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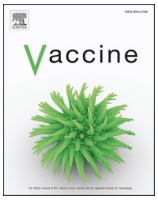

Short communication

Saccharomyces cerevisiae as a platform for vaccination against bovine mastitis

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ABSTRACT

Mastitis is a major issue for the dairy industry. Despite multiple attempts, the efficacy of available mastitis vaccines is limited and this has been attributed to their incapacity to trigger robust cell-mediated immunity. Yeasts have recently been identified as promising antigen vectors capable of inducing T-cell responses, surpassing the antibody-biased mechanisms elicited by conventional adjuvanted vaccines. In this study, we combine *in vitro, ex vivo,* and *in vivo* approaches to evaluate the potential of the yeast *Saccharomyces cerevisiae* as a platform for novel vaccines against bovine mastitis. We demonstrate that *S. cerevisiae* is safe for intramuscular and intramammary immunisation in dairy cows. Vaccination resulted in a significant increase of IFNγ and IL-17 responses against the yeast platform but not against the vaccine antigen. These observations highlight that strategies to counterbalance the immunodominance of *S. cerevisiae* antigens are necessary for the development of successful vaccine candidates.

1. Introduction

Intramammary infections caused by bacteria (mastitis) represent the most frequent disease of dairy cows. Besides exerting detrimental effects on animal welfare and farm profitability, these infections are the leading cause of antibiotic use in dairy farming [1]. Significant effort has been made in recent decades to develop vaccines for mastitis. However, the efficacy of available options remains controversial, as they fail to prevent infection and promote only limited economic benefits in field trials [2]. Commercial and candidate vaccines have been primarily designed to elicit antibody production, but accumulating evidence shows that induction of strong cell-mediated immune mechanisms, particularly T-helper 1 (Th1) and Th17-type immunity, is necessary for the protection of the mammary gland (MG) [3].

Yeasts have recently been identified as promising workhorses for vaccine development [4–7]. Due to the immunogenic nature of their conserved cell wall carbohydrates, whole yeast-based vaccines do not require adjuvants and have proven to induce Th1, Th17 and cytotoxic T-cell responses *in vivo*, surpassing the antibody-biased responses elicited by conventional adjuvanted vaccines [8]. In this study, we combine *in vitro, ex vivo,* and *in vivo* approaches to evaluate the potential of the yeast *Saccharomyces cerevisiae* as a platform for novel vaccines against bovine mastitis.

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2. Material and methods

2.1. Ethics statement and animal experimentation

Animal handling and experimentation were conducted with the approval of the Ethics Committee of Val de Loire (France, DGRIs agreements APAFIS#7094–2,016,082,518,447,444 v3, APAFIS#29498–2,021,020,410,061,759 v2 and APAFIS #35905–2,022,060,113,398,545 v1) in strict accordance with all applicable provisions established by the European directive 2010/63/UE.

2.2. Whole-blood and primary mammary cells stimulation

EDTA-treated blood samples were collected by jugular venipuncture from eight Holstein cows in lactation bred in the Unité Expérimentale de Physiologie Animale (UEPAO INRAE, France). Whole blood was stimulated with 10^6 CFU of heat-inactivated *S. cerevisiae* EBY100 (ATCC strain #MYA-4941, 56 °C for 30 min), *S. aureus* (strain 169.32, isolated from a case of subclinical mastitis [9]) or *E. coli* (strain P4, isolated from a case of clinical mastitis [10]) as previously described [11]. Bacterial strains were heat-inactivated at 70 °C for 30 min. Bovine primary mammary epithelial cells were prepared as described in [9] and stimulated with 10^5 CFU of heat-inactivated EBY100, *S. aureus* or *E. coli*. Cultures were kept for 48 h at 38.5 °C with 5 % CO_2 and supernatants were collected for cytokine measurement by ELISA.

2.3. Ex vivo stimulation of mammary tissue

Teats were collected from MGs with no signs of disease from four slaughtered dairy cows in a French commercial abattoir. Teat explants were processed and stimulated with 10^6 CFU of heat-inactivated EBY100, *S. aureus* or *E. coli* as previously described [12]. Supernatants were collected 24 h after stimulation and cytokine production was measured by ELISA.

2.4. Molecular cloning and yeast-based vaccine production

Gallus gallus ovalbumin (OVA) coding sequence (depleted of its first 50 aminoacids) was inserted into the pYD1 vector backbone (Invitrogen) in-frame with the Aga2p coding sequence to construct pYD1-OVA. The recombinant DNA construct was cloned and amplified in *E. coli* DH5a (New England Biolabs). *S. cerevisiae* strain EBY100 was cultivated in YPD broth to OD_{600} of 0.6–0.8 and routinely transformed with 1 µg of pYD1-OVA using the LiAc/SS carrier DNA/PEG method to generate EBY100-OVA. To produce a prototype vaccine, EBY100-OVA was inoculated in minimal broth (0.67 % Yeast Nitrogen Base with ammonium sulfate, 2 % raffinose and dropout aminoacids mixture (76 mg/l each) without tryptophan) and OVA surface expression was induced with 2 % galactose at an OD_{600} of 0.5 for 6 h. Afterwards, yeast cells were heat-inactivated at 56 °C for 30 min, centrifuged at 3500 rpm for 10 min at 10 °C. Yeast pellet was washed twice with wash buffer (DPBS without Ca^{+2} and Mg^{+2}, 2 % FBS, 2 mM EDTA) and kept dry at −20 °C until vaccination. For the preparation of vaccine doses, the required amount of yeast cells was resuspended in 3 ml of DPBS.

2.5. Vaccination with EBY100-OVA and clinical monitoring

Six non-pregnant Holstein cows (dried for 4 months) were recruited for an immunisation protocol carried out at the Plateforme d'Infectiologie Experimentale (PFIE INRAE, France). Selected animals showed low somatic cell counts in udder quarter milk (< 200 000 cells/ml) and absence of intramammary infection by major pathogens (*Staphylococcus aureus*, *Escherichia coli* or streptococci) before dry-off. For prime immunisation, 2.10^9 CFU of EBY100-OVA were administered intramuscularly at the prescapular region. After 60 days, animals were administered with an intramammary booster as follows: rear quarters received 10^9 CFU of EBY100-OVA, the front right quarter received 10^8 CFU of EBY100-OVA and the front left quarter was used as control. After immunisations, the presence of systemic and local reactogenicity signs was evaluated and recorded by a single operator using a scoring grid (**Supplementary File 1**). As a complement, the presence of subcutaneous edema at the MG cistern was monitored by ultrasound using an Esaote Piemedical MyLab30 ultrasound device (Hospimedi France).

2.6. Vaccination with recombinant OVA

Three Holstein cows in lactation bred in the Unité Experimentale du Pin (UEP, INRAE) were immunised intramuscularly in the prescapular region with 50 µg of pyrogen-free ovalbumin (Calbiochem) dissolved in 0.8 ml phosphate-buffered saline and emulsified in 1.2 ml of Montanide™ ISA 61 VG (Seppic). After 30 days, PBMCs were isolated from blood samples and stored in liquid nitrogen until analysis.

2.7. Evaluation of blood immune response to vaccination

Serum and PBMCs were routinely isolated from blood samples collected at the indicated time points and kept in liquid nitrogen. PBMCs were defrosted and stimulated at the same time as follows: 3.10^5 cells were mixed with 200 µl of WBA medium (RPMI 10 % FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 nM non-essential amino acids, 50 µM β-mercaptoethanol, 10 mM HEPES) containing 10^6 CFU of heat-inactivated EBY100, EBY100-OVA (only day 30) or 1 µg of pyrogen-free ovalbumin (Calbiochem) in a 96 well round bottom plate (Nunc). Empty medium was used as control. Cultures were kept for 48 h at 38.5 °C with 5 % CO_2 and supernatants were collected for cytokine measurement by ELISA. For the estimation of total antibody titers to OVA and EBY100 in blood serum, microtiter plates (Nunc Immunoplate Maxisorp) were coated by overnight incubation with 2 µg/ml of ovalbumin or EBY100 protein lysate (100 µl/well) in phosphate-buffered saline (PBS). Then, ELISA assays were carried out as described in [11].

2.8. Intramammary stimulation and somatic cells count

At day 100 (40 days after intramammary booster), control and boosted mammary quarters of the cows immunised with EBY100-OVA were administered with 10 µg of pyrogen-free ovalbumin (Calbiochem) dissolved in 3 ml of DPBS containing 1 mg of pyrogen-free bovine serum albumin (Sigma). Ten millilitres of mammary secretion were collected before (day 100) and 24 h after stimulation (day 101) for the measurement of somatic cell counts using an automated cell counter (Fossomatic).

2.9. Western blot and ELISA

EBY100 and EBY100-OVA cultivated in the presence of galactose (OD_{600} 1.0) were lysed with YeastBuster Protein Extraction Reagent (Merck) following the manufacturer's condition. Ten micrograms of total protein were separated by electrophoresis and blotted under standard conditions using a rabbit polyclonal antibody anti-OVA antibody (produced by our team, 0.5 µg/ml) and a goat anti-rabbit IgG (H + L) antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, 1:5000). Membranes were scanned in a Fusion FX imager (Vilber). ELISA assays were performed as follows: IFNγ (Mabtech, 3119-1H-20), IL-8 (Mabtech, 3114-1H-6), TNFα (Kingfisher, DIY0675B-003), IL-6 (Kingfisher, DIY0670B-003). IL-17 was measured as described in [11].

2.10. Flow cytometry and immunofluorescence

EBY100-OVA cultivated in the presence of galactose or raffinose was heat-inactivated at 54 or 56 °C for 30 min. Yeast cells were washed with FACS buffer (DPBS without Ca^{2+} and Mg^{2+}, 2 mM EDTA, 2 % FBS) and

incubated with an anti-Xpress (AB_2556552 Invitrogen, 1:200) antibody for 30 min. A goat anti-mouse IgG (H + L) conjugated to Alexa Fluor 555 (AB_2535844, Thermo Fischer, 2 µg/ml) was used as secondary antibody. Stained yeast cells were examined using a BD LSR Fortessa cytometer and data were analysed with the Kaluza software (Beckman Coulter). For immunofluorescence, EBY100-OVA cultivated in the presence of galactose or raffinose (heat-inactivated at 56 °C for 30 min) was incubated in suspension with a rabbit polyclonal antibody anti-OVA antibody (produced by our team, 5 µg/ml) and a donkey anti-rabbit IgG (H + L) secondary antibody conjugated to Alexa Fluor 594 (Jackson ImmunoResearch, 1:100) diluted in FACS buffer. Stained samples were mounted between a slide and coverslip and immediately examined under a fluorescence microscope (EVOS M5000, Thermo Fischer). Images were processed using ImageJ 1.54d (NIH, USA).

2.11. Statistical analyses

Data were analysed and plotted using GraphPad Prism, version 6.0 (GraphPad Software Incorporation). Kruskal–Wallis test was used to compare groups and pairwise comparisons (treated *versus* control) were carried out using Dunn's test. In time course analyses, data were analysed using Friedman's test and pairwise comparisons (indicated time point *versus* day 0) were carried out using the Dunn's test. Data shown represent the median and interquartile range. Black (treated *versus* control) and red (indicated time points *versus* day 0) asterisks denote statistically significant difference. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

3. Results and discussion

Initially, we checked the capacity of *S. cerevisiae* to stimulate the bovine immune system. Our results show that *S. cerevisiae* strain EBY100 is properly sensed by bovine blood cells and induces the production of markers of inflammation and T-cell response, similar to those of mastitis-causing bacteria (Fig. 1A-B). Next, we tested whether a vaccine formulation based on *S. cerevisiae* would be suitable for intramammary (IMM) administration. High levels of proinflammatory cytokines have been reported to compromise the integrity of mammary epithelial cells (MECs), leading to a loss of milk production [13]. Thus, we used primary cell cultures and a recently reported *ex vivo* model [12] to evaluate the inflammatory response of MECs and teat explants upon exposure to yeast (Fig. 1C). As shown in **Fig. 1D-E**, *S. cerevisiae* induced a lower release of pro-inflammatory cytokines than mastitis-causing bacteria. It has been reported that receptors enabling the recognition of yeast cell wall sugars are mostly found on the surface of antigen-presenting cells such as dendritic cells and macrophages [14], whereas MECs are particularly equipped to recognise bacteria-associated molecular patterns [15]. Therefore, antigen vectorisation by yeast might represent an effective strategy to trigger adaptive immunity in the MG while circumventing an overproduction of inflammatory mediators by MECs.

To test this hypothesis *in vivo*, we developed a yeast-based vaccine formulation and tested its immunogenicity in dairy cows. An ovalbumin (OVA) coding sequence was inserted into the expression vector pYD1 [16] to construct pYD1-OVA (Fig. 2A). EBY100 was transformed with pYD1-OVA to generate EBY100-OVA, which expresses the Aga2p-OVA fusion protein on its surface upon induction by galactose (Fig. 2B). Next, we used heat-inactivated EBY100-OVA as a model vaccine. The presence of OVA on EBY100 surface after heat-inactivation was validated by flow cytometry, western blot analysis and immunofluorescence (Fig. 2C-E).

Six dairy cows were immunised with EBY100-OVA by an intramuscular injection followed by an intramammary booster, as described in Fig. 3A. For the booster, each mammary quarter was administered with 0, 10^8 or 10^9 (two quarters) CFU of EBY100-OVA. None of the animals showed fever (Fig. 3B**)** or other reactogenicity events after priming. After booster, clinical examination showed only mild to moderate inflammation in the MG 24 to 48 h post immunisation (Fig. 3C**, Supplementary File 2**). To evaluate the systemic immune response to the

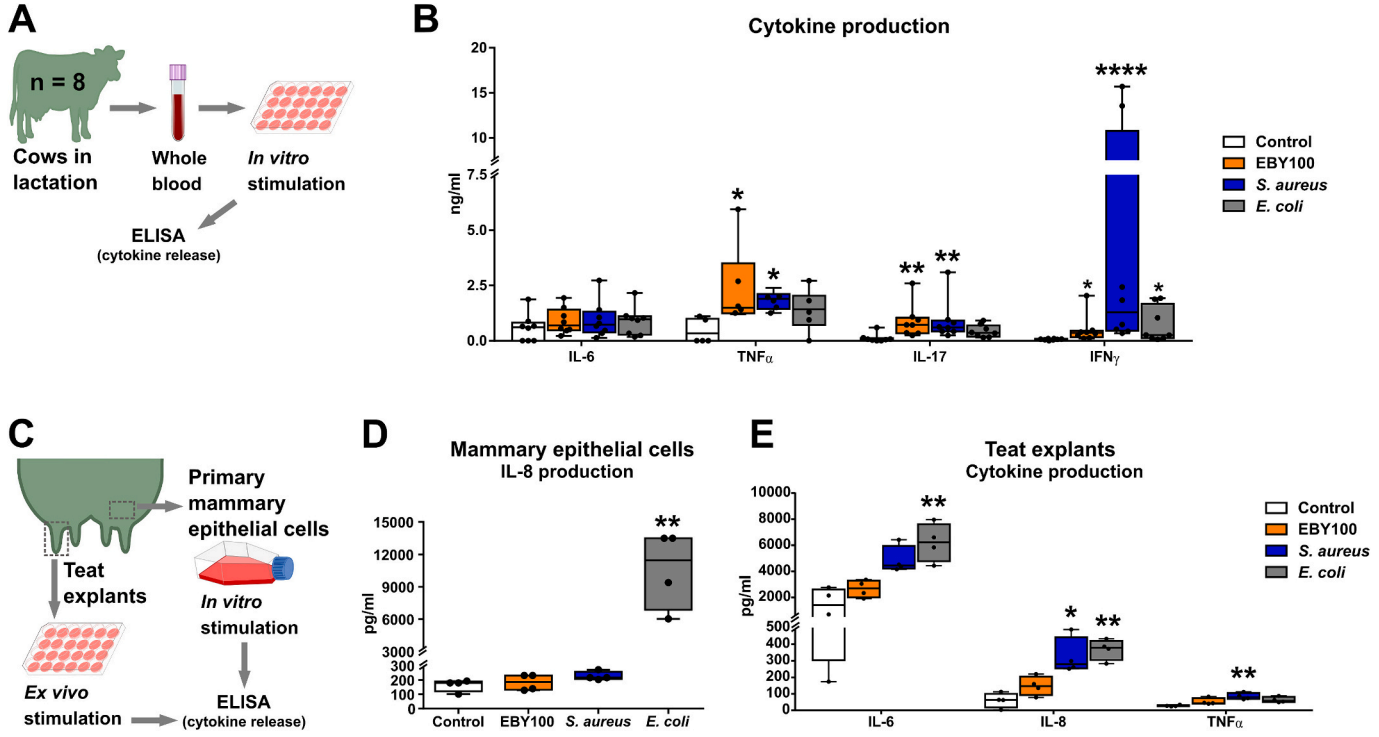

Fig. 1. – Stimulation of bovine cells and mammary tissue with *Saccharomyces cerevisiae*. **A.** Blood from 8 cows was stimulated *in vitro* with heat-killed *S. cerevisiae* (strain EBY100) or heat-killed *Staphylococcus aureus* and *Escherichia coli*. **B.** Evaluation of cytokine release by ELISA. **C.** *In vitro* and *ex vivo* models used to estimate the inflammatory potential of *S. cerevisiae* to bovine mammary gland. Stimulations were carried out as described in **A**. **D-E**. Evaluation of cytokine release by ELISA. Asterisks denote statistically significant difference (treated *versus* control).

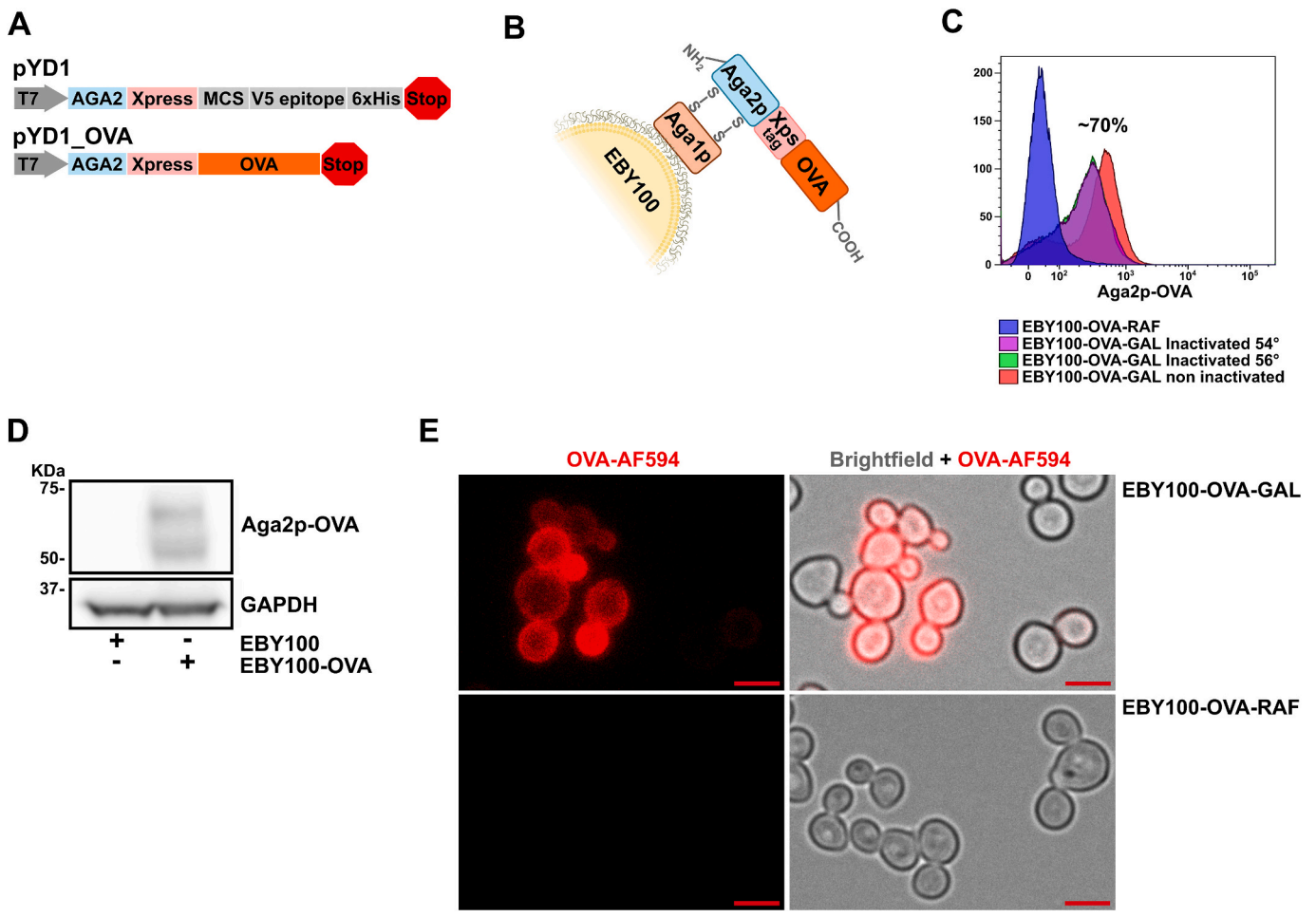

Fig. 2. – Production of a model yeast-based vaccine. A. Diagram depicting pYD1 and pYD1-OVA constructs. **B.** Representation of Aga2p-OVA expression by yeast surface display. **C.** Flow-cytometry analysis of OVA surface expression by EBY100-OVA after galactose (GAL) induction and heat-inactivation. EBY100-OVA non-inactivated or grown in raffinose (RAF) were used as controls. **D.** Western-blot analysis of OVA expression by EBY100-OVA. EBY100 was used as a negative control. **E.** Analysis of OVA surface expression by EBY100-OVA after GAL induction and heat-inactivation by immunofluorescence using a rabbit anti-OVA polyclonal antibody. OVA staining is highlighted in red. Red bars correspond to 5 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vaccine vector and model antigen separately, PBMCs were stimulated *in vitro* with empty EBY100 or recombinant OVA and the production of IFNγ and IL-17 was estimated. As shown in Fig. 3D-E, vaccination resulted in a significant increase in IFNγ and IL-17 responses against EBY100 but not against OVA. Similarly, a significant increase in serum anti-EBY100 but not anti-OVA antibody titers was observed after vaccination (Fig. 3F). We also evaluated the cell numbers in mammary secretion before and after stimulation of the MG with recombinant OVA (Fig. 3G-H). The results indicated that EBY100-OVA could not induce an OVA-specific neutrophilic inflammation in the MG, contrary to our previous observations in cows immunised with OVA emulsified in an oil-in-water adjuvant [11]. To clarify these observations, we repeated immunisation with adjuvanted OVA in three other cows (Fig. 3I) and analysed the response of their PBMCs to *in vitro* stimulation in comparison with PBMCs from EBY100-OVA immunised cows. Interestingly, cells from OVA-immunised cows showed IFNγ and IL-17 responses to EBY100-OVA and OVA (Fig. 3J), whereas cells from EBY100-OVA-immunised animals responded only to EBY100 and EBY100-OVA (Fig. 3K). We also observed that OVA-immunised but not EBY100-OVA cows showed OVA-specific antibodies in serum 30 days after vaccination (**Supplementary File 3**).

Altogether, these results indicate that *S. cerevisiae* antigens are dominant over OVA. Similar observations have been reported upon vaccination with other immunogenic antigen vectors. *Listeria monocytogenes*-based vaccines failed to prime immune responses to cancer antigens due to the immunodominance of its T-cell epitopes [17] and the immunogenicity of adenovirus vaccine vectors has been associated with limited T-cell response to transgenic antigens [18].

The safety, affordability and adjuvant capacity of yeasts represent a significant opportunity to develop novel vaccines against mastitis. Nevertheless, this study indicates that strategies to counterbalance the immunodominance of *S. cerevisiae* antigens are mandatory to ensure the success of this organism as a vaccine platform. Such strategies include the improvement of *S. cerevisiae* as a heterologous protein factory by the construction of optimised DNA delivery systems (by promoter engineering or codon optimisation) and modification of its secretory pathway to increase protein production levels (by enhancing and optimising protein translocation, glycosylation and trafficking) [19,20].

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CRediT authorship contribution statement

Célya Danzelle: Writing – review & editing, Validation,

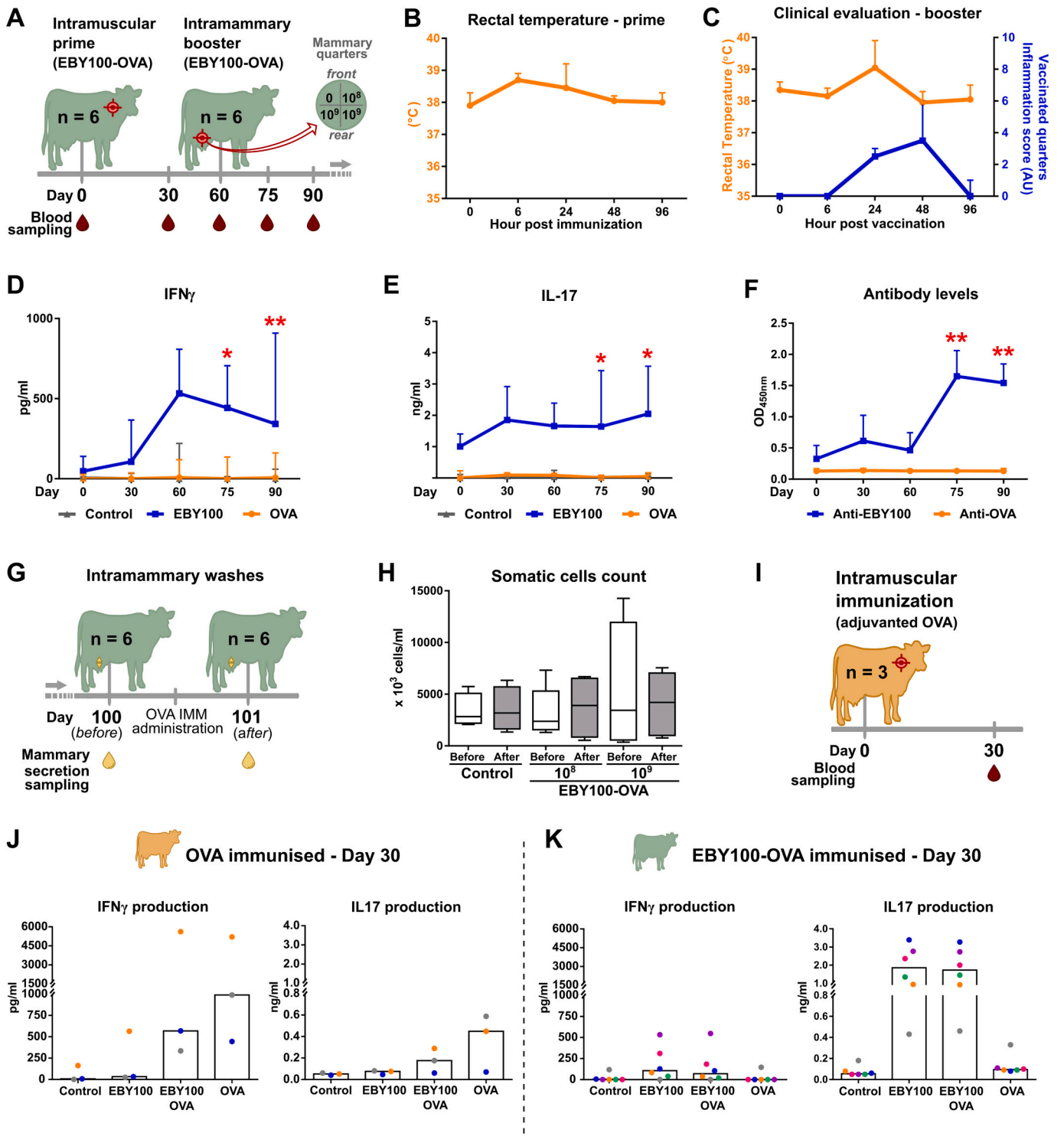

Fig. 3 – In. *vivo* evaluation of a yeast-based vaccine against mastitis. A. Scheme of immunisation with EBY100-OVA. **B.** Monitoring of rectal temperature after prime. C.. Rectal temperature and mammary gland evaluation after booster. D-E. PBMC from vaccinated animals were isolated in the indicated days and stimulated *in vitro* with empty EBY100 or OVA. IFNγ (**D**) and IL-17 (**E**) release in supernatants was measured by ELISA. **F.** Analysis of serum total antibodies anti-EBY100 and anti-OVA by ELISA. **G.** Scheme depicting intramammary stimulations of cows immunised as described in **A** with recombinant OVA. **H.** Somatic cells count in mammary gland secretion before and after intramammary stimulations. **I.** Scheme of immunisation with adjuvanted-recombinant OVA. **J-K.** PBMC from OVA (**J**) or EBY100-OVA (**K**) immunised cows were stimulated *in vitro* with empty EBY100, EBY100-OVA or OVA. IFNγ and IL-17 release was analysed by ELISA. Data shown in **B**—**F, H** and **K** were obtained from the same cows immunised with EBY100-OVA (**A**) and subsequently stimulated *via* the intramammary route with recombinant OVA (**G**). Asterisks denote statistically significant difference (indicated time points *versus* day 0).

Methodology, Investigation. **Patricia Cunha:** Supervision, Methodology, Investigation, Formal analysis. **Pablo Gomes Noleto:** Methodology, Investigation, Formal analysis. **Florence B. Gilbert:** Writing – review & editing, Resources, Methodology, Formal analysis, Data curation. **Kamila Reis Santos:** Methodology, Investigation. **Christophe Staub:** Investigation. **Anne Pinard:** Investigation. **Alain Deslis:** Investigation. **Sarah Barbey:** Methodology, Investigation. **Pierre Germon:** Writing – review & editing, Resources. **Johan-Owen De Craene:** Methodology, Investigation. **Pascal Rainard:** Writing – review & editing, Resources. **Marc Blondel:** Resources, Methodology. **Rodrigo Prado Martins:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rodrigo Prado Martins reports financial support, administrative support, article publishing charges, and equipment, drugs, or supplies were provided by French National Research Agency. Kamila Reis Santos reports financial support was provided by Campus France. Pablo Gomes Noleto reports financial support was provided by French National Institute for Agricultural Research INRAE. Celya Danzelle reports financial support was provided by University of Tours. Rodrigo Prado Martins has patent pending to INRAE, Université de Tours. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2024.126385.

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