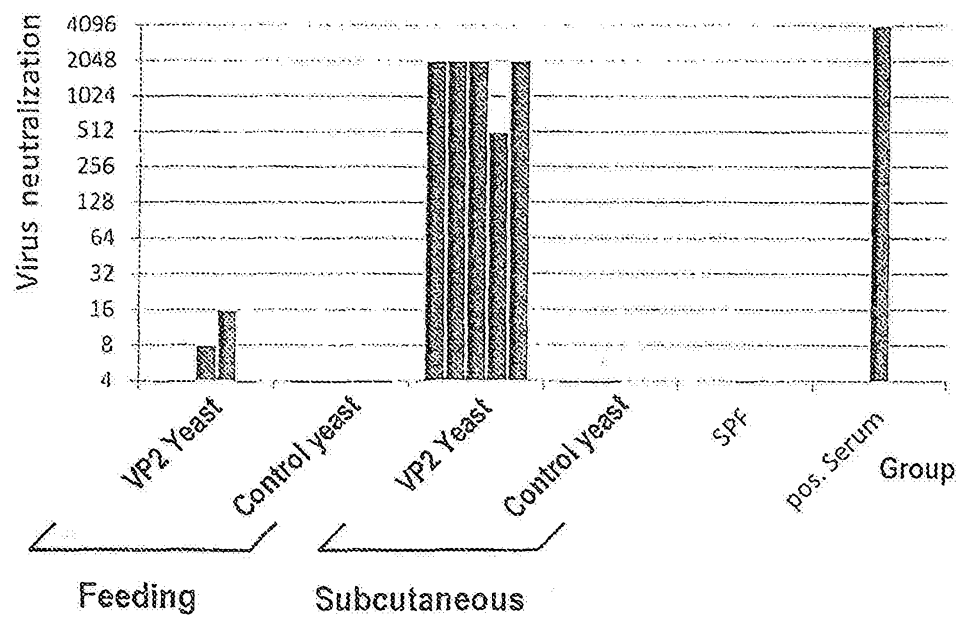
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Figure 4B



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Figure 4C



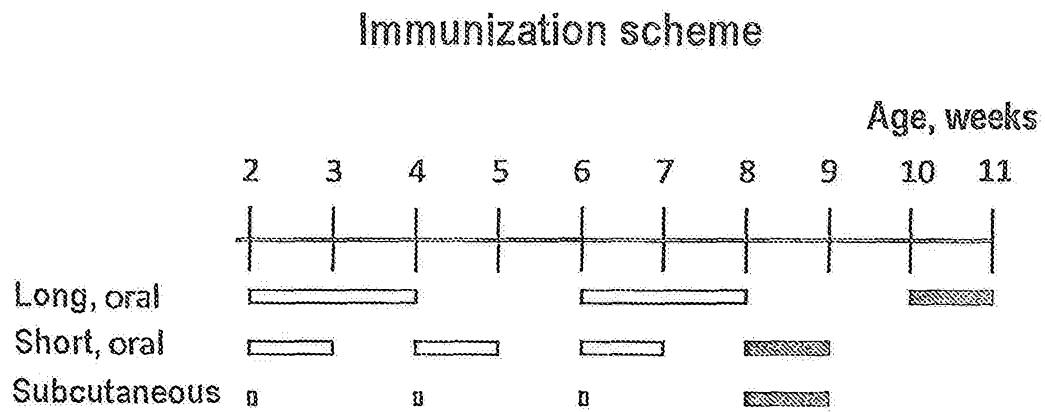
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Figure 4D

	Group	ELISA	Virus neutralization number positive
Feeding	VP2 Yeast	0.13 ± 0.031	40 %
	Control yeast	0.11 ± 0.006	0 %
Subcutaneous	VP2 Yeast	0.22 ± 0.05	100 %
	Control yeast	0.13 ± 0.015	0 %

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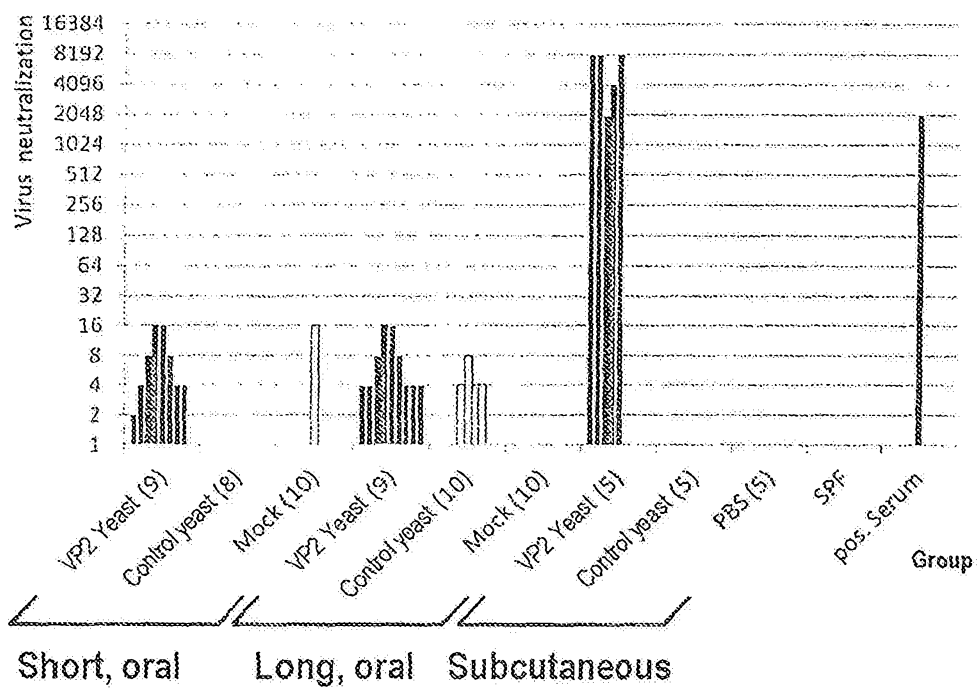
Figure 5A



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Figure 5B

Serum neutralisation assay



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Figure 5C

Group	ELISA (Title)	Virus neutralization	Mortality	Lesions Score
VP2 Yeast, short (8)	1	6.89 ± 5.75	0/9	4, 4, 4, 4, 4, 4, 4, 4
Control yeast, short (8)	1	0	3/8	4, 4, 4, 4, 4, 4, 4, 4
Mock, short (10)	1	1.6 ± 5.06	1/10	4, 4, 4, 4, 4, 4, 4, 4, 4, 4
VP2 Yeast, long (9)	35.56 ± 36.12	7.56 ± 5.08	0/9	3, 4, 4, 4, 4, 4, 4, 4, 4
Control yeast, long (10)	9.4 ± 19.6	2 ± 2.91	1/10	4, 4, 4, 4, 4, 4, 4, 4, 4, 4
Mock, long (10)	4 ± 2.58	0	0/10	4, 4, 4, 4, 4, 4, 4, 4, 4, 4
VP2 Yeast, long, Saponin (10)	68.8 ± 70.87	5.8 ± 5.46	0/10	2, 4, 4, 4, 4, 4, 4, 4, 4, 4
Control yeast, long, Saponin (9)	33.7 ± 38	0.89 ± 1.76	1/9	4, 4, 4, 4, 4, 4, 4, 4, 4
Mock, long, Saponin (9)	1	0	0/9	4, 4, 4, 4, 4, 4, 4, 4, 4
VP2 Yeast (5)	2707 ± 823.4	3072 ± 1448.2	0/5	1, 1, 1, 1, 3
Control yeast (5)	1	> 8	1/5	4, 4, 4, 4, 4, 4
PBS (5)	1	> 8	1/5	4, 4, 4, 4, 4, 4

Feeding

Subcutaneous

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Figure 6

