



(12)

Oversættelse af europæisk patentskrift

Patent- og
Varemærkestyrelsen

(51) Int.Cl.: **C 12 N 15/81 (2006.01)** **A 61 K 39/00 (2006.01)** **A 61 K 39/02 (2006.01)**
A 61 K 39/12 (2006.01) **G 01 N 33/569 (2006.01)**

(45) Oversættelsen bekendtgjort den: **2018-11-05**

(80) Dato for Den Europæiske Patentmyndigheds
bekendtgørelse om meddelelse af patentet: **2018-07-18**

(86) Europæisk ansøgning nr.: **12823073.7**

(86) Europæisk indleveringsdag: **2012-12-12**

(87) Den europæiske ansøgnings publiceringsdag: **2015-03-11**

(86) International ansøgning nr.: **DE2012001205**

(87) Internationalt publikationsnr.: **WO2013107436**

(30) Prioritet: **2011-12-13 DE 102011121069**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **Martin-Luther-Universität Halle-Wittenberg, Universitätsplatz 10, 06108 Halle/Saale, Tyskland**

(72) Opfinder: **BEHRENS, Sven-Erik, Springkrautweg 14, 06120 Halle/Saale, Tyskland**
BREUNIG, Karin, Lindenstrasse 7, Berlin 14109, Tyskland

(74) Fuldmægtig i Danmark: **AWA Denmark A/S, Strandgade 56, 1401 København K, Danmark**

(54) Benævnelse: **Vaccinering ved hjælp af rekombinant gær ved fremkaldelse af en beskyttende humoral immunreaktion mod definerede antigener**

(56) Fremdragne publikationer:
EP-A1- 1 752 468
WO-A1-90/15140
WO-A2-2010/054649
VILLEGAS PEDRO ET AL: "Infectious bursal disease subunit vaccination.", AVIAN DISEASES DEC 2008, Bd. 52, Nr. 4, Dezember 2008 (2008-12), Seiten 670-674, XP002695513, ISSN: 0005-2086
PITCOVSKI J ET AL: "Development and large-scale use of recombinant VP2 vaccine for the prevention of infectious bursal disease of chickens", VACCINE, ELSEVIER LTD, GB, Bd. 21, Nr. 32, 1. Dezember 2003 (2003-12-01), Seiten 4736-4743, XP004469693, ISSN: 0264-410X, DOI: 10.1016/S0264-410X(03)00525-5
STUBBS A C ET AL: "WHOLE RECOMBINANT YEAST VACCINE ACTIVATES DENDRITIC CELLS AND ELICITS PROTECTIVE CELL-MEDIATED IMMUNITY", NATURE MEDICINE, NATURE PUBLISHING GROUP, NEW YORK, NY, US, Bd. 7, Nr. 5, 1. Mai 2001 (2001-05-01), Seiten 625-629, XP008073223, ISSN: 1078-8956, DOI: 10.1038/87974 in der Anmeldung erwähnt
STUBBS A C ET AL: "Recombinant yeast as a vaccine vector for the induction of cytotoxic T-lymphocyte responses", CURRENT OPINION IN MOLECULAR THERAPEUTICS, CURRENT DRUGS, LONDON, GB, Bd. 4, Nr. 1, 1. Februar 2002 (2002-02-01), Seiten 35-40, XP008092368, ISSN: 1464-8431 in der Anmeldung erwähnt
WANSLEY ELIZABETH K ET AL: "Vaccination with a recombinant *Saccharomyces cerevisiae* expressing a

tumor antigen breaks immune tolerance and elicits therapeutic antitumor responses", CLINICAL CANCER RESEARCH, THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, Bd. 14, Nr. 13, 1. Juli 2008 (2008-07-01), Seiten 4316-4325, XP002587490, ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-08-0393 in der Anmeldung erwähnt

BATHURST I C: "PROTEIN EXPRESSION IN YEAST AS AN APPROCH TO PRODUCTION OF RECOMBINANT MALARIA ANTIGENS", AMERICAN JOURNAL OF TROPICAL MEDICINE & HYGIENE, AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE, US, Bd. 50, Nr. 4, SUPPL, 1. Januar 1994 (1994-01-01), Seiten 20-26, XP001088993, ISSN: 0002-9637 in der Anmeldung erwähnt

ARNOLD MARINA ET AL: "Protective Vaccination against Infectious Bursal Disease Virus with Whole Recombinant *Kluyveromyces lactis* Yeast Expressing the Viral VP2 Subunit", PLOS ONE, Bd. 7, Nr. 9, September 2012 (2012-09), XP002695514, ISSN: 1932-6203

JORRIT-JAN KRIJGER ET AL: "A novel, lactase-based selection and strain improvement strategy for recombinant protein expression in *Kluyveromyces lactis*", MICROBIAL CELL FACTORIES, BIOMED CENTRAL, LONDON, NL, Bd. 11, Nr. 1, 20. August 2012 (2012-08-20), Seite 112, XP021128994, ISSN: 1475-2859, DOI: 10.1186/1475-2859-11-112

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

5 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

10 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

15 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*,
30 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,

5 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,

10 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 15 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 20 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 25 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).

30 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 5 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 10 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 15 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 20 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 25 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 30 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, 5 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 10 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 15 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).

20 **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 25 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. 30 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 5 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**).

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 15 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 20 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 25 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'.

30 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 a recombinant yeast of the species *Kluyveromyces lactis* which carries as a foreign gene a gene that codes for a VP2

5 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,

10 *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.

15 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

20 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

25 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

30 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*

5 *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.

10 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)).

15 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.

20 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.

25 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.

30 *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 *KIGal4* 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 5 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 10 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 15 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.: 20 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 25 *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 30 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 15 administering the recombinant yeasts orally/mucosally or subcutaneously. In a 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 5 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 10 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, 15 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 20 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 25 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 30 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 5 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 15 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 20 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

20 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

25 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

30 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 *KIGa4* 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_Ascl_fwd (5'-GGCGCGCCGATGACAAACCTGCAAGATC-3') (**SEQ ID NO.: 7**), containing an *Ascl* restriction cleavage site, and

VP2_NotI_rev (5'-ATAAGAATGCGGCCGCTCACACAGCTATCCTCCTTATG-3')

30 (**SEQ ID NO.: 8**), containing an *NotI* restriction cleavage site, and

The following oligonucleotide pair was used to generate VP2-T2S:

IBDV_S:T_Ascl_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.

5

10 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, 15 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 20 potassium acetate; 5mM magnesium acetate; 0.1% Triton X100; 10% glycerol; 1mM sodium fluoride; 20mM glycerol phosphate; 10mM MgCl₂; 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 25 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 5 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 10 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 15 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 20 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 25 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 30 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 5 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. 10 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 15 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 20 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 25 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 30 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

5 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).

10

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

15 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).*

20 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).

25 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.*

30 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 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 10 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 15 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	Deoxyribonucleic 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 adeninivorans</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-E.coli</i> shuttle vector, Gene bank Accession U03501 (Botstein et al., 1979)

Reference List

Backhaus, K. et al. Milk and sugar: Regulation of cell wall synthesis in the milk yeast *Kluyveromyces lactis*. *European Journal of Cell Biology* **90**, 745-750 (2011).

5

Bathurst, I.C. Protein Expression in Yeast as an Approach to Production of Recombinant Malaria Antigens. *The American Journal of Tropical Medicine and Hygiene* **50**, 20 -26 (1994).

10 Botstein D, Falco, S.C., Stewart, S.E., Brennan, M., Scherer, S., Stinchcomb, D.T., Struhl, K. & Davis, R.W. Sterile host yeast (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. *Gene* **8**, 17-24 (1979).

Breunig et al. Regulation of primary carbon metabolism in *Kluyveromyces lactis*.

15 *Enzyme Microb. Technol* **26**, 771-780 (2000).

Chen, X.J. & Clark-Walker, G.D. Specific mutations in alpha- and gamma-subunits of F1-ATPase affect mitochondrial genome integrity in the petite-negative yeast *Kluyveromyces lactis*. *EMBO J* **14**, 3277-3286 (1995).

20 Clark-Walker, G.D. The F1-ATPase inhibitor Inh1 (IF1) affects suppression of mtDNA loss-lethality in *Kluyveromyces lactis*. *FEMS Yeast Research* **7**, 665-674 (2007).

Donnini, C. et al. Improved Production of Heterologous Proteins by a Glucose 25 Repression-Defective Mutant of *Kluyveromyces lactis*. *Appl Environ Microbiol* **70**, 2632-2638 (2004).

Engler-Blum, G., Meier, M., Frank, J. & Muller, G.A. Reduction of Background Problems in Nonradioactive Northern and Southern Blot Analyses Enables Higher 30 Sensitivity Than 32P-Based Hybridizations. *Analytical Biochemistry* **210**, 235-244 (1993).

Gellissen G., Hollenberg C.P. Application of yeasts in gene expression studies: a comparison of *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Kluyveromyces lactis* -- a review. *Gene* **190**(1), 87-97 (1997).

5 Granzow, H. et al. A second form of infectious bursal disease virus-associated tubule contains VP4. *J Virol* **71**, 8879-8885 (1997).

Jagadish, M.N., Laughton, D.L., Azad, A.A. & Macreadie, I.G. Stable synthesis of viral protein 2 of infectious bursal disease virus in *Saccharomyces cerevisiae*. *Gene* **108**, 275-279 (1991).

10 Icard, A.H., Sellers, H.S., & Mundt, E. Detection of infectious bursal disease virus isolates with unknown antigenic properties by reverse genetics. *Avian Dis.* **52**, 590-8. (2008)

15 Kuger, P., Gödecke, A. & Breunig, K.D. A mutation in the Zn-finger of the GAL4 homolog LAC9 results in glucose repression of its target genes. *Nucleic Acids Res* **18**, 745-751 (1990).

20 Lu, Y. et al. Mutation-Selective Tumor Remission with Ras-Targeted, Whole Yeast-Based Immunotherapy. *Cancer Research* **64**, 5084 -5088 (2004).

25 Raab, D., Graf, M., Notka, F., Schödl, T. & Wagner, R. The GeneOptimizer Algorithm: using a sliding window approach to cope with the vast sequence space in multiparameter DNA sequence optimization. *Syst Synth Biol* **4**, 215-225 (2010).

30 Raschke, W.C. & Ballou, C.E. Characterization of a yeast mannan containing N-acetyl-D-glucosamine as an immunochemical determinant. *Biochemistry* **11**, 3807-3816 (1972).

Sauger, I. et al. Structural Polymorphism of the Major Capsid Protein of a Double-Stranded RNA Virus: An Amphipathic [alpha] Helix as a Molecular Switch. *Structure* **13**, 1007-1017 (2005).

Schröder, A., van Loon, A.A.W.M., Goovaerts, D. & Mundt, E. Chimeras in noncoding regions between serotypes I and II of segment A of infectious bursal disease virus are viable and show pathogenic phenotype in chickens. *Journal of General Virology* 81, 533 -540 (2000).

5

Stubbs, A.C. et al. Whole recombinant yeast vaccine activates dendritic cells and elicits protective cell-mediated immunity. *Nat. Med* 7, 625-629 (2001).

10 Stubbs, A.C. and Wilson, C.C. Recombinant yeast as a vaccine vector for the induction of cytotoxic T-lymphocyte responses. *Curr. Opin. Mol. Ther.* 4: 35-40 (2002)

15 Uccelletti, D., Farina, F., Mancini, P. & Palleschi, C. KIPMR1 inactivation and calcium addition enhance secretion of non-hyperglycosylated heterologous proteins in *Kluyveromyces lactis*. *Journal of Biotechnology* 109, 93-101 (2004).

Van Ooyen, A.J.J. et al. Heterologous protein production in the yeast *Kluyveromyces lactis*. *FEMS Yeast Research* 6, 381-392 (2006).

20 Wansley E.K. et al., Vaccination with a recombinant *Saccharomyces cerevisiae* expressing a tumor antigen breaks immune tolerance and elicits therapeutic antitumor responses. *Clin. Cancer Res.* 14: 4316-4325 (2008).

25 Zenke et al. Gal80 proteins of *Kluyveromyces lactis* and *Saccharomyces cerevisiae* are highly conserved but contribute differently to glucose repression of the galactose regulon. *Molecular and Cellular Biology* 13:7566-7576 (1993)

SEQUENCE LISTING

<110> Martin-Luther-Universität Halle-Wittenberg

<120> Vaccination with Recombinant Yeast by Producing a Protective Humoral Immune Response Against Defined Antigens

<130> MLU_Breu_1

<160> 10

<170> BiSSAP 1.0

<210> 1

<211> 1371

<212> DNA

<213> Birnaviridae

<220>

<221> source

<222> 1..1371

<223> /mol_type="DNA"
/organism="Birnaviridae"

<400> 1

atgacaaacc tgcaagatca aacccaacag attgttccgt tcatacggag ctttctgtat 60

ccaacaaccg gaccggcggtc cattccggac gacaccctgg agaaggcacac tctcaggtca 120

gagacctcga cctacaattt gactgtgggg gacacaggggt cagggctaat tgtctttttc 180

cctggattcc ctggctcaat tgtgggtgct cactacacac tgcagggcaa tgggaactac 240

aagttcgatc agatgctcct gactgcccag aacctaccgg ccagttacaa ctactgcagg 300

ctagttagtc ggagtctcac agtgagggtca agcacacttc ctggtggcgt ttatgcacta 360

aacggcacca taaacgcccgt gaccttccaa ggaaggctga gtgaactgac agatgttagc 420

tacaatgggt ttagtctgc aacagccaaac atcaacgaca aaattggaa cgtccatgt 480

gggaaagggg tcaccgtcct cagcttaccc acatcatatg atcttggta tgtgaggctt 540

ggtagccccca ttcccgcaat agggcttgac caaaaatgg tagccacatg tgacagcagt 600

gacaggccccca gagtctacac cataactgca gccgatgatt accaattctc atcacagtac 660

caaccagggtg gggtaacaat cacactgttc tcagccaaaca ttgatgccat cacaaggctc 720

agcgttgggg gagagctcgt gtttcaaaca agcgtccacg gccttgtact gggcgccacc 780

atctacctca taggctttga tgggacaacg gtaatcacca gggctgtggc cgcaaacaat 840

gggctgacga ccggcaccga caaccttatg ccattcaatc ttgtgattcc aacaaacgag	900
ataaccaggc caatcacatc catcaaactg gagatagtga cctccaaaag tggtggtcag	960
gcaggggatc agatgtcatg gtcggcaaga gggagcctag cagtgacgat ccatggtggc	1020
aactatccag gggccctccg tcccgtaacg ctagtggcct acgaaagagt ggcaacagga	1080
tccgtcgta cggtcgctgg ggtgagcaac ttcgagctga tcccaaattcc tgaacttagca	1140
aagaacctgg ttacagaata cggccgattt gacccaggag ccatgaacta cacaaaattg	1200
atactgagtg agagggaccg tcttggcatc aagaccgtct ggccaacaag ggagtacact	1260
gactttcgtg aataacttcat ggaggtggcc gacctaact ctccccctgaa gattgcagga	1320
gcattcggct tcaaagacat aatccgggcc ataaggagga tagctgtgtg a	1371

<210> 2
 <211> 456
 <212> PRT
 <213> Birnaviridae

<220>
 <221> SOURCE
 <222> 1..456
 <223> /mol_type="protein"
 /organism="Birnaviridae"

<400> 2
 Met Thr Asn Leu Gln Asp Gln Thr Gln Gln Ile Val Pro Phe Ile Arg
 1 5 10 15
 Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro Asp Asp Thr
 20 25 30
 Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr Asn Leu Thr
 35 40 45
 Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro Gly Phe Pro
 50 55 60
 Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn Gly Asn Tyr
 65 70 75 80
 Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro Ala Ser Tyr
 85 90 95
 Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg Ser Ser Thr
 100 105 110
 Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn Ala Val Thr
 115 120 125
 Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr Asn Gly Leu
 130 135 140
 Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn Val Leu Val
 145 150 155 160

Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr Asp Leu Gly
 165 170 175
 Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu Asp Pro Lys
 180 185 190
 Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val Tyr Thr Ile
 195 200 205
 Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln Pro Gly Gly
 210 215 220
 Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile Thr Ser Leu
 225 230 235 240
 Ser Val Gly Gly Glu Leu Val Phe Gln Thr Ser Val His Gly Leu Val
 245 250 255
 Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr Thr Val Ile
 260 265 270
 Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly Thr Asp Asn
 275 280 285
 Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile Thr Gln Pro
 290 295 300
 Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser Gly Gly Gln
 305 310 315 320
 Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu Ala Val Thr
 325 330 335
 Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val Thr Leu Val
 340 345 350
 Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val Ala Gly Val
 355 360 365
 Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys Asn Leu Val
 370 375 380
 Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr Thr Lys Leu
 385 390 395 400
 Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val Trp Pro Thr
 405 410 415
 Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val Ala Asp Leu
 420 425 430
 Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys Asp Ile Ile
 435 440 445
 Arg Ala Ile Arg Arg Ile Ala Val
 450 455

<210> 3
 <211> 1371
 <212> DNA
 <213> Birnaviridae

<220>
 <221> source
 <222> 1..1371
 <223> /mol_type="DNA"
 /organism="Birnaviridae"

<400> 3
 atgtctaacc tgcaagatca aacccaacag attgttccgt tcatacggag ccttctgtatg

60

ccaacaaccg gaccggcgtc cattccggac gacaccctgg agaagcacac ttcaggtca	120
gagacctcga cctacaattt gactgtgggg gacacagggt cagggctaat tgtcttttc	180
cctggattcc ctggctcaat tgtgggtgct cactacacac tgcagggcaa tgggaactac	240
aagttcgatc agatgctcct gactgcccag aacctaccgg ccagttacaa ctactgcagg	300
ctagttagtc ggagtctcac agtgaggta agcacacttc ctggggcgt ttatgcacta	360
aacggcacca taaacgccgt gaccccaa ggaagcctga gtgaactgac agatgttagc	420
tacaatgggt ttagtctgc aacagccaa acatcaacgaca aaattggaa cgtccttagta	480
ggggaaagggg tcaccgtcct cagcttaccc acatcatatg atcttggta tgtgaggctt	540
ggtgacccca ttcccgcaat agggcttgac ccaaaaatgg tagccacatg tgacagcagt	600
gacaggccca gagtctacac cataactgca gccgatgatt accaattctc atcacagtac	660
caaccaggta gggtaacaat cacactgttc tcagccaaaca ttgatgccat cacaaggctc	720
agcggtgggg gagagctcgt gtttcaaaca agcgccacg gcctgtact gggcgcacc	780
atctaccta taggcttta tggacaacg gtaatcacca gggctgtggc cgcaaacaat	840
gggctgacga cggcaccga caaccttatg ccattcaatc ttgtgattcc aacaaacgag	900
ataacccagc caatcacatc catcaaactg gagatagtga cctccaaaag tggtggtcag	960
gcaggggatc agatgtcatg gtcggcaaga gggagcctag cagtgacgat ccatggtggc	1020
aactatccag gggccctccg tcccgccacg ctagtggcct acgaaagagt ggcaacagga	1080
tccgtcgta cggtcgctgg ggtgagcaac ttcgagctga tcccaaatacc tgaactagca	1140
aagaacctgg ttacagaata cggccgattt gacccaggag ccatgaacta cacaatttg	1200
ataactgagtg agagggaccg tcttggcatc aagaccgtct ggccaaacaag ggagtacact	1260
gactttcgta aataacttcat ggaggtggcc gacctaact ctccctgaa gattgcagga	1320
gcattcggt tcaaagacat aatccggcc ataaggagga tagctgtgtg a	1371

<210> 4
 <211> 456
 <212> PRT
 <213> Birnaviridae
 <220>

<221> SOURCE

<222> 1..456

<223> /mol_type="protein"
/organism="Birnaviridae"

<400> 4

Met	Ser	Asn	Leu	Gln	Asp	Gln	Thr	Gln	Gln	Ile	Val	Pro	Phe	Ile	Arg
1				5				10						15	
Ser	Leu	Leu	Met	Pro	Thr	Thr	Gly	Pro	Ala	Ser	Ile	Pro	Asp	Asp	Thr
					20			25						30	
Leu	Glu	Lys	His	Thr	Leu	Arg	Ser	Glu	Thr	Ser	Thr	Tyr	Asn	Leu	Thr
	35					40						45			
Val	Gly	Asp	Thr	Gly	Ser	Gly	Leu	Ile	Val	Phe	Phe	Pro	Gly	Phe	Pro
	50				55						60				
Gly	Ser	Ile	Val	Gly	Ala	His	Tyr	Thr	Leu	Gln	Gly	Asn	Gly	Asn	Tyr
	65					70			75			80			
Lys	Phe	Asp	Gln	Met	Leu	Leu	Thr	Ala	Gln	Asn	Leu	Pro	Ala	Ser	Tyr
				85					90					95	
Asn	Tyr	Cys	Arg	Leu	Val	Ser	Arg	Ser	Leu	Thr	Val	Arg	Ser	Ser	Thr
				100				105				110			
Leu	Pro	Gly	Gly	Val	Tyr	Ala	Leu	Asn	Gly	Thr	Ile	Asn	Ala	Val	Thr
	115					120					125				
Phe	Gln	Gly	Ser	Leu	Ser	Glu	Leu	Thr	Asp	Val	Ser	Tyr	Asn	Gly	Leu
	130				135				140						
Met	Ser	Ala	Thr	Ala	Asn	Ile	Asn	Asp	Lys	Ile	Gly	Asn	Val	Leu	Val
	145					150				155				160	
Gly	Glu	Gly	Val	Thr	Val	Leu	Ser	Leu	Pro	Thr	Ser	Tyr	Asp	Leu	Gly
				165				170			175				
Tyr	Val	Arg	Leu	Gly	Asp	Pro	Ile	Pro	Ala	Ile	Gly	Leu	Asp	Pro	Lys
				180				185			190				
Met	Val	Ala	Thr	Cys	Asp	Ser	Ser	Asp	Arg	Pro	Arg	Val	Tyr	Thr	Ile
				195				200			205				
Thr	Ala	Ala	Asp	Asp	Tyr	Gln	Phe	Ser	Ser	Gln	Tyr	Gln	Pro	Gly	Gly
				210				215			220				
Val	Thr	Ile	Thr	Leu	Phe	Ser	Ala	Asn	Ile	Asp	Ala	Ile	Thr	Ser	Leu
	225				230					235				240	
Ser	Val	Gly	Gly	Glu	Leu	Val	Phe	Gln	Thr	Ser	Val	His	Gly	Leu	Val
				245				250			255				
Leu	Gly	Ala	Thr	Ile	Tyr	Leu	Ile	Gly	Phe	Asp	Gly	Thr	Thr	Val	Ile
				260				265			270				
Thr	Arg	Ala	Val	Ala	Ala	Asn	Asn	Gly	Leu	Thr	Thr	Gly	Thr	Asp	Asn
				275				280			285				
Leu	Met	Pro	Phe	Asn	Leu	Val	Ile	Pro	Thr	Asn	Glu	Ile	Thr	Gln	Pro
				290				295			300				
Ile	Thr	Ser	Ile	Lys	Leu	Glu	Ile	Val	Thr	Ser	Lys	Ser	Gly	Gly	Gln
	305				310					315				320	
Ala	Gly	Asp	Gln	Met	Ser	Trp	Ser	Ala	Arg	Gly	Ser	Leu	Ala	Val	Thr
					325				330			335			
Ile	His	Gly	Gly	Asn	Tyr	Pro	Gly	Ala	Leu	Arg	Pro	Val	Thr	Leu	Val
				340				345			350				
Ala	Tyr	Glu	Arg	Val	Ala	Thr	Gly	Ser	Val	Val	Thr	Val	Ala	Gly	Val
				355				360			365				

Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys Asn Leu Val
 370 375 380
 Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr Thr Lys Leu
 385 390 395 400
 Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val Trp Pro Thr
 405 410 415
 Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val Ala Asp Leu
 420 425 430
 Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys Asp Ile Ile
 435 440 445
 Arg Ala Ile Arg Arg Ile Ala Val
 450 455

<210> 5
 <211> 1371
 <212> DNA
 <213> Birnaviridae

<220>
 <221> source
 <222> 1..1371
 <223> /mol_type="DNA"
 /organism="Birnaviridae"

<400> 5
 atgtccaaact tacaagacca aacccaaacaa atcgtccctt ttatcagatc cttattaatg 60
 cctactaccg gtcctgcttc tattcctgat gacacccgttgg aaaaacacac cttgagatcc 120
 gaaacttcaa cctataactt gactgtcggt gacactgggtt ctggtttaat cgtttcttc 180
 cctggttttc ctggttcaat tgtcggtgcc cactataacct tacaaggtaa cggttaactat 240
 aagttcgatc aaatgttgtt gaccgccaa aatttgcctg cctcctataa ctattgtaga 300
 ttggtttcta gatcttaac cgtcagatca tccactttgc ctggtggtgt ctatgtttg 360
 aacggtacaa tcaacgctgt cacattcaa ggttccctgt ccgaattgac cgatgtctcc 420
 tataacggtt taatgtccgc tactgcaat atcaatgaca aaattggtaa cgtcttagtc 480
 ggtgaagggtg ttactgtttt gagtttgcca acctctttagt acttgggtta tgtcagattg 540
 ggtgacccta ttccctgctat cggttttagac ccaaaaatgg ttgccacttg tgactctgt 600
 gatagaccaa gagtctatac catcaactgct gccgatgact atcaattctc ctcccaatata 660
 caacctggtg gtgtcactat cacccgttcc tctgccaaca tcgacgctat aacatcttg 720
 tccgtcggtg gtgaatttgtt attccaaacc tccgtccatg gtttagtatt gggtgccacc 780
 atctatgttga ttggtttcga cggtacaacc gtcattacta gagccgttgc tgccaacaat 840

ggtttaacca ctggtaactga caacttgcattt ccattcaact tggtaatccc taccaacgaa 900
atcacacaac caatcacatc catcaaattt gaaattgtca cctccaaatc cggtgtcaa 960
gccggtgacc aaatgtcatg gagtgctaga ggttcatttt ccgttaaccat ccacggtggt 1020
aactatcctg gtgccttgag acctgtcaact ttagtgcctt atgaaagagt tgctacttgt 1080
tccgtcgatca ctgttgcgg tgtttcaaacc ttcaatttga tcccaaaaccc agaattggcc 1140
aaaaacttgg ttaccgaata tggtagattt gaccctggtg ctatgaacta tacaaaattt 1200
atcttatccg aaagagacag attgggtatc aaaaactgtct ggcctacttag agaatataacc 1260
gacttttagag aatatttcat ggaagtcgcc gactttaaattt ccccaatttgaatcgccggt 1320
gcctttggtt ttaaggacat cattagagcc attagaagaaa tagccgtctg a 1371

<210> 6
<211> 456
<212> PRT
<213> Birnaviridae

```
<220>
<221> SOURCE
<222> 1..456
<223> /mol_type="protein"
      /organism="Birnaviridae"
```

<400> 6
 Met Ser Asn Leu Gln Asp Gln Thr Gln Gln Ile Val Pro Phe Ile Arg
 1 5 10 15
 Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro Asp Asp Thr
 20 25 30
 Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr Asn Leu Thr
 35 40 45
 Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro Gly Phe Pro
 50 55 60
 Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn Gly Asn Tyr
 65 70 75 80
 Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro Ala Ser Tyr
 85 90 95
 Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg Ser Ser Thr
 100 105 110
 Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn Ala Val Thr
 115 120 125
 Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr Asn Gly Leu
 130 135 140
 Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn Val Leu Val
 145 150 155 160

Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr Asp Leu Gly
 165 170 175
 Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu Asp Pro Lys
 180 185 190
 Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val Tyr Thr Ile
 195 200 205
 Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln Pro Gly Gly
 210 215 220
 Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile Thr Ser Leu
 225 230 235 240
 Ser Val Gly Gly Glu Leu Val Phe Gln Thr Ser Val His Gly Leu Val
 245 250 255
 Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr Thr Val Ile
 260 265 270
 Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly Thr Asp Asn
 275 280 285
 Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile Thr Gln Pro
 290 295 300
 Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser Gly Gly Gln
 305 310 315 320
 Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu Ala Val Thr
 325 330 335
 Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val Thr Leu Val
 340 345 350
 Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val Ala Gly Val
 355 360 365
 Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys Asn Leu Val
 370 375 380
 Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr Thr Lys Leu
 385 390 395 400
 Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val Trp Pro Thr
 405 410 415
 Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val Ala Asp Leu
 420 425 430
 Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys Asp Ile Ile
 435 440 445
 Arg Ala Ile Arg Arg Ile Ala Val
 450 455

<210> 7
 <211> 28
 <212> DNA
 <213> synthetic construct

<220>
 <221> source
 <222> 1..28
 <223> /mol_type="DNA"
 /note="Oligonucleotide for PCR amplification"
 /organism="synthetic construct"

<400> 7

ggcgcgccga tgacaaacct gcaagatc	28
<210> 8 <211> 38 <212> DNA <213> synthetic construct	
<220> <221> source <222> 1..38 <223> /mol_type="DNA" /note="Oligonucleotid für PCR Amplifikation" /organism="synthetic construct"	
<400> 8	
ataagaatgc ggccgctcac acagctatcc tcctttag	38
<210> 9 <211> 35 <212> DNA <213> synthetic construct	
<220> <221> source <222> 1..35 <223> /mol_type="DNA" /note="Oligonucleotid für die PCR-Amplifikation" /organism="synthetic construct"	
<400> 9	
ggcgcgccga tgtctaacct gcaagatcaa accca	35
<210> 10 <211> 8157 <212> DNA <213> synthetic construct	
<220> <221> source <222> 1..8157 <223> /mol_type="DNA" /note="plasmid vector" /organism="synthetic construct"	
<400> 10	
agggtggcaact tttcggggaa atgtgcgcgg aacccttatt tgtttatttt tctaaataca	60
ttcaaatatg tatccgctca tgagacaata accctgataa atgcttcaat aatattgaaa	120

aaggaagagt atgagtattc aacatttccg tgcgcctt attcccttt ttgcggcatt	180
ttgccttcct gttttgctc acccagaaac gctggtaaa gtaaaagatg ctgaagatca	240
gttgggtgca cgagtgggtt acatcgaact ggatctcaac agcggtaaga tccttgagag	300
ttttcgcccc gaagaacgtt ttccaatgat gagcactttt aaagttctgc tatgtggcgc	360
ggtattatcc cgtattgacg cggggcaaga gcaactcggt cgccgcatac actattctca	420
aatgacttg gttgagtact caccagtcac agaaaagcat cttacggatg gcatgacagt	480
aagagaatta tgcagtgtg ccataaccat gagtgataac actgcggcca acttacttct	540
gacaacgatc ggaggaccga aggagctaac cgcttttg cacaacatgg gggatcatgt	600
aactcgctt gatcggtggg aaccggagct gaatgaagcc ataccaaacg acgagcgtga	660
caccacgatg cctgtagcaa tggcaacaac gttgcgaaa ctattaactg gcgaactact	720
tactctagct tcccggcaac aattaataga ctggatggag gcggataaag ttgcaggacc	780
acttctgcgc tcggcccttc cggctggctg gtttattgct gataaatctg gagccgggtga	840
gcgtgggtct cgcggtatca ttgcagcact ggggccagat ggtaagccct cccgtatcgt	900
agttatctac acgacgggga gtcaggcaac tatggatgaa cggaaatagac agatcgctga	960
gataggtgcc tcactgatta agcattggta actgtcagac caagtttact catatatact	1020
ttagattgat taaaacttc attttaatt taaaaggatc taggtgaaga tccttttga	1080
taatctcatg accaaaatcc cttaacgtga gtttgcgttc cactgagcgt cagacccgt	1140
agaaaaagatc aaaggatctt cttgagatcc ttttttctg cgcgtaatct gctgcttgca	1200
aacaaaaaaaaa ccaccgctac cagcgggtgt ttgtttgcgc gatcaagagc taccaactct	1260
ttttccgaag gtaactggct tcagcagagc gcagatacca aatactgtac ttcttagtgta	1320
gccgtagttt ggccaccact tcaagaactc tgttagcaccg cctacatacc tcgctctgct	1380
aatccgttta ccagtggctg ctgccagtgg cgataagtgc tgtcttaccg ggttggactc	1440
aagacgatag ttaccggata aggcgacgcg gtcgggtga acgggggtt cgtgcacaca	1500
gcccagctt gaggcaacga cctacaccga actgagatac ctacagcgtg agctatgaga	1560
aagcgccacg cttcccgaag ggagaaaggc ggacaggtat cggtaagcg gcagggtcgg	1620
aacaggagag cgcacgaggg agcttccagg gggaaacgcc tggtatctt atagtcctgt	1680

cgggtttcgc	cacctctgac	ttgagcgtcg	atttttgtga	tgctcgtag	ggggggggag	1740
cctatggaaa	aacgccagca	acgcggcctt	tttacggttc	ctggcctttt	gctggccttt	1800
tgctcacatg	ttctttcctg	cgttatcccc	tgattctgtg	gataaccgta	ttaccgcctt	1860
tgagttagct	gataccgctc	gccgcagccg	aacgaccgag	cgcagcgagt	cagtgagcga	1920
ggaagcggaa	gagcgcccaa	tacgcaaacc	gcctctcccc	gcgcgttggc	cgattcatta	1980
atgcagctgg	cacgacaggt	ttcccgactg	gaaagcgggc	agtgagcgca	acgcaattaa	2040
tgtgagttag	ctcactcatt	aggcacccca	ggctttacac	tttatgctcc	cggctcgtat	2100
gttgtgtgga	attgtgagcg	gataacaatt	tcacacagga	aacagctatg	accatgatta	2160
cgc当地	gcaattaacc	ctcactaaag	ggaacaaaag	ctgggtaccg	ggcccgac	2220
ctaaccattc	aatgattca	taactatctc	ctagccagaa	ttcgttacccca	actcttggga	2280
aatcaggagg	ctgatattcg	ccagtaagct	tcatagaagt	gttcaagttt	attttggtag	2340
caaagatcgt	gtacttctga	acagtctcaa	acccatagta	aaatacaact	ggggatatac	2400
gagagttAAC	cgtgactaca	gctagagaac	cattagaacc	tttttcgaca	ctcactccat	2460
ggatgtttg	cttcattaaa	tcaatattgt	acttcttcca	gttcttaaag	tcccttaggtt	2520
catcattatt	cgttggaggt	ctccagaaag	tgattgaaga	accctcaaac	ttgctggaaa	2580
tttccttacc	tttgacctt	aggcttcaa	ttttacccaa	caatttgc	aagataaaat	2640
gcaatccact	ggattcaact	gagacataac	gtttaccgtc	gttgcatttc	gcagctttt	2700
ctgctgtctc	tgtacaaaaa	tcgggtacct	tcaatggaa	ttcagcttgg	ccccaggcaa	2760
tttcatgacc	tgcctttaga	acaccagcat	catcttcaa	cacggcaaca	acataagttg	2820
tatcagaagg	aatagtaaca	gattcttctg	gctttaaaga	tggAACgtcg	attgtcttcc	2880
ccgtgtcctt	gtcgataaac	aataagtgg	ctgtcgtaat	gaagtcgtgc	ttatttgc	2940
ttgttacaga	tccgtgcga	attttaat	gaacgggttc	aataaccttc	ttataactcta	3000
caaggcccgg	agtaggatta	tgctcactgt	tacacaaacc	atccatgatg	aacactccgt	3060
catgaacctc	ttccttaaag	tcaccaccat	aagcataagc	tttatgcaac	ttaccatctg	3120
cagtactaac	atttcgaat	tcaataccgt	gatttgc	ttcccgagata	aagccaccc	3180
ggtaaaactt	ctccttgc	gatattctt	caaagagcca	ggaccgttac		3240

ccattgcatttgcgtactca cacaagatca aaggcttttc aaacttacca ttttcatcag 3300
tgtggttctt cctccacctt tccataattt caaatgttgg gtacatgaaa ctaaagatat 3360
ctgcactcaa agcggttcaag tcaccctcat aatgcacaag tctggtagga tccaaattgtt 3420
taattaactt gtacatggct ttgtggtttc tgccataaca agcttcgtta cccaggacc 3480
agataataat cgaaggatga ttgacatctc ttaggacaag ttggaaagct ctgtctaagt 3540
acgcgaccc tcgtactctggta ttatctgata agtaatgggc attaacatcg tagagtttat 3600
tttttagtatac tggatattca gcctccaagt tcgtatgacg attaaatggc tcttgaacac 3660
catgagtttc aagatctgcc tcgtcaatga cccagaagcc cagcttatcg aagaggtcat 3720
acacacctttagg atgggttggta taatgcgagt tacgaacagc attgatgtta aacttcttca 3780
ttagaatcaa gtccctaaca acaaaatcta atggcacagc tctaccgaac cttggatggt 3840
gatcatgtct gttgacacctt ctaaagagaa tgcgtttgcc attaacagta atgttaccgt 3900
ccttcaactc cacttgcgtc aaaccaacat ggtgcttaat agattgaatc acactgccat 3960
cagatccaaat taaatccaaac tggtacttgt acaaagtagg attttctgcg gtccaatgtt 4020
ctggggcctt gacgttgcattt ttgaaagctg tttcttcgtt cttttgggt gagaaggaaa 4080
taaattcttt agttgaaaaaa gtcgtgttcc cattctcctc gttcaacaaa gagcttgcatt 4140
cgtaaacttt agatccatct tcagggtcgt aaagtgtgaa attgatgtga tcataagaag 4200
aaccctggac atcaactttc acagaaagct ctgcattccgt atactgagag tccacaaaaag 4260
ttgttagtgac cctaacgtct tcaatatggg ctttcttagg caatttttagt aaagaaacgt 4320
ctctgtaaat accagagagc caccattgtat tgggtcctc gatataagt gaaatggacc 4380
acttggaaac cttgacgacc actaagtttt cggccctcaga aacgtacttt tggatatacaa 4440
attcagcccc gttacgggac cccttattga aacccacata ttgaccatta acataaagct 4500
cgtaacaattt gtccacaccc tcaaattctca atctgtgctc gaacgactca atcgatttcg 4560
aatctaaattc aaaagttcta gcataaacac cagtaggatt tacagtggaa ggatttggaa 4620
tgtcgattgg gatagggtac tgtacgttcg tgtaaattgg tttaccgtac ttccagtctt 4680
cctgaagttc ccaatggat ggcacagaaa tggtgctcca tttctttgcc gtttcccagt 4740
ctaaattctt agcatccgga gcgtcaagag gtgcattcaaaa caacgcacaaa gccccaggcc 4800

cattgagaga ttcgaaaata tcctgatcat agtagtaagc cctagtaggc aatctat	4860
cgtgaacctt tttggggttc cttaaattct caggaataag gcaagccatg gtgccgtc	4920
gccgagatat tgtgtacact ggatcaaata ataacacttt caaagtgact aaatcaca	4980
tgtcccaaga tatactatag ctctctgtt aaccttata ttgtcaaaaa gggacaatg	5040
atgaaagtac aaacacaaac acaaacacaa tggaagggag tgtccagggt ggtgattc	5100
gactgtactg attcgacgga gttttatgg atttcgttga agtggtaaa gtgaataatt	5160
cttgaattga gaggaacaaa gagtgatcaa aataacgaa tggagaggc cgagcgatg	5220
ataatgtacg attcggaaaga ctatgagccg gctgaacctg aggttatggc ccactaacgt	5280
cctggttgac aagagtagtc atgtaataca aacgtaaatg tgatatttaa tagaatataa	5340
gtagatatag taaaaaagaa gaagaagaat agaaagaata agggatttag aaatttagag	5400
tcattttaaa caattgataa cttgggttaa agctcgaagt tttgttata gtagttttt	5460
tttttggttt agttggtttgc ttcaatagta taaggtaaca gggtgcgaga caaacgttgc	5520
aacacttttc atctcccccc gctaattcacc tagtcgagag ctcgtttcg acactggatg	5580
gcggcgtag tatcgaatcg acagcagtat agcgaccagc attcacatac gattgacgca	5640
tgatattact ttctgcgcac ttaacttcgc atctggcag atgatgtcga ggcgaaaaaa	5700
aatataaattc acgctaacat ttgataaaa tagaacaact acaatataaa aaaactatac	5760
aaatgacaag ttcttgaaaa caagaatctt ttatgtca gtactgagtc gaggccggc	5820
ctggccaccc gggtagag gcgcgcgtc gacggtagc cttctcgatg agtatgtgt	5880
tttatttttt tttatttttt ttgtccaaat tctgtcttt cctaaatttc aagtgttag	5940
cttggtatcc gctcacaatt ccagcttttgc tctttcacc tttccaaact acaagcgca	6000
cataacaaaa gaataataat tctcctaaga aacacaagcc tcataacat ttcgagttag	6060
ggaagaacat ttctctcat gatacacatt gattcgagct attaaatacc ttttctcaa	6120
tcgaaatctc aagtaaaaaca gcaatgaaaa cattacgtaa ctaaagggtgt tcaccactag	6180
aaatcatacc ttccacactc gacttcaagt agtgaatggt gtagcaacaa agtccaaata	6240
ccaatgtcaa ccaagtaacc gaccgcacta ctagaaaaag acgctgtgc tcggaccaca	6300
aatttccgct acactttca caactatact gaagatacaa aaaacgtgtg tgggtatggc	6360

tggctaccag gtcgcctgggt taaaccaagt caacgtgata catatgtacg ttccaaacact	6420
aagcctaccc taagttcgg ctcacaggct aggctattat taacatgcaa gacaagggag	6480
aagcaaagca aagaccaacc gaaacccacc agagcaccct gaactttgcg gtgaacagaa	6540
ttccgcaaca tatctgagga taccatgatc tcgtttcct actccatatg ggaatcaccc	6600
actgttgtcc gtaaatatga ccaaattcct accttgcattc ctcacgaata atcgcagtcc	6660
gaaaagccgt tccaaaagcc agtccacagt ccatcaattt gatatgtatg tttttttttt	6720
ttcaaaactga cacactaacg gtgtggaatg cgaagagtga gcttaccctt ctccctttt	6780
ctagcagtac ttgcctacct acctactcta ctacgctgcc atattgtcta acattcggt	6840
ttctctatctt ctacctggcc tggatggctc cgtctcgccc gcctcacaca catacattcc	6900
tccccctctc gcctgccccca taataattaa acaagttAAC aaaaggcgTT acctttccg	6960
catcctctcc aatctcatac gattccctt tcatccgact tacccaaacaa gatacaggat	7020
ctcagtggaa gatccttcctt gccctccctg tctgttgtct actctacatg cgacttggaa	7080
ggccaaagga ctatcgcatg attattcgcc gggAACCGC gagttccctg ctctttctt	7140
tcaaaccagg cagcaaaccA ggtgaacaca ctctgatgtA gtgcagtccc taagtcctt	7200
gaagattcgg ggagcttagt acccacgcga atgtaacaaa agaacatttA ctttgtggg	7260
gggtggaaaa gtcgattagg atcttgcagc acagaaactg cgccgggtt tttttcatct	7320
tggagaagca actggctaaa ttgcacacAA acaaaaactg aaaaatggaa aataaaaaat	7380
gaaaaagcaa gctgaattcg aagaaggag aattccgcct tctgcaacca cactaatgg	7440
tggtagtcaa tagatacgca tttagaaggTT actattttat gagtcgatcc ccgcgggtgg	7500
gctccaattt cccctatagt gagtcgtatt acgcgcgcTC actggccgTC gttttacaac	7560
gtcgtgactg ggaaaaccct ggcgttaccc aacttaatcg ccttgcagca catccccctt	7620
tcgcccagctg gcgtaatagc gaagaggccc gcaccgatcg cccttcccaa cagttgcgcA	7680
gcctgaatgg cgaatggcgc gacgcgcct gtacgcgcgC attaagcgcg gcgggtgtgg	7740
tggttacgcg cagcgtgacc gctacacttg ccagcgcctt agcgcccgCT ctttcgcTT	7800
tcttcccttc ctttctcgcc acgttgcgcg gctttccccg tcaagctcta aatcgggggc	7860
tcccttttagg gttccgattt agtgcTTAC ggcacctcgA ccccaaaaaaa cttgattagg	7920

gtgatggttc acgttagtggg ccatcgccct gatagacggt ttttcgcccct ttgacgttgg	7980
agtccacgtt ctttaatagt ggactcttgt tccaaactgg aacaacactc aaccctatct	8040
cggcttattc ttttgattta taagggattt tgccgatttc ggcctattgg ttaaaaaatg	8100
agctgattta acaaaaattt aacgcgaatt ttaacaaaat attaacgctt acaattt	8157

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-
5 genet, 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:

10 *Kluyveromyces lactis* DSM 25405;
Kluyveromyces lactis DSM 25406, og
Kluyveromyces lactis DSM 25407.

15 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 fremmedgen-
udtrykkelsen 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 vaccinering.

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 indi-
viduer.

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
vaccinering 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, kendtegnet 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, kendtegnet ved, at fremmedgenet
5 koder for proteinet IBDV VP2-T2S med aminosyresekvensen ifølge SEQ ID NR. 4 eller pro-
teinet IBDV oVP2-T2S med aminosyresekvensen ifølge SEQ ID NR. 6.

Drawings

Figure 1

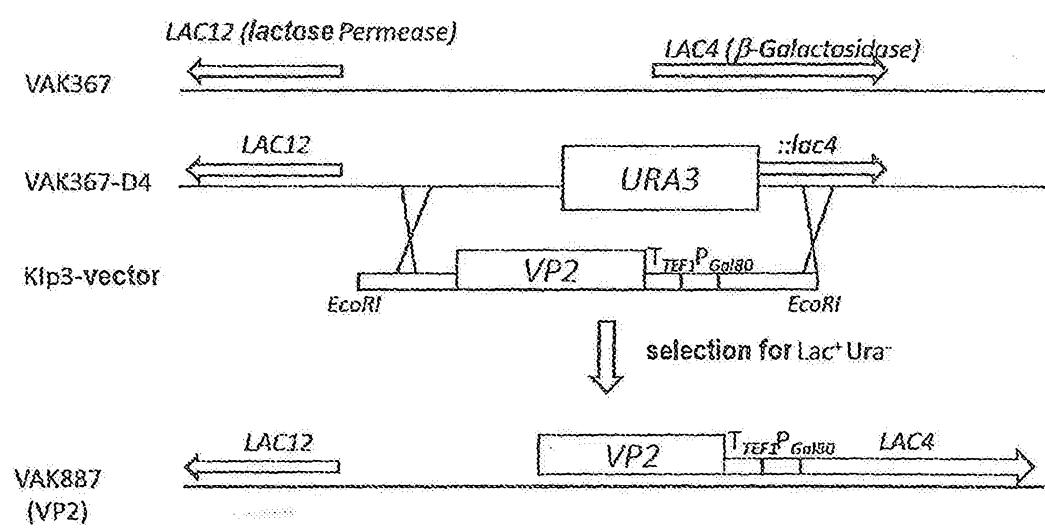


Figure 2A

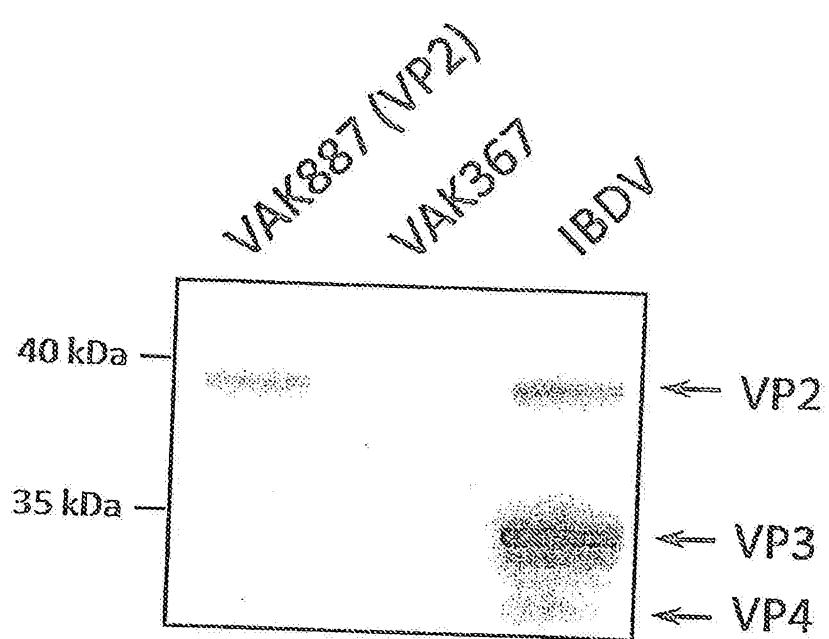


Figure 2B

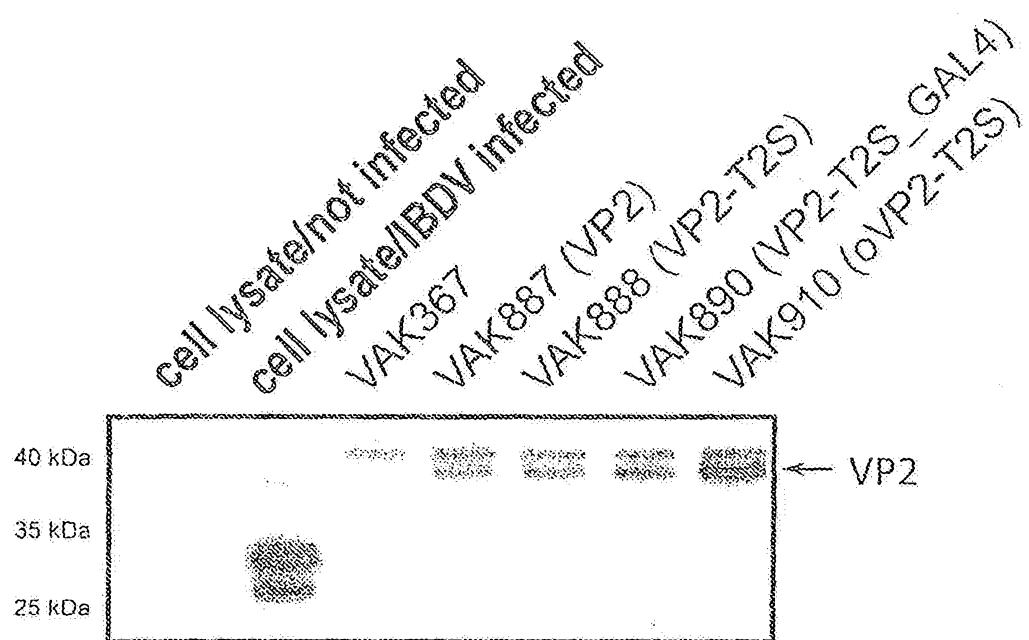
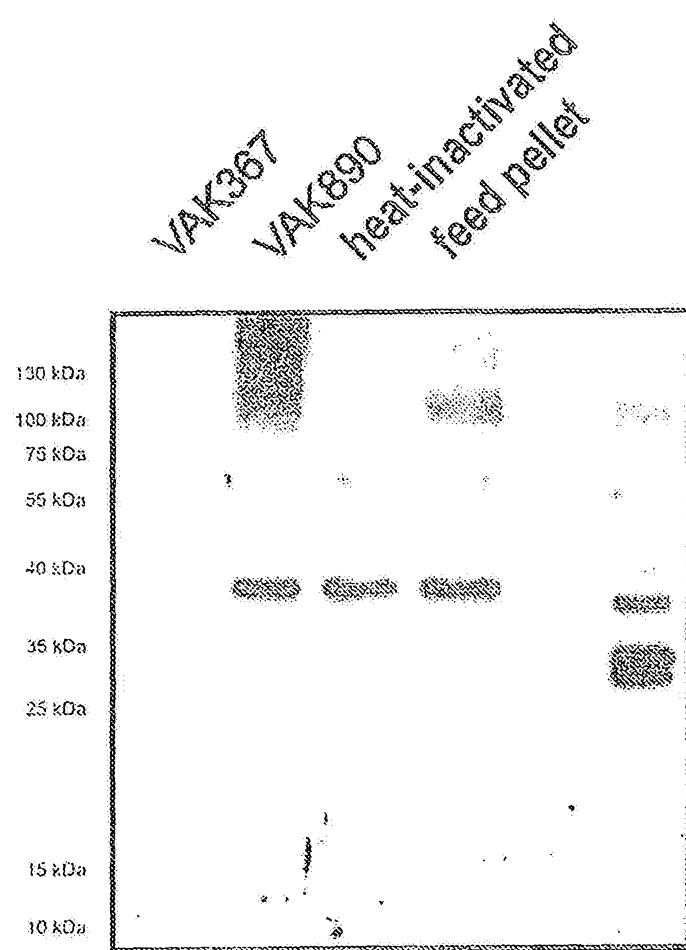
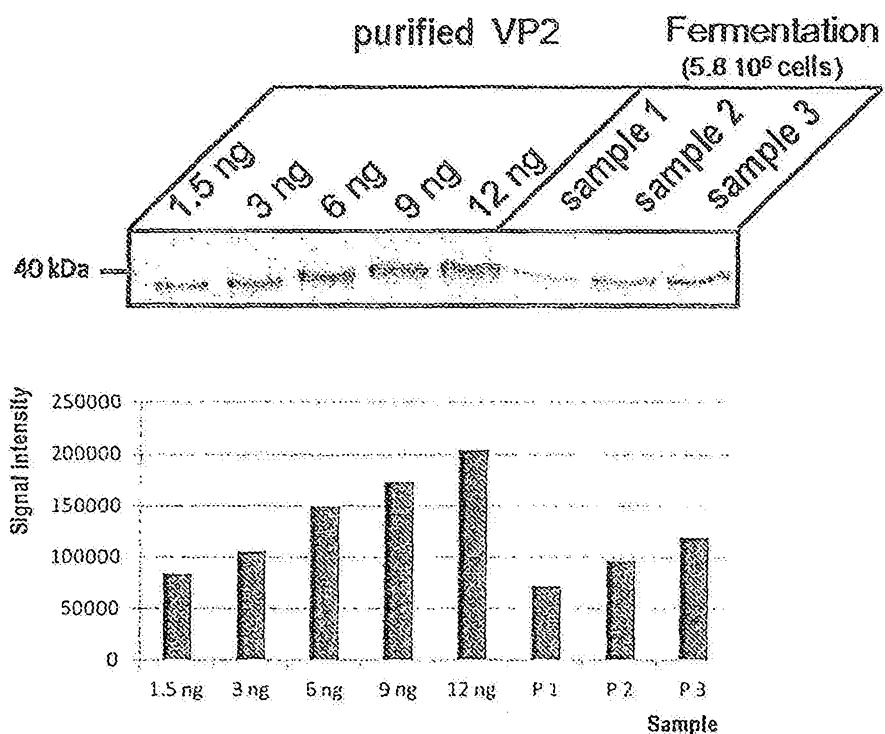


Figure 3A



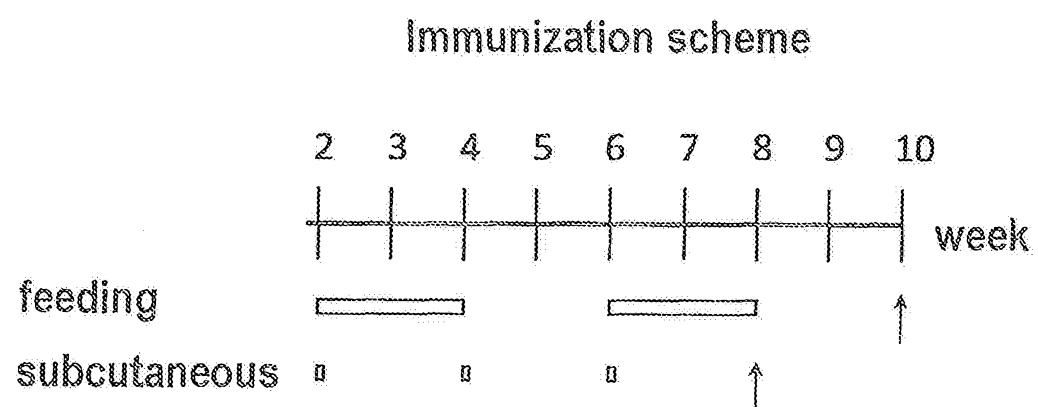
5/13

Figure 3B



6/13

Figure 4A



7/13

Figure 4B

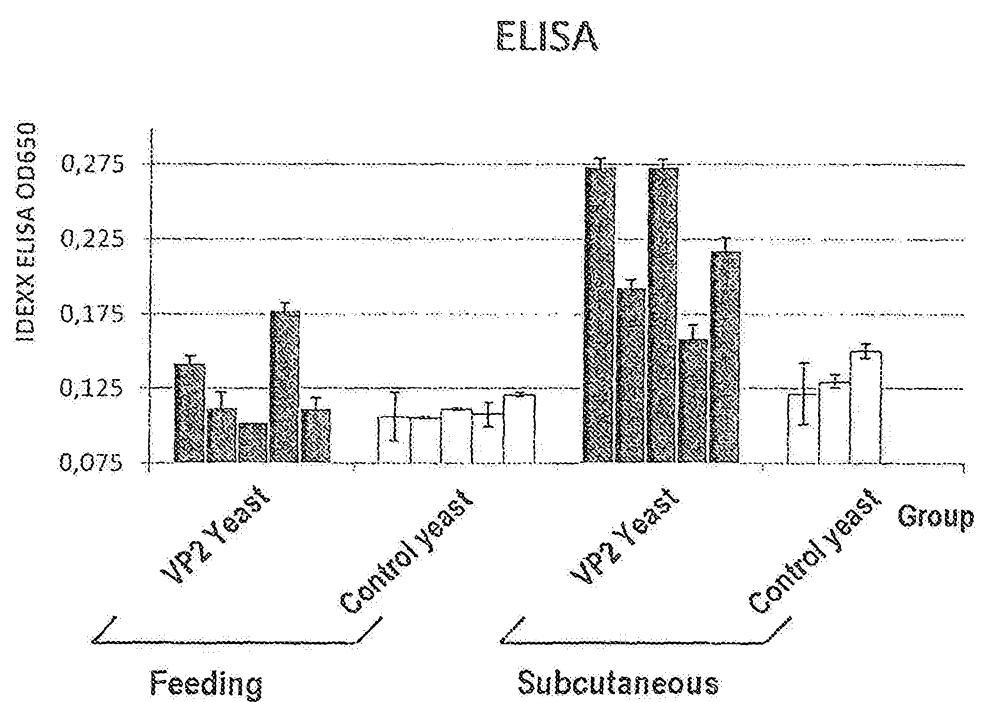
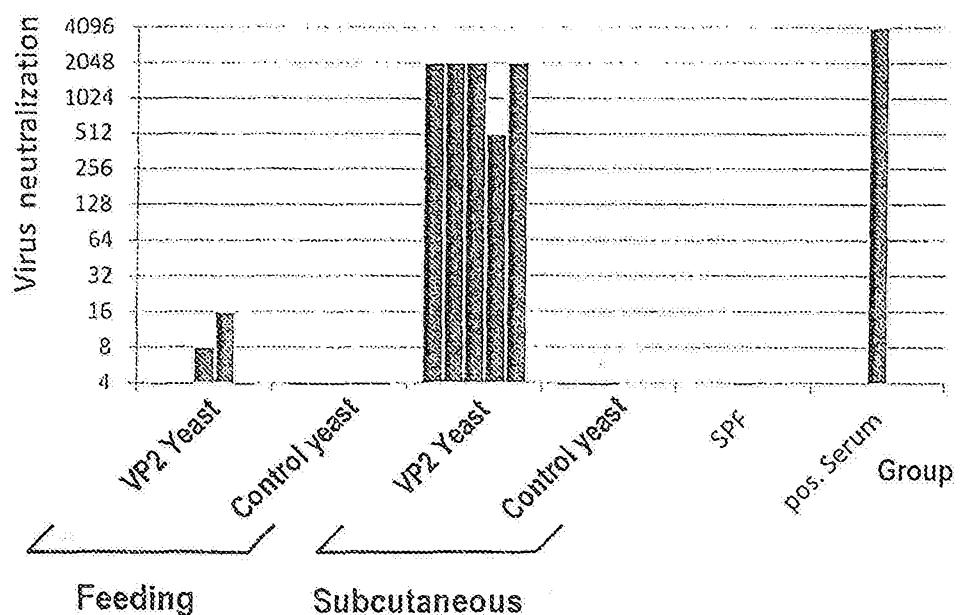


Figure 4C



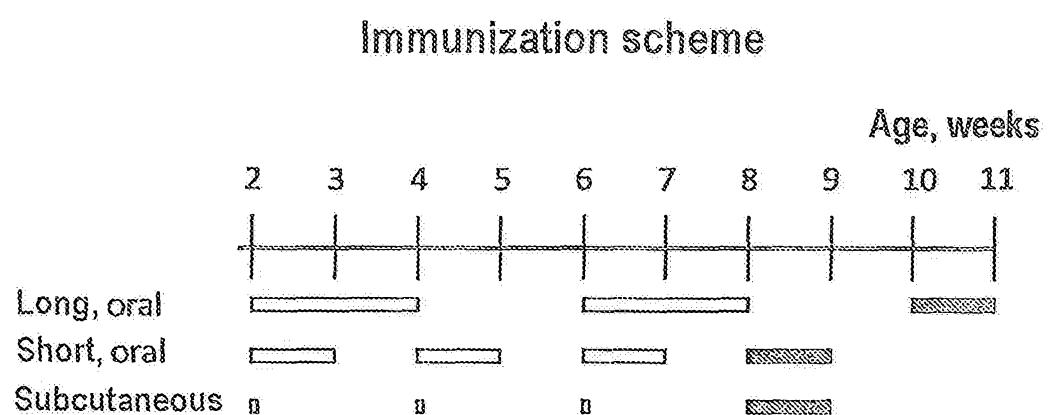
9/13

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 %

10/13

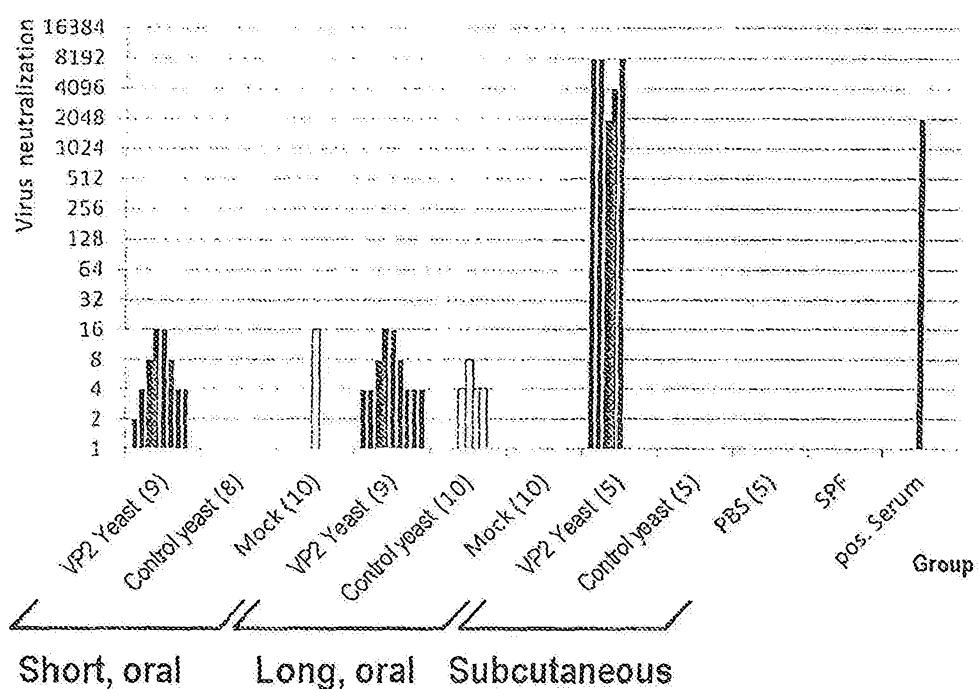
Figure 5A



11/13

Figure 5B

Serum neutralisation assay



12/13

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
VP2 Yeast, long (8)	35.56 ± 36.12	7.56 ± 5.08	0/9	3, 3, 4, 4, 4, 4, 4, 4
Control yeast, long (10)	9.4 ± 19.6	2 ± 2.91	1/10	3, 4, 4, 4, 4, 4, 4, 4
Mock, long (10)	4 ± 2.58	0	0/10	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
Control yeast, long, Saponin (8)	33.7 ± 38	0.89 ± 1.76	1/9	4, 4, 4, 4, 4, 4, 4, 4
Mock, long, Saponin (9)	1	0	0/9	4, 4, 4, 4, 4, 4, 4, 4
VP2 Yeast (5)	2707 ± 823.4	3072 ± 1443.2	0/5	2, 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

13/13

Figure 6

