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Expression of avian influenza haemagglutinin (H5) and chicken interleukin 2 (chIL-2) under control of the *ptcB* promoter in *Lactococcus lactis*

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Gram-positive and nonpathogenic lactic acid bacteria (LAB) are considered to be promising candidates for the development of new, safe systems of heterologous protein expression. Recombinant LAB has been shown to induce specific local and systemic immune response against selected pathogens, and could be a good alternative to classical attenuated carriers. The main goal of our study was to express the avian influenza haemagglutinin (H5) and chicken interleukin 2 (chIL-2) in *Lactococcus lactis*. Results of this study were anticipated to lead to construction of lactococcal strain(s) with potential vaccine properties against the avian influenza A (H5N1) virus. Expression of the cloned *H5* gene, its His-tagged variant and *chIL-2* gene, under the control of the *ptcB* gene promoter was attested by RT-PCR on transcriptional level and Western or dot blot analysis on translational level, demonstrating that system can be an attractive solution for production of heterologous proteins. The results of the preliminary animal trial conducted in mice are a promising step toward development of a vaccine against avian bird flu using *Lactococcus lactis* cells as antigen carriers.

Key words: *Lactococcus lactis*, *ptcB* promoter, heterologous gene expression, avian influenza H5N1, H5 haemagglutinin, chicken interleukin-2

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INTRODUCTION

Lactic acid bacteria (LAB) are non-invasive and non-pathogenic Gram-positive bacteria with a GRAS (generally regarded as safe) status, widely used for food-processing and preservation. Due to their long and safe association with humans, rapid growth, small genomes and simple metabolism, LAB are considered to be promising candidates for controlled and targeted administration of vaccine antigens to the mucosal immune system (Le Loir *et al*., 1998; Geoffroy *et al*., 2000; Enouf *et al*., 2001; Bermudez-Humaran *et al*., 2002). For this, bacteria are specifically engineered to obtain recombinants, which can induce a specific local and systemic immune response against selected pathogens. The properties of many LAB include: gastric acid and bile salts tolerance, antagonistic properties against pathogenic microorganisms and ability to adhere to the gut epithelium, which makes them useful for oral immunization (Titgemeyer & Hillen, 2002; Mota *et al*., 2006). *Lactococcus lactis* is a non-invasive and non-commensal lactic acid bacterium with low potential to trigger immunotolerance or side effects upon prolonged use (Perdigon *et al*., 2001). Furthermore, *L. lactis* elicits only a weak immune response against itself, despite its ability to act as an immunoadjuvant to accompanying antigens (Drouault *et al*., 1999; Guillemin *et al*., 2009). During the past two decades a remarkable progress has been made toward the molecular characterization of *L. lactis* and development of genetic engineering tools (Gasson & Vos, 1994; Underdown & Mestecky, 1994; Wood & Warner, 2003; Wegmann *et al*., 2007; Wells & Mercenier, 2008). Moreover, *L. lactis* has been extensively engineered for production of heterologous proteins, including some antigens of bacterial or viral origin (Sanchez *et al*., 2008). Promising results have been obtained for various antigens administered via different routes using *L. lactis* as a delivery vehicle (Ribeiro *et al*., 2002; Ramasamay *et al*., 2006).

The study presented here describes preliminary experiments using *L. lactis* engineered for production of the major avian influenza virus (H5N1) antigen - viral haemagglutinin (H5) and chicken interleukin 2 (chIL-2), based on the *ptcB* promoter system. Haemagglutinin is the major protein of the influenza virus capable of inducing antibody production in the infected host. ChIL-2 is a glycoprotein produced by T-type lymphocytes under the influence of specific and nonspecific mitogens. It induces proliferation of the T helper and suppressor as well as cytotoxic cells and enhances the activity of NK cells. Due to such properties, it can be used in designed vaccines as a natural adjuvant (Lillehoj *et al*., 2001). Our study sets the basis for development of a *L. lactis* strain(s) with potent vaccine properties against avian influenza A (H5N1) virus.

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Abbreviations: sLAB, lactic acid bacteria; H5, haemagglutinin of highly pathogenic H5N1 avian influenza virus; chIL-2, chicken interleukin 2; CDM, chemically defined medium; NBT/BCIP, nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyphosphate; HPR, horseradish peroxidase; CcpA protein, Catabolite Control Protein A.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacteria and plasmids used in this study are listed in Table 1. Bacterial cells were cultured at 30°C in M17 broth (Terzaghi & Sandine, 1975) containing 0.5% glucose (GM17) without shaking or on GM17 plates with 1.5% agar. Erythromycin (Boehringer GmbH, Mannheim, Germany) was added to a concentration of 5 μg ml^{-1}, where necessary. For induction of protein synthesis, transformed cells were cultured overnight at 30°C in a chemically defined medium (CDM) containing 0.5% cellobiose and erythromycin (5 μg ml^{-1} final concentration).

DNA manipulation and transformation. The analysis was carried out by CODONW 1.4.2 (http://codonw.sourceforge.net/). Codon usage in the nucleotide sequence of both H5 and chIL-2 appeared to be compatible with codons preferred by *L. lactis*. DNA molecular cloning and restriction enzyme analysis were performed according to general procedures (Staats *et al.*, 1994). *L. lactis* cells were made electrocompetent for transformation with the pIL253:P*ptcB* vector or its recombinant variants as already described (Holo & Nes, 1989; Wells *et al.*, 1993). Plasmid DNA from *L. lactis* was isolated using PureYield™ plasmid mini-prep system (Promega) and a protocol modified for *L. lactis* where lysozyme was added (10 mg×ml^{-1}) prior to the lysis step and incubating the cell suspension for 30 min at 37°C to generate protoplasts.

Amplification of *H5* and *chIL-2* DNA genes by polymerase chain reaction (PCR). *H5* [H5N1 A/swan/Poland/305-135V08/2006 strain from AIV EpiFluDatabase [http://platform.gisaid.org]; Accession No. EP1156789], *H5* with a C-terminal His-tag and *chIL-2* [GenBank AF017645] genes were amplified using a standard PCR technique employing cDNA as template and specific primers listed in Table 1.

Cloning of avian influenza virus *H5* and chicken *IL-2* genes in pIL253:P*ptcB* vector. Recombinant plasmids were created by ligation of pIL253:P*ptcB* vector with the 1.8-kb H5 gene PCR product or its his-tagged derivative, previously digested with BamHI/XhoI restrictases. Construct carrying additionally the *chIL-2* gene downstream of the H5 gene was generated by digesting the 434-bp *chIL-2* PCR product with BoxI and ligating it with pIL253:P*ptcB*:*H5*, prepared in the same way. Recombinant constructs were selected by colony PCR, with one primer specific for the insert, and the second one - specific for the plasmid sequence. Inserts in each clone were sequenced (GS FLX Titanium 454, Roche) to confirm their identity with the templates used.

Isolation of total RNA from *L. lactis*. RNA samples were isolated from logarithmic-phase *L. lactis* cultures grown on CDM medium supplemented with 0.5% cellobiose using commercial RNeasy mini Kit (Qiagen). RNA samples were stored at –80ºC prior to further use.

Reverse transcription analysis. Reverse transcription (RT) reaction was performed with the SuperScript™ III reverse transcriptase kit (Invitrogen), according to the manufacturer's instructions, using an appropriate reverse primer (Table 1). Specific cDNA was amplified using a standard PCR technique and a pair of specific primers (Table 1).

Protein isolation. Recombinant strains harboring H5- and chIL-2-coding genes were grown in CDM medium, to an optical density OD_{600} equal 0.6. Crude extracts were obtained by disrupting the cells at high speed using the Mini Bead Beater (MBB-8) device and glass beads (106-μm diameter; Sigma) 3 × for 1 min with 1-min intervals, during which the cells were kept on ice. Cellular

Table 1. Bacterial strain, plasmids and primers used in this study.

Bacterial strain	Properties	reference
IL1403	Laboratory, wild type, plasmid-free strain	(Chopin *et al*, 1984)
Plasmids	Properties	reference
pIL253:P*ptcB*	Em^R, pIL253-derivative with *ptcB* gene promoter region	IBB PAS collection
pIL253:P*ptcB:H5*	Em^R, pIL253-derivative with *ptcB* gene promoter region, encoding *H5* gene of H5N1	this work
pIL253:P*ptcB:H5His*	Em^R, pIL253-derivative with *ptcB* gene promoter region encoding *H5* gene of H5N1 fused with His-tag nucleotide sequence at C' terminal end	this work
pIL253:P*ptcB:H5:chIL2*	Em^R, pIL253-derivative with *ptcB* gene promoter region encoding *H5* gene of H5N1 and chIL2	this work
Primer	Nucleotide sequence	
HAF	5'**CGGGATCCCG**AAGGAGTATTTCTATGGA GAATATAGTGCTTCTTT3'	
HAR	5'**CCGCTCGAG**TTAAATGCAAATTCT3'	
HAHisR	5'**CCGCTCGAG**TTATTAATGATGATGATGAT GATGAATGCAAATTCTGCGTTG3'	
chIL-2F	5'**GCGACGAGCGTC**AAAAGGAGGTATTTCTA TGATGTGCAAATGA3'	
chIL-2R	5'**CGGACGAGCGTC**GCGACCGGTTTATTATTTTTGCAG3'	

*Conditions for DNA amplification were determined depending on each primer and length of the amplified sequence. Sequences recognized by particular restriction enzymes are shown in bold.

debris and glass beads were removed by centrifugation for 10 min at 8 000 rpm. Protein concentrations were determined using Nano Drop (Thermo Scientific).

SDS/PAGE analysis of protein extracts. Equal volumes of 2× loading buffer (125 mM Tris/HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.01% bromophenol blue) and protein extracts were combined. Subsequently, samples were incubated for 5 min at 95°C and then cooled on ice for 5 min. Next, protein samples were analyzed by SDS/PAGE (110 V, 500 mA, 2 h). Inclusion bodies obtained by over-expression of H5-coding gene in *E. coli* were used as a positive control for protein analysis. Negative standard protein samples obtained from IL1403 [pIL253:P*ptcB*] were used for data normalization.

Western-blot analysis for H5 antigen. Proteins separated by SDS/PAGE were transferred onto nitrocellulose Hybond-C Extra membranes (Amersham) at 45 A and constant V for 1 h using semi-dry blotter (Hoefer). Membranes were blocked overnight at 4°C with TS buffer (20 mM Tris, 0.13 mM NaCl, pH 8.0), containing 3% skim milk. Immunoblotting was carried out using commercial monoclonal mouse antibodies against the H5 antigen (Abcam), diluted at 1:1000.

Immunodetection was performed with secondary rabbit or mouse antibodies conjugated with alkaline phosphatase (Sigma) at dilution 1:30 000 and NBT/BCIP Stock Solution (Roche) as above.

Dot blot detection of chIL2. Five μl of total protein extracts were deposited on a Hybond- C Extra nitrocellulose membrane (Amersham). The dot-blot membrane was air-dried for 5 min, blocked at 37°C for 20 min in TS buffer (20 mM Tris, 0.13 mM NaCl, pH 8.0) containing 3% skim milk, and then incubated with chIL-2-specific commercial monoclonal antibodies (AbDserotec), at dilution 1:1000. Immunodetection was performed with secondary mouse antibodies conjugated with alkaline phosphatase (Sigma) at dilution 1:30 000 and NBT/BCIP Stock Solution (Roche) as recommended by the suppliers. Negative standard protein samples obtained from IL1403 [pIL253:P*ptcB*) were used for data normalization. Recombinant chicken interleukin-2 (PAP005 AbDSerotec) served as a positive control.

Mouse immunization and sample collection. All work was performed ethically in compliance with the First Warsaw Local Ethics Committee for Animal Experimentation. Ten-week-old female BALB/c mice (Mossakowski Medical Research Centre of Polish Academy of Sciences, Warsaw, Poland), counting four groups of 5 mice each, were immunized intragastrically (i.g.) with 10^9 CFU of recombinant *L. lactis* expressing a single H5 antigen (group 1), both H5 and chIL-2 proteins (group 2), with bacterial protein extract from IL1403 [pIL253:P*ptcB:H5*] (group 3), and with *L. lactis* carrying the empty vector [pIL253:P*ptcB*] — control group. Same doses were administered to animals for the first three days of the trial, and then four times at 1-week intervals. Blood samples from the orbital sinus were collected four days after first three doses, six days after each subsequent booster, and 7 and 14 days after the last immunization. To obtain serum, whole blood samples were left to coagulate at room temperature for approximately 15 to 30 minutes. The clot was rimmed using an applicator stick and then the sample was centrifuged twice at 4°C for approximately 10 min at 10 000 *g*. Obtained serum samples were stored at –80°C until assayed.

Enzyme-linked immunosorbent assay (ELISA). Collected sera (15 μl) were assayed for the presence of antibodies against H5 antigen by an ELISA method, using MaxiSorp plates (Nunc) coated with mammalian cell-expressed H5 protein (17–530 aa, ΔRRRKKR) of A/Bar-headed Goose/Qinghai/12/05 (H5N1)) strain of influenza A virus (3 μg×ml^{-1}) (Immune Technology). Secondary labeling and its detection were done with reagents purchased from Sigma-Aldrich. Serum samples from i.g. immunized mice with H5 harbored by *L. lactis* were tested in parallel with serum from sham-immunized mice (negative controls). Monoclonal antibodies to avian influenza A virus (H5N1) HA (US Biological, Acris Antibodies) were used as positive controls. Experimental samples, diluted 1:25 in 2% BSA-PBS, positive controls and blanks (sample diluent) were applied onto the plates previously coated with H5 (3 μg×ml^{-1} in PBS, overnight, 2–8°C) and then incubated overnight at 2–8°C. Antibody classes were detected with goat generated and horseradish peroxidase (HRP)-labeled antibodies against mouse IgG (γ-chain specific) and IgA (α-chain specific) at 1:500 dilution (1 h, 37°C). In all tests performed, TMB was used as the substrate. After incubation for 30 min at room temperature, the reaction was stopped by the addition of 0.5 M sulfuric acid. The absorbance was quantified at 450 nm with subtraction of the mean OD readings of blanks. Samples were considered to be positive for anti-H5 antibodies if the OD readings were 2SD above the mean OD readings of samples from control mice (cut-off value).

RESULTS

Recombinant H5 and chIL-2 expression in *L. lactis*

Expression of *H5, H5* His-tag and *chIL-2* genes under the control of the *ptcB* promoter was attested by RT-PCR. The main products of 1.8 kb and 434 bp corresponding respectively to H5- and chIL-2-encoding regions were identified (data not shown). No positive signals were noted for the negative control. Subsequently, specific positive signals on the translational level were detected in immunoenzyme reactions. However, Western blot analysis performed for the H5 protein revealed intracellular proteolysis of the antigen, visible as a ladder on the nitrocellulose membrane. This degradation was also visible and confirmed for the His-tagged protein

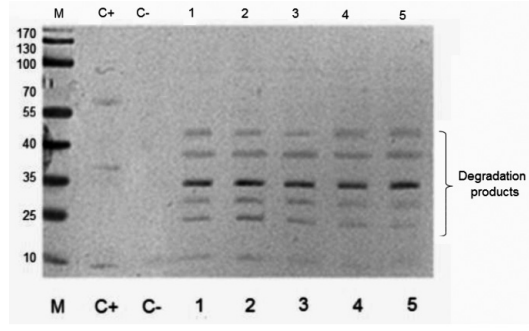

Figure 1.Western blot analysis of H5 production under *ptcB* promoter with anti-His commercial antibodies.
Immunoblotting was carried out using H5 specific commercial monoclonal mouse antibodies (Abcam) diluted at 1:1000. Immunodetection was performed with secondary mouse antibodies conjugated with alkaline phosphatase (Sigma) at dilution 1:30 000 and a NBT/BCIP Stock Solution (Roche) as recommended by the suppliers. Intracellular proteolysis of the antigen was visible as a characteristic ladder of bands on the nitrocellulose membrane (lanes 1, 2, 3, 4, 5 — protein extracts of IL1403 [pIl253:P*ptcB:H5His*] strain). No specific products were observed for negative control — protein extract of IL1403 [pIl253:PptcB] (C–).

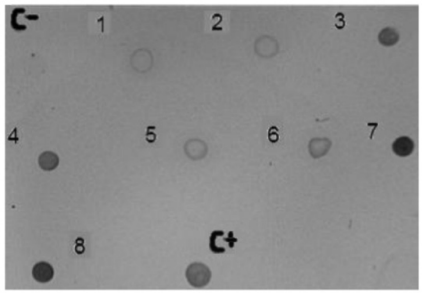

Figure 2.Dot blot analysis for chIL-2 production in the IL1403 [pIL253:*PptcB:H5:IL-2*] strain.
The dot-blot membrane was air-dried for 5 min, blocked with 10% nonfat dry milk blocking buffer at 37°C for 20 min, and then incubated with the primary antibody using a chIL-2 specific commercial monoclonal antibodies (AbDserotec), at dilution 1:1000, and with secondary mouse antibodies conjugated with alkaline phosphatase (Sigma) at dilution 1:30 000. Immunodetection was performed with NBT/BCIP Stock Solution (Roche) as recommended by the suppliers. Negative standard protein samples IL1403 [pIL253:P*ptcB*] were used for data normalization. C– (negative control), 1–2 and 5–6 soluble fraction; 3–4 and 7–8 insoluble, membrane fraction, C+ (positive control).

(Fig. 1). For the chIL-2 protein (Fig. 2), both the soluble and insoluble fraction samples gave positive signals, with a definitely stronger signal for samples enriched with the membrane fraction. The negative control did not react with the antibody.

ELISA detection of H5-specific serum antibody

Immunogenicity of *L. lactis*-expressed H5 antigen was investigated in the mouse model. Recombinant bacteria in the form of live vector vaccine or protein extracts were administered by intragastric gavage using the same time scheme. In mice immunized with *L. lactis* expressing the H5 antigen alone (group 1), only weak serum IgG and IgA responses were observed (Fig. 3A and B). Only a single mouse responded slightly stronger with an IgG peak after administration of the third dose (Fig. 3A). Higher serum antibody responses were shown for mice in group 2, vaccinated with *L. lactis* co-expressing H5 and chIL-2. Antigen-specific IgG antibodies were detected in serums collected after the third and subsequent immunizations (Fig. 4A) and for IgA isotype at different time points of the experiment (Fig. 4B). Three out of four mice in group 2 responded more effectively with serum IgG after the fourth booster (Fig. 4A). For two mice this was accompanied by increased IgA levels (Fig. 4B). The strongest response was induced using the protein extract from H5-producing *L. lactis* (group 3), yet, this effect was observed for only one out of four mice. In this case, IgG antibodies against H5 were detected in the serum after the fourth immunization and peaked 7 days after application of the fifth dose (Fig. 5A and B). This was not accompanied by IgA response. The remaining mice from group 3 did not develop anti-H5 antibodies. Samples from mice sham-immunized with the control strain gave low OD values, which were constant during the whole experiment. This is indicated in the figures as dashed cut-off lines. In summary, four mice developed more pronounced H5-specific IgG antibodies in sera after three-four boosters of recombinant *L. lactis* expressing H5 (Fig. 3A and Fig. 4A).

DISCUSSION

In this study, a new system of protein production in *L. lactis* has been tested. The idea was based on carbon catabolite repression operating in Gram-positive bacteria that is exerted through binding of the CcpA protein to *cre*, *a cis*-acting catabolite responsive element (Aleksandrzak-Piekarczyk *et al.*, 2005). CcpA can act as an activator of the promoter sequence of the regulated gene or an inhibitor. The promoter sequence of the chromosomal *L. lactis ptcB* gene, containing a *cre* sequence, was used to create the pIL253:P*ptcB* vector to potentially increase heterologous protein production in transformed *L. lactis* strains. The activity and efficiency of the *ptcB* promoter region was already described by Aleksandrzak-Piekarczyk *et al.*, 2011. To investigate the efficiency in immunoprotection against avian influenza virus, three *L. lactis* strains expressing genes for avian influenza virus H5 protein and chicken IL-2 from the pIL253:P*ptcB* vector were analyzed. For this, recombinant bacteria in the form of live vector vaccine or protein extract were administered to BALB/c mice by intragastric gavage using the same time scheme. Serum antibody responses of IgG and IgA isotypes were monitored. These antibodies are considered to be one of the most effective de-

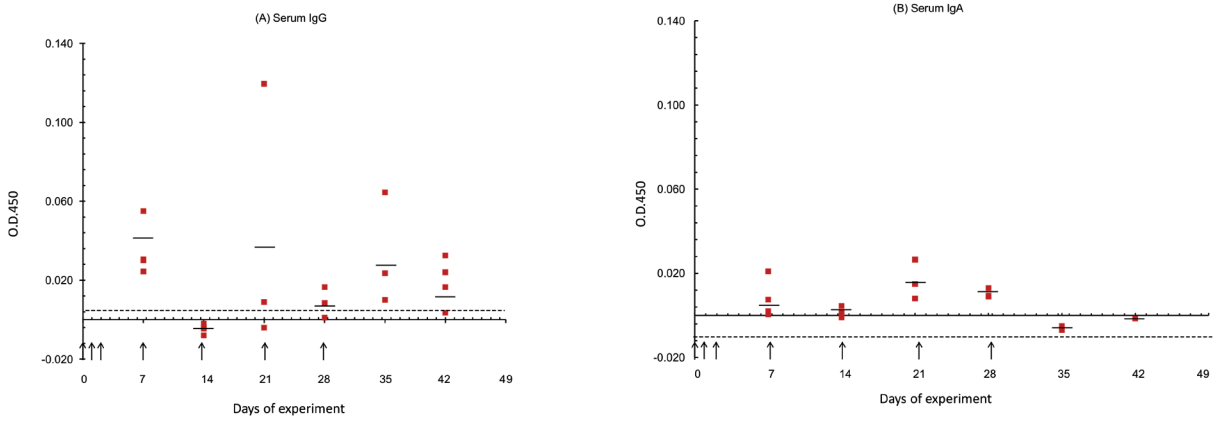

Figure 3. Serum antibody response after immunization with *L. lactis* expressing H5 antigen.
Five mice were immunized seven times with 10^9 CFU of *L. lactis* [pIL253:P*ptcB:H5*] on days indicated by arrows. Serum samples were collected at various time points of the experiment. Levels of H5-specific IgG (**A**) and IgA (**B**) antibodies were determined in samples diluted 1:25. Results obtained for immunized mice at each time point of blood sampling are shown individually (squares) and as mean OD values (dashes). The mean OD readings higher by 2 SD than control mice samples were considered as the cut-off indicated by the dashed line.

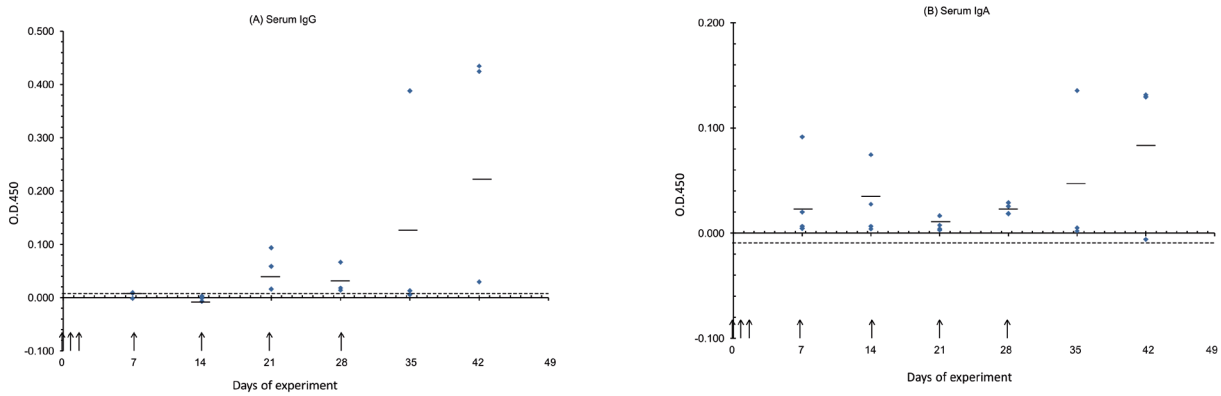

Figure 4. Serum antibody response after immunization with *L. lactis* expressing H5 and chIL-2.
Five mice were immunized seven times with 10^9 CFU of *L. lactis* [pIL253:P*ptcB:H5:IL-2*] on days indicated by arrows. Serum samples were collected at various time points of the experiment. Levels of H5-specific IgG (**A**) and IgA (**B**) antibodies were determined in samples diluted 1:25. Results obtained for immunized mice at each time point of blood sampling are shown individually (diamonds) and as mean OD values (dashes). The mean OD readings higher by 2 SD than control mice samples were considered as the cut-off indicated by the dashed line.

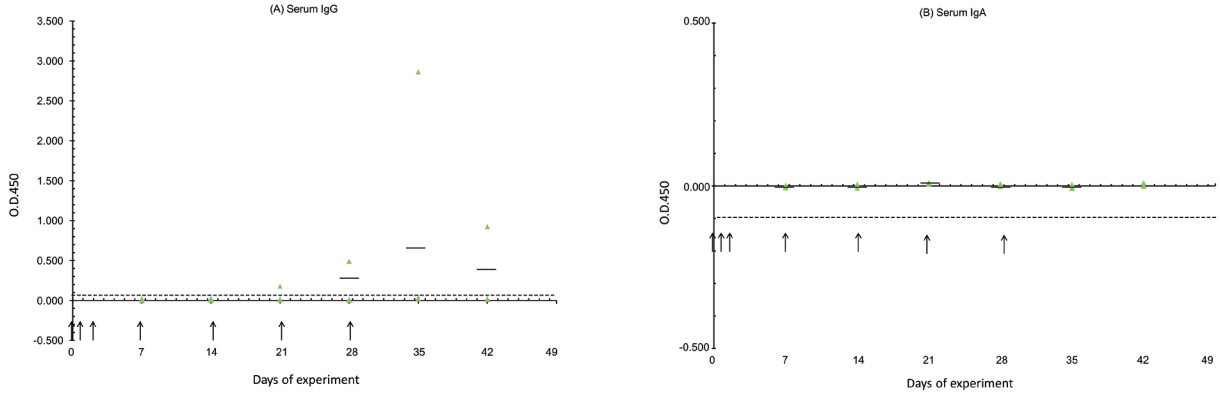

Figure 5. Serum antibody response after immunization with protein extract from *L. lactis* expressing H5.
Five mice were immunized seven times on days indicated by arrows with protein extract obtained from IL1403 [pIL253:P*ptcB:H5*] corresponding to 10^9 CFU. Serum samples were collected at various time points of the experiment. Levels of H5-specific IgG (**A**) and IgA (**B**) antibodies were determined in samples diluted 1:25. Results obtained for immunized mice at each time point of blood sampling are shown individually (triangles) and as mean OD values (dashes). The mean OD readings higher by 2 SD than control mice samples were considered as the cut-off indicated by the dashed line.

fense mechanisms against respiratory infections and a vital attribute of any vaccine designed to prevent infections (Lamm, 1997; Xi n*et al.*, 2003). Preliminary animal trial showed the capability of recombinant *L. lactis* strains producing the H5 antigen to evoke an immune response via the mucosal route. Moreover, antibodies induced with *L. lactis*-expressed H5 recognize recombinant H5 of mammalian-cell origin, which has been shown to preserve essential epitopes of the native protein and its oligomeric structure (data not shown). The best immunization results were obtained with the use of a strain co-expressing H5 and chIL-2, as mice from this group gave the clearest response to vaccination as judged by IgG and IgA level of anti-H5 antibodies (Fig. 4A and B). Overall, the enhanced immune response observed in this animal group was presumably related to the presence of chIL-2, anticipated to play a role of an adjuvant. In particular, chIL-2 has been studied widely as an immuno-enhancer due to its role in activating T cell proliferation. The unsatisfactory number of responder mice and overall low levels of serum H5-specific antibodies were probably, and at least partially, due to the low amounts of properly folded H5, caused by strong intracellular proteolysis of expressed antigen inside bacterial cells. The proteolytic pattern of degradation observed for the heterologous protein is far different from that which occurs during a natural viral infection of host cells. Such effect often leads to production of many undesirable epitopes which can be a response-limiting factor. On the other hand, strong serum antibody response is difficult to induce solely by the mucosal route and several experiments are often needed to establish an effective immunization scheme.

In summary, our group demonstrated here the use of *L. lactis* as a biological host for production of viral antigen and chicken cytokine. The tested *ptcB* promoter region might be an attractive system for production of heterologous proteins. The results of the preliminary animal trial conducted in mice are a promising step toward development of a vaccine against avian bird flu using *L. lactis* cells as antigen carriers.

Competing interests

The authors declare that they have no competing interests.

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