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# Insect larvae biofactories as a platform for influenza vaccine production

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ABSTRACT

Increased production capacity is one of the most important priorities for seasonal and pandemic influenza vaccines. In the present study, we used a baculovirus-insect larvae system (considered small, living biofactories) to improve the production of recombinant influenza virus H1N1 hemagglutinin (HA). Insect larvae produced four-fold more HA protein than insect cells per biomass unit (1 g of fresh larvae weight). A single infected *Trichoplusia ni* larva produced up to 113 μg of soluble and easily purified recombinant HA, an amount similar to that produced by $1.2 \times 10^8$ Sf21 insect cells infected by the same baculovirus. The use of the KDEL endoplasmic reticulum retention signal fused to the HA protein further increased recombinant protein production. Larvae-derived HA was immunogenically functional in vaccinated mice, inducing the generation of hemagglutination inhibition antibodies and a protective immune response against a lethal challenge with a highly virulent virus. The productivity, scalability and cost efficiency of small, living biofactories based on insect larvae suggest a broad-based strategy for the production of recombinant subunit vaccines against seasonal or pandemic influenza as an alternative to fermentation technologies.



## Introduction

Vaccines are central both to the effective control of seasonal influenza outbreaks and to pandemic preparedness. Embryonated hen's-egg technology for vaccine production has been used as the unique, licensed method of addressing seasonal and pandemic influenza since the 1950s [1]. This process takes place nearly year round, achieving infection of up to 500,000 embryonated hen's eggs per day (World Health Organization, www.who.int). However, producing the 300 million doses of influenza vaccine needed annually worldwide requires more than 350 million chicken eggs [1]. In addition, there are well-recognized disadvantages of using egg-based technology for influenza vaccine production. The process requires specialized manufacturing facilities, which could be difficult to scale up rapidly in response to seasonal and pandemic influenza situations. In addition, it is usually necessary to adapt candidate vaccine viruses for high-yield growth in eggs, a process that can be time consuming and that is not always successful; additionally, this process can select for receptor variants that may have suboptimal immunogenicity [2]. Furthermore, animal diseases may affect chicken flocks, which could be an important issue in a pandemic caused by an avian influenza virus strain because such an outbreak could disrupt the supply of eggs to vaccine manufacturers. Another notable disadvantage of these vaccines is that some individuals have egg allergies.

Big efforts have been undertaken in recent years to use alternative methods in order to obtain new virus sources for vaccine production. Through these efforts, some of the problems plaguing vaccine production have been solved using cultured mammalian cell lines, including MDCK and PER.C6 [3–5], in vaccine manufacturing. On the other hand, the development of recombinant hemagglutinin-based vaccines using the baculovirus-insect cell expression system has been previously explored [6–8] and carried out in phase I and phase II human clinical trials involving over 1200 subjects, which have demonstrated safety, immunogenicity and efficacy [8,9]. However, the production of bioreactor-based influenza vaccines requires specialized manufacturing facilities, may have higher costs than vaccines produced using hen's eggs, and may encounter difficulties in rapidly scaling up the process in response to a pandemic situation. The glycosylation pattern of HA varies depending on whether it is produced in eggs or in insect cells. However, this fact does not seem to interfere with the protective antibody response, suggesting that minor changes in glycosylation are not relevant to the immunogenicity and appropriate folding of the antigen [10].

Although new protein targets, such as M2, are being extensively studied as broad vaccines, hemagglutinin (HA) has been the key protective antigen in seasonal influenza vaccines for the last four decades because its structure and the basis of its efficacy are

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thoroughly understood. HA changes antigenically to evade the immune response, and on average, the prevalent influenza strains in circulation acquire three to four amino-acid changes to HA per year, mostly in the regions of HA that are recognized by protective antibodies. Mutations accumulate over time, and the virus evolves into an antigenically distinct strain approximately every three to five years [11]. This phenomenon requires that manufacturers regularly update their vaccine strains. Chimeric virus-like particles (VLPs), composed of the HA, NP and M1 proteins of the same or different serotypes [12–14], are good immunogens but need to be updated for the purpose of producing vaccines against new virus strains. Moreover, the purification of VLPs may introduce additional problems of antigen stability and contamination with baculovirus or baculovirus DNA.

The use of insect larvae as living biofactories for protein production has been explored as an alternative to cell-culture technology. A wide variety of recombinant proteins have been efficiently expressed in insect larvae using recombinant baculoviruses as vectors, including enzymes [15,16], antibodies [17,18], hormones [19,20], vaccines [21–25], cytokines [26–28] and diagnostic proteins [29–39]. Most of these antigens were processed correctly after synthesis, and their antigenic activities remained intact in soluble larval protein extracts. The advantages of using insect larvae as a recombinant protein production system include the dramatic reduction in production costs with respect to insect cell cultures, increased recombinant protein yields, the absence of high-tech fermentation procedures, reduced development times and easy production scale-up. These advantages make the use of insect larvae as biofactories a legitimate alternative to standard cell-culture fermentation systems.

In the present work, we evaluated the production of recombinant HA derived from the A/PR/8/34 influenza virus (H1N1) by various recombinant baculoviruses in both insect larvae and cultured cells. To increase the production of recombinant HA, we used two different techniques. First, the signal peptide from honey bee melittin was incorporated into the HA protein to facilitate efficient translocation of recombinant proteins into the endoplasmic reticulum in insect cells [40]. Second, we fused the reticulum endoplasmic retention signal sequence KDEL (Lys-Asp-Glu-Leu) to HA. This strategy has been previously used to increase soluble heterologous protein expression in plants [41], *Escherichia* coli [42,43], *Saccharomyces* [44] and also in the baculovirus expression vector system in insect cell cultures [45]. All versions of HA contained a 6X-His tag to facilitate protein detection and purification. Finally, we evaluated the immunogenicity and protective value of larvae-derived HA in mice.

## Material and methods

### *Influenza virus*

In this study, the mouse-adapted H1N1 A/PR/8/34 strain (kindly provided by Professor Juan Ortin, CNB, Madrid) was used. Full-length genomic RNA was obtained by infecting MDCK cells under biosafety level-2 conditions (BSL-2).

### *Recombinant baculoviruses*

The nucleotide sequence encoding the globular domains of HA (aa 18–529) from the A/PR/8/34 influenza virus was amplified from virus RNA using the PCR primers 5′GGATCCACATGTAATGGGGAAGGCAAACCTACTGGTCCTG and 5′TAGCTAGCAATCT GATAGATCCCCATTGA. The amplified sequence was cloned into the intermediate plasmid pGem-T (Promega, Madison, WI, USA) and sequenced. Next, the HA insert was subcloned into the following vectors: (A) a pFastBac plasmid containing the insect signal peptide sequence derived from the honey bee melittin (MKFLVNVALVFMVVYISYIYADPS) at its 5′ end and a 6X-His tag at its 3′ end; or (B) a pFastBac plasmid carrying a 6X-His tag and the endoplasmic reticulum retention amino-acid sequence KDEL at its 3′ end. The resulting plasmids were termed pFBMelHAhis and pFBHAhisKDEL, respectively (Fig. 1), and were used to generate the recombinant baculoviruses BacMelHAhis and BacHAhisKDEL with the Bac-to-Bac® baculovirus expression system (Invitrogen, USA), following the manufacturer's instructions. The baculovirus vector BacNI (a baculovirus with no foreign gene) was used as a control. Recombinant baculoviruses were propagated and amplified in Sf21 insect cells to reach infective titers of approximately $10^8$ pfu (plaque-forming units) $ml^{-1}$, and baculovirus stocks were stored at 4 °C for daily use and −80 °C for long-term storage.

### *Infection of insect larvae and Sf21 insect cells*

*Trichoplusia ni* (*T.* ni; Cabbage looper) larvae were reared following a previously described methodology [33,36]. For all experiments, fifth-instar larvae (last instar larvae before pupation), weighing each larva approximately 120–130 mg, were injected near the proleg (forward of the body cavity) with 5 μl of recombinant baculovirus diluted to reach the number of pfu per dose selected. To determine the optimal dose of recombinant baculoviruses for larvae infection, groups of 120 fifth-instar larvae were infected with $5 \times 10^4$, $5 \times 10^3$ or $5 \times 10^2$ pfu of the different baculoviruses and were processed at 60, 72, 84 or 96 h postinfection (hpi). The larvae collected at each time point were weighed to obtain the total biomass and were frozen immediately to be stored at −20 °C until they were processed for recombinant protein quantification. Insect cells (Sf21) were also infected *in vitro* with recombinant baculoviruses at an MOI of 5. Cells were harvested 72 hpi and were frozen until processing.

### *Analysis of protein extracts*

Total soluble, non-denatured proteins (TSNDPs) from frozen *T. ni* larvae infected by the baculoviruses were obtained by homogenization using a Bag Mixer® blender (www.interscience.fr) for 2 min. Extraction buffer was composed by PBS 1×, Triton X-100 at 0.01%, complete protease inhibitor cocktail (Roche, Germany), and DTT 25 mM. Total soluble protein (TSNDPs) from Sf21 baculovirus-infected cells was obtained using the same extraction buffer and doing two cycles of freezing with liquid nitrogen and thawing

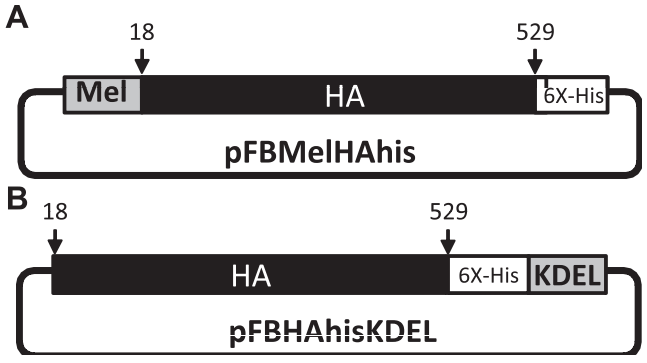


**Fig. 1.** Schematic structure of the pFastBac constructs used for generating baculoviruses expressing hemagglutinin ($HA_{18–529}$) from the H1N1 A/PR/8/34 virus. (A) Construct pFastBac, containing the $HA_{18–529}$ gene fused at the 5′ end with the signal peptide from honey bee melittin (Mel). (B) Construct pFastBac, containing the $HA_{18-529}$ gene fused at the 3′ end with the reticulum endoplasmic retention signal sequence KDEL. Both constructs contained a 6X-His-tag-encoding sequence to facilitate protein detection and purification.

at 37 °C. In both cases, the total concentration of extracted proteins was quantified by the Bradford method [46]. The TSNDPs obtained from identical insect biomasses infected with either MelHAhis or HAhisKDEL baculoviruses were denatured with 2× Laemmli buffer, and samples were resolved by SDS–PAGE.

For western blot (WB) assays, various quantities of TSNDP per lane were resolved by SDS–PAGE and transferred onto nitrocellulose membranes (Bio-Rad, USA). For protein size determination, BenchMark™ protein and BenchMark™ prestained protein ladders (Invitrogen, USA) were also included. Membranes were blocked overnight at 4 °C with PBS-0.05% Tween 20 (PBST) and 4% skim milk (blocking buffer, BF) and incubated at room temperature (RT) for 1 h using an anti-6X-His mAb (1:2000 in BF, Clontech, USA). Blots were then washed three times with PBST, and anti-mouse IgG-alkaline phosphatase (AP)-labeled conjugate (Bio-Rad, USA), diluted 1:3000 in BF, or anti-mouse IgG-horseradish peroxidase (HRP)-labeled conjugate (KPL, UK), diluted 1:4000 in BF, was added for 1 h as a secondary antibody. After extensive washing with PBST, protein bands were detected using the NBT/BCIP substrate (Roche, Germany) or an ECL western blotting detection system on Hyperfilm ECL films (Amersham, USA).

The quantification of soluble recombinant HA proteins expressed in *T. ni* larvae or Sf21 cells was carried out by measurement of band densitometry with the TINA v2.0 software package (v2.09a) and the ChemiDoc™ XRS Gel Imaging System (Biorad, USA). A 6X-His-tagged, purified protein standard (the 22.5 kDa Cap protein from the porcine circovirus type 2) was used. These analyses were performed in triplicate, and the standard deviation was calculated. Statistical differences were analyzed using Student's *t* test.

To detect recombinant HA protein glycosylation, 100 μg of TSNDPs per lane were resolved in a 10% SDS–PAGE gel. The gel was stained with a Glycopro Detection Kit (Sigma, USA), following the manufacturer's instructions. This kit enables the detection of protein bands containing at least 25–100 ng of carbohydrates through staining vicinal diol groups that are found mainly in peripheral sugars and sialic acids.

### *Recombinant HA protein purification*

Recombinant HAhisKDEL was purified from infected larvae using $Co^{2+}$-based immobilized metal affinity chromatography (IMAC) resins (TALON®, Clontech, USA). Approximately twenty larvae (approximately 5 g) were homogenized with 6.5 ml/g of biomass of binding buffer (10 mM imidazole, 20 mM sodium phosphate, 500 mM NaCl, 0.5% Triton X-100). Protein extracts were centrifuged for 15 min at 4000*g*. Supernatants were purified with a 22-μm filter (Miracloth, Calbiochem®, Merck, UK). Next, the samples were centrifuged for 15 min at 15,000*g*. Supernatants were then mixed and incubated with the pre-equilibrated TALON® resin. After an incubation of 2 h at 4 °C, the resin was washed twice with washing buffer (20 mM imidazole, 20 mM sodium phosphate, 50 mM NaCl). Finally, the resin was packed into a column, and elution was performed with 500 mM imidazole, collecting 1-ml fractions that were analyzed by a Bradford assay to detect the eluted protein. After dialysis, quantification of purified protein was carried out by SDS–PAGE and densitometry was measured with the TINA v2.0 software package (v2.09a) using the BSA standard curve as a comparison. In addition, purified HA was further quantified through gel-based electrophoresis in microfluidic chips using the Experion™ Automated Electrophoresis System (Bio-Rad, USA).

### *Specific antibody determination*

The HA-specific IgG response was evaluated by ELISA. Purified HAhisKDEL in 50 mM carbonate/bicarbonate buffer at pH 9.6 was used to coat ELISA microplates (Polysorp, Nunc, Denmark) overnight at 4 °C at a concentration of 500 ng/well. Plates were washed with PBS-Tween 20 (0.1%, PBST) four times and incubated for 1 h at 37 °C with constant agitation with blocking solution (PBST-2% BSA, 100 μl/well). Two-fold dilutions of serum samples, from 1:50 to 1:12,800, in blocking buffer were incubated for 1 h at 37 °C with agitation. Next, plates were washed again four times with PBST, and 100 μl/well of anti-mouse, IgG-HRP-conjugated monoclonal antibody (GE Healthcare, USA) diluted 1:2000 in blocking solution was added. For substrate reactions, plates were washed four times with PBST, and 100 μl/well of 1 mM 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, KPL, USA) was added to the plates. The peroxidase reaction was allowed to react for 15 min at room temperature to develop immunocomplexes, and the plates were read at 405 nm in an ELISA microplate reader (Multiskan EX, Thermo Electron Corp, USA). The cutoff threshold for discriminating negative and positive sera was set at two times the mean of the $OD_{405}$ derived from three negative control sera that were included in each assay.

### *Hemagglutination inhibition tests*

Hemagglutination inhibition tests were carried out for each duplicate, according to the World Organization for Animal Health (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.08_SWINE_INFLUENZA.pdf). Briefly, red blood cells (RBCs) were taken from a minimum of three SPF (specific pathogen-free) chickens and pooled in an equal volume of Alsever's solution. RBCs were washed three times in PBS before use as a 1% (packed cell v/v) suspension. One hemagglutination unit (HAU) was determined by mixing two-fold dilutions of A/PR/8/34 virus with 1% (v/v) RBCs. The hemagglutination inhibition test was carried out with four HAUs and using serum samples that were previously treated with receptor-destroying enzyme II (RDE, Denka Seiken Co., Ltd., Tokyo, Japan). Sera showing inhibition titers $\geqslant$1:40 were considered protective [47–50].

### *Mice immunization and virus challenge*

Female (6–8 weeks old) BALB/C mice were purchased from Harlan Laboratories (Barcelona, Spain). All immunization studies were performed under the guidelines of the European Community (86/609). Four mice were immunized with a TSNDP extract from infected larvae containing 19 μg of HAhisKDEL protein. A second group ($n = 4$) of mice were immunized with 10 μg of purified HAhisKDEL protein. A control group of mice ($n = 3$) were immunized with TSNDP extract from larvae infected with the control baculovirus BacNI (an identical amount was used for an experiment with non-purified HA protein), and a second control group ($n = 6$) was immunized with phosphate-buffered saline (PBS). Vaccine formulations were prepared in PBS with incomplete Freund's adjuvant. Mice received three vaccine doses through the intraperitoneal (i.p.) route on days 0, 14 and 28, and they were bled before each immunization and fifteen days after the last immunization. After the last bleeding, the mice were challenged intra-nasally with $4 \times LD_{50}$ of the highly virulent H1N1 A/PR/8/34 viral strain. Animals were monitored daily for fifteen days post-challenge for clinical symptoms and weight-loss measurements.

## Results

### *Improved baculovirus-based production of recombinant hemagglutinin through the use of insect larvae*

The ectodomain (aa 18–529) of hemagglutinin (HA0) from the H1N1 A/PR/8/34 influenza strain was expressed together with

either the signal peptide sequence derived from honey bee melittin or fused with the endoplasmic reticulum retention amino-acid sequence KDEL. Primary sequences were cloned into the baculovirus transfer vector pFastBac, and the plasmids were named pFBMelHAhis and pFBHAhisKDEL, respectively (Fig. 1). The plasmids were used to obtain two recombinant baculoviruses (BacMelHAhis and BacHAhisKDEL), as described in the methods section.

The titrated, recombinant baculoviruses were used to infect *T. ni* larvae to produce two versions of recombinant HA protein. Coomassie blue staining of infected larvae extracts indicated several major insect proteins with electrophoretic mobilities of 70–80 kDa, corresponding to the hexamerin family of proteins [51,52]. Below this band, another band was observed, corresponding to the recombinant HAhisKDEL protein (Fig. 2A). However, the HAhis induced by BacMelHAhis could not be easily observed (Fig. 2A). By contrast, both insect-derived versions of HA were detected by WB using an anti-PR/8 virus polyclonal antibody obtained from mice infected with a sub-lethal dose of virus (Fig. 2B). Differences in the intensities of the reacting bands were observed between the HA proteins induced by BacHAhisKDEL and BacMelHAhis recombinant baculoviruses. The HA protein fused to KDEL showed a stronger reactive band in soluble insect extracts than did the HA protein expressed with the melittin signal peptide (Fig. 2B).

To determine whether the HA proteins expressed in larvae were glycosylated, TSNDP fractions from *T. ni* larvae infected with BacMelHAhis and BacHAhisKDEL were resolved through SDS–PAGE and reacted with a Glycopro Detection Kit. A distinct stained band, corresponding to HA expressed by the recombinant baculovirus BacHAhisKDEL, was detected (Fig. 2C). A low-intensity band was detected in larval extracts producing the MelHAhis version of the HA protein, probably due to the lower expression level of this recombinant HA protein.

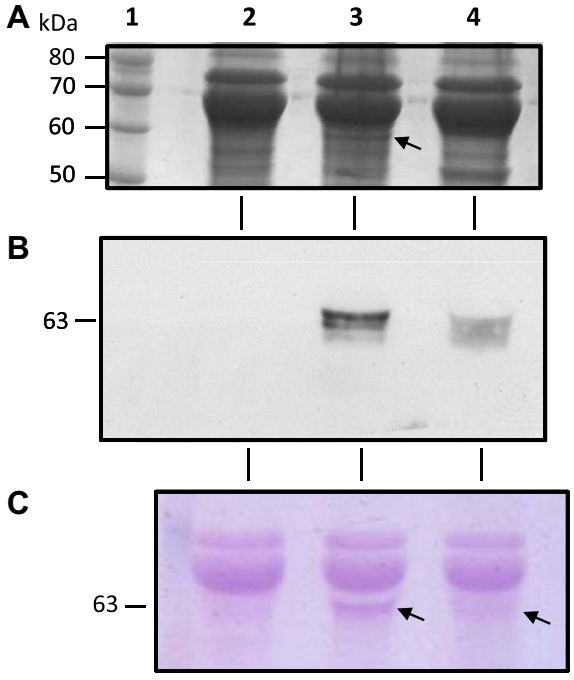


**Fig. 2.** Characterization of larvae-derived $HA_{18-529}$ protein isoforms by SDS–PAGE 10%, loading 80 μg of TSNDPs from infected larvae per lane. Lanes: (1) BenchMark™ protein ladder; (2) BacNI-infected larvae control extract; (3) BacHAhisKDEL-infected larvae extract; and (4) BacMelHAhis-infected larvae extract. (A) Coomassie blue staining of proteins resolved; (B) WB of various extracts using a polyclonal antibody (1:100 dilution) against the PR/8 virus; (C) Glycosylation pattern of the larvae-derived extracts. Larvae were infected using 5000 pfu per insect, and protein was recovered at 72 h post-infection.

To optimize recombinant HAhisKDEL protein production in larvae, we conducted experiments using various baculovirus infection doses (500, 5000 and 50,000 pfu per larva), and insects were harvested at 60, 72, 84 or 96 hpi. In these experiments, we determined both the HA production level and the insect biomass recovered (measured as larvae surviving after baculovirus infection and average weight of infected larvae). Larvae were processed, and TSNDPs were analyzed by SDS–PAGE and WB using anti-6X-His tag antibodies (see Section 2.4). Taking into consideration the recombinant protein productivity per infected larva, the insect biomass recovered and the virus used for infections, we established an optimal inoculation dose of $5 \times 10^4$ pfu per larva and 72 h for the duration of infection (Fig. 3). Subsequent experiments were performed under these conditions.

### *Comparison of HA protein production yields by recombinant baculoviruses in T. ni larvae and insect cell cultures*

To quantify the recombinant HA proteins expressed in larvae by the baculoviruses BacMelHAhis and BacHAhisKDEL, 40 μg of TSNDPs were analyzed via 10% SDS–PAGE and submitted to WB analysis using an anti-6X-His tag monoclonal antibody (Fig. 4A). Previously determined quantities of purified His-tagged protein measuring 22.5 KDa (Cap protein from porcine circovirus type 2) were used as protein standards. An analysis of reactive bands by densitometry indicated that 40 μg of TSNDPs contained at least 0.41 μg of HAhisKDEL and 0.21 μg of MelHAhis (1.03% and 0.52% of the TSNDP fraction from larvae, respectively) (Fig. 4B). These data translate into 451 μg of HAhisKDEL and 231 μg of MelHAhis per gram of insect biomass, respectively. Quantities corresponded to the arithmetic mean of three independent experiments of protein expression, with standard errors of 0.19 for HAhisKDEL and 0.10 for MelHAhis (Fig. 4B). These data indicate that we were able to duplicate the level of HA production with the HA protein fused to the KDEL retention signal.

To compare the HA productivity in larvae with that obtained in cell cultures, Sf21 insect cells were infected with each baculovirus at an MOI of 5. Cells were processed 72 hpi with the same protein-extraction buffer used to obtain TSNDPs from larvae, and 40 μg of the extracted soluble proteins were resolved through SDS–PAGE and analyzed by WB using an anti-6X-Histag monoclonal antibody (Fig. 4A). A similar analysis carried out with TSNDPs from larvae demonstrated HA accumulation levels in cells of approximately 0.035 and 0.105 μg in 40 μg of total soluble protein from cells infected by BacMelHAhis and BacHAhisKDEL, respectively (Fig. 4B). This result corresponds to 0.087% and 0.26% of the total soluble protein, respectively.

A similar analysis was carried out with the ChemiDoc™ XRS Gel Imaging System. Results by this methodology indicated 3.25-fold greater productivity in larvae than in Sf21 cells in the same quantity of total soluble protein extracts for the HAhisKDEL protein and 8.7 times greater production of the MelHAhis protein (Fig. 4C). This analytical technique also determined that the KDEL sequence increased HA production in Sf21 cells approximately fivefold (Fig. 4C). Although some differences were found using the aforementioned quantification methodologies, both techniques indicated that the larval expression system and the use of the KDEL sequence represents a clear improvement in recombinant HA production.

Table 1 summarizes the production percentages of the various versions of HA in larvae and Sf21 cells. In both production systems, the KDEL sequence increased HA productivity (by 198% in larvae and by 298% in Sf21 cells compared with the same HA sequence without the KDEL retention signal). The comparison of HA productivity in larvae vs. cells suggested that insects can increase HA productivity by approximately 400% and 600% compared with Sf21

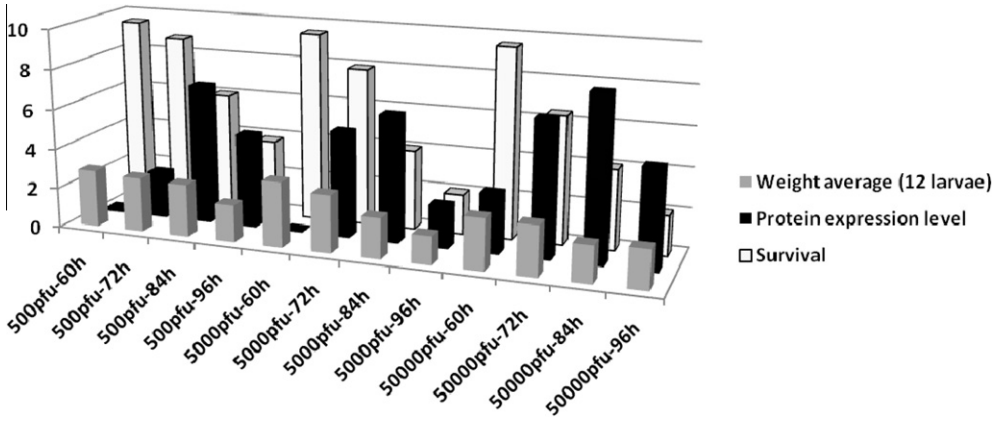


**Fig. 3.** Optimization of HA protein (HAhisKDEL) production in *T. ni* larvae. This figure shows the three different parameters studied: the weight average of infected larvae ($n$ = 12), larval survival after infection with various virus doses (500, 5000, 50,000 pfu) and protein expression levels determined by ELISA (OD × 1000). Larvae were analyzed at the post-infection time points of 60, 72, 84 and 96 h.

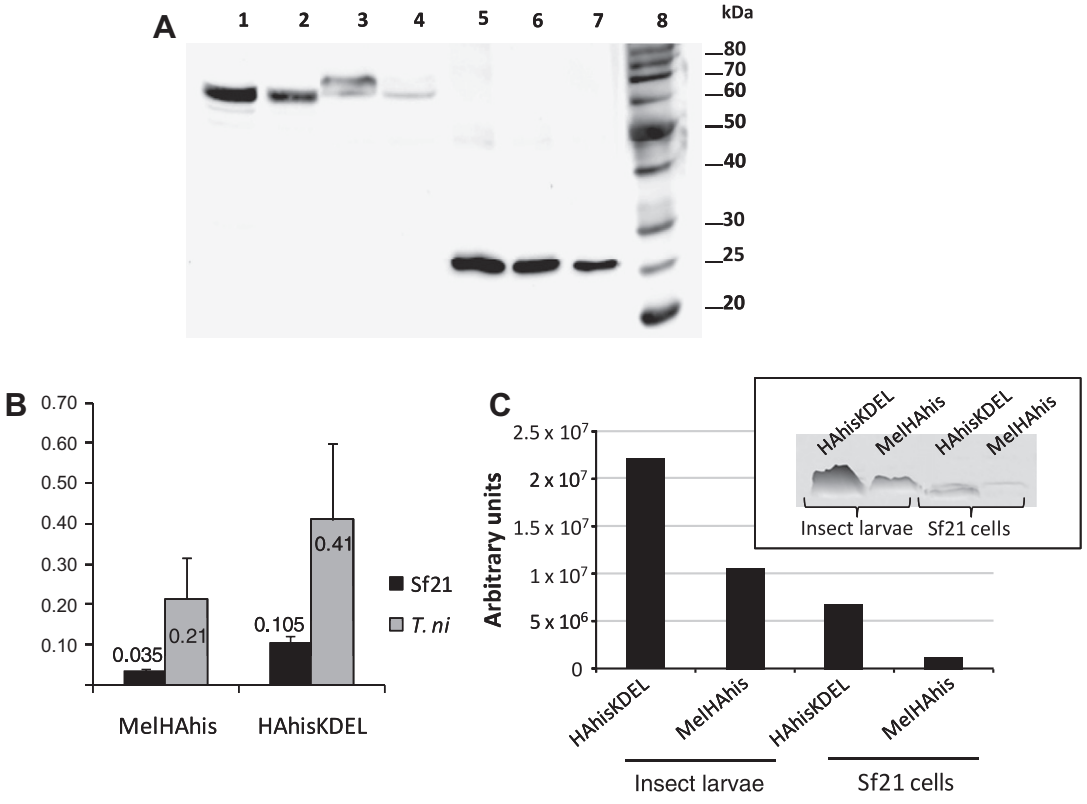


**Fig. 4.** Quantification of the expression of recombinant HAhisKDEL and MelHAhis proteins produced in *T. ni* larvae and Sf21 cells. Panel A: representative WB, using an anti-6X-His tag antibody, of 40 μg TSNDPs from infected larvae or cells resolved by SDS–PAGE. (1) TSNDPs from larvae extract containing the HAhisKDEL recombinant protein; (2) TSNDPs from larvae extract containing the MelHAhis recombinant protein; (3) TSNDPs from Sf21 cells extract containing the HAhiskDEL recombinant protein; (4) TSNDPs from Sf21 cells extract containing the MelHAhis recombinant protein; (5), (6) and (7) Various amounts (0.46, 0.23 and 0.12 μg, respectively) of purified porcine circovirus His-tagged Cap control protein; (8) BenchMark™ protein ladder. Panel B: representation of the quantification of HAhisKDEL and MelHAhis recombinant protein expression in larvae and Sf21 cells, as determined by densitometry of reacting bands in various WBs with the anti-6X-His tag antibody. Results are expressed as μg of HA/40 μg of TSNDPs. Panel C: comparison of HAhisKDEL expression in insect larvae and Sf21 cells by WB using the anti-6X-His tag antibody in a ChemiDoc™ XRS Gel Imaging System (Bio-Rad, USA).

cells for HAhisKDEL and MelHAhis proteins, respectively, per biomass unit (1 g of fresh larvae). Considering the production of MelHAhis by a recombinant baculovirus in cells as the baseline of HA productivity, we were able to increase HA productivity by nearly 1184% by combining the use of the KDEL sequence and the insect larvae production platform. Almost all results for comparisons of HA productivities were statistically significant (see Table 1).

Finally, the larvae-derived HA protein (HAhisKDEL) was purified by His-tag affinity chromatography from TSNDP larvae extracts. Two major bands of similar molecular weights and several minor bands of higher electrophoretic mobilities, probably corresponding to degradation products, were detected by Coomassie blue staining (Fig. 5A). The resulting purified protein from a single purification step was analyzed by microfluidic chips using the Experion™ Auto-

**Table 1**
Summary of recombinant expression data obtained from insect larvae and Sf21 cells using the MelHAhis and HAhisKDEL constructs.

| | MelHAhis | HAhisKDEL | $\Delta_a$ |
|---|---|---|---|
| Sf21 | 0.087 | 0.26 | 298[c] |
| *T. ni* | 0.52 | 1.03 | 198[c] |
| $\Delta_b$ | 597[c] | 396 | |

The numbers represent the percentages of total soluble protein that correspond to recombinant HA.
$\Delta_a$ – Increased percentage of HA production between HAhisKDEL/MelHAhis intra-Sf21 and intra-*T. ni* groups.
$\Delta_b$ – Increased percentage of HA production between *T. ni*/Sf21 using MelHAhis and HAhisKDEL constructs.
[c] Denotes values defined as statistically significant ($p \leqslant 0.05$) through Student's *t* test.

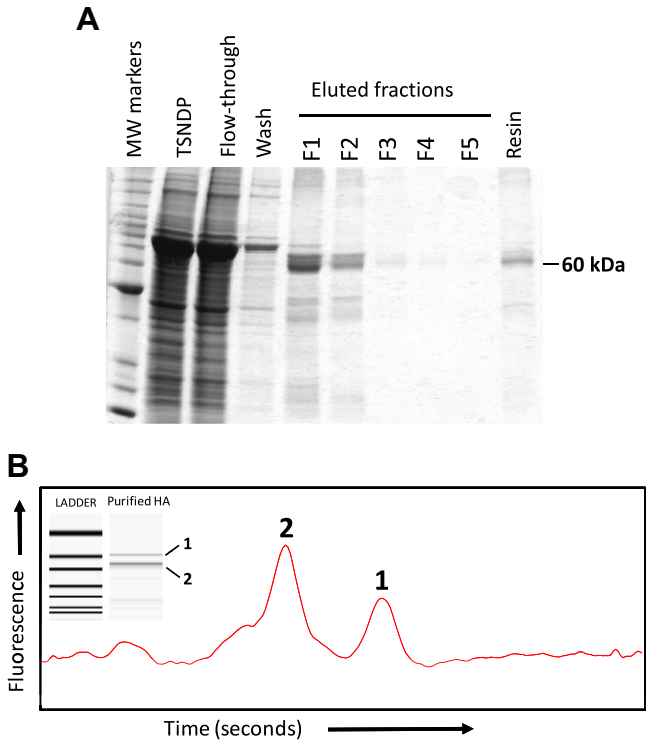


**Fig. 5.** Protein profiles of the HAhisKDEL purification process from TSNDPs extracted from larvae using cobalt IMAC affinity chromatography. (A) Analysis by SDS–PAGE and Coomassie blue staining of the various fractions obtained through the purification process. (B) Analysis of F1 fraction, shown in panel A, by gel-based electrophoresis in microfluidic chips using Experion™ (Automated Electrophoresis System).

mated Electrophoresis System (Fig. 5B). More than 72.5% of the purified HA protein had the expected electrophoretic mobility (two major bands), indicating a low level of protein degradation following harvest from larvae. The results indicate that it is possible to purify approximately 2 mg of HA from 5 g of insect biomass.

*Immune response and protection against influenza in mice immunized with larvae-derived HA*

To determine whether the HA produced in larvae is processed adequately to generate functional and protective antibodies in immunized mice, various groups of animals were vaccinated with TSNDP extracts from larvae or purified HA. HA-specific humoral responses in mice were determined by HA-based ELISA (Fig. 6). All animals that were immunized with crude preparations ($n = 4$) developed specific antibodies after the first antigen dose (Fig. 6A). At day 51 post-immunization, all vaccinated animals belonging to this group reached antibody titers between 1/6400 and 1/12,800 (Fig. 6B). Sera from this group of mice were analyzed for their ability to inhibit the hemagglutination induced by the virus and indicated inhibition at titers higher than a 1:64 dilution (Fig. 6B), which is considered protective against influenza disease. Immunized mice were analyzed to investigate whether the larvae-derived HA induced long-lasting antibody immune responses by measuring specific IgG levels after more than 365 days. ELISA results indicated only a two-fold reduction in antibody titers at one year after the last immunization dose (data not shown).

A second group of mice ($n = 4$) that were immunized with the purified, larvae-derived HA formulation also developed specific antibodies early after immunization (Fig. 6C), presenting high antibody titers after the second dose (the antibodies elicited by only one dose were not determined in this group). After three immunization doses with the purified protein, the antibody titers exhibited by all animals were between 1/50,000 and 1/100,000 (Fig. 6D). All control mice that were immunized with extracts from BacNI-infected larvae possessed no specific cross-reactive immune responses against the HA protein by ELISA (data not shown).

To evaluate the *in vivo* protection of larvae-derived HA, mice immunized with the purified HAhisKDEL protein were challenged with a lethal dose of the A/PR/8/34 virus ($4 \times LD_{50}$) fifteen days after the last immunization dose. All of the mice survived with no significant weight loss. By contrast, a control group that was mock-immunized with PBS ($n = 6$) and challenged with the same dose of virus presented clinical symptoms of influenza and died or were euthanized between 7 and 10 days post-infection (Fig. 7).

## Discussion

Traditional, egg-based vaccines have been successfully utilized for more than 50 years to prevent influenza. These vaccines are reliable, effective, and affordable. However, the production cycle of egg-based vaccines is lengthy, heavily dependent on egg supply and unable to respond quickly to sudden demands, as occurs in an influenza pandemic [6]. To replace or supplement egg-based vaccines, alternative vaccines must be equally effective, reliable, economical and capable of being developed and delivered quickly.

It has been extensively demonstrated that the use of insect larvae as living biofactories is a suitable method for the inexpensive production of recombinant antigens and subunit vaccines, generically termed "insectigens" [23–25,33,36,39,53]. With respect to cells cultured in fermentators, insects have many advantages related to the costs of goods, manipulation, amount of infection inoculum required for infection and the infrastructure needed to produce recombinant proteins. In this work, we have demonstrated that the *T. ni* larvae expression system, in combination with the AcNPV baculovirus vector, constitute an efficient, readily scalable alternative to conventional baculovirus-based methods for the production of recombinant HA proteins for use in future vaccine formulations.

In the present work, we expressed the HA protein (specifically, the globular domain, $HA0_{18-529}$) from the H1N1 A/PR/8/34 virus in insects through two different approaches. One method consisted of the use of the signal peptide of honey bee melittin (MelHAhis), which can enable efficient translocation of proteins into the endoplasmic reticulum and could increase the amount of soluble protein in the cytosol [40]. In the other approach, HA was fused to the endoplasmic reticulum retention signal KDEL (HAhisKDEL), which, by inducing retention in the endoplasmic reticulum, could reduce degradation, increasing the quantity of intact HA that accumulates in cells. In both cases, a 6X-His tag was added to facilitate the detection and purification of recombinant HA.

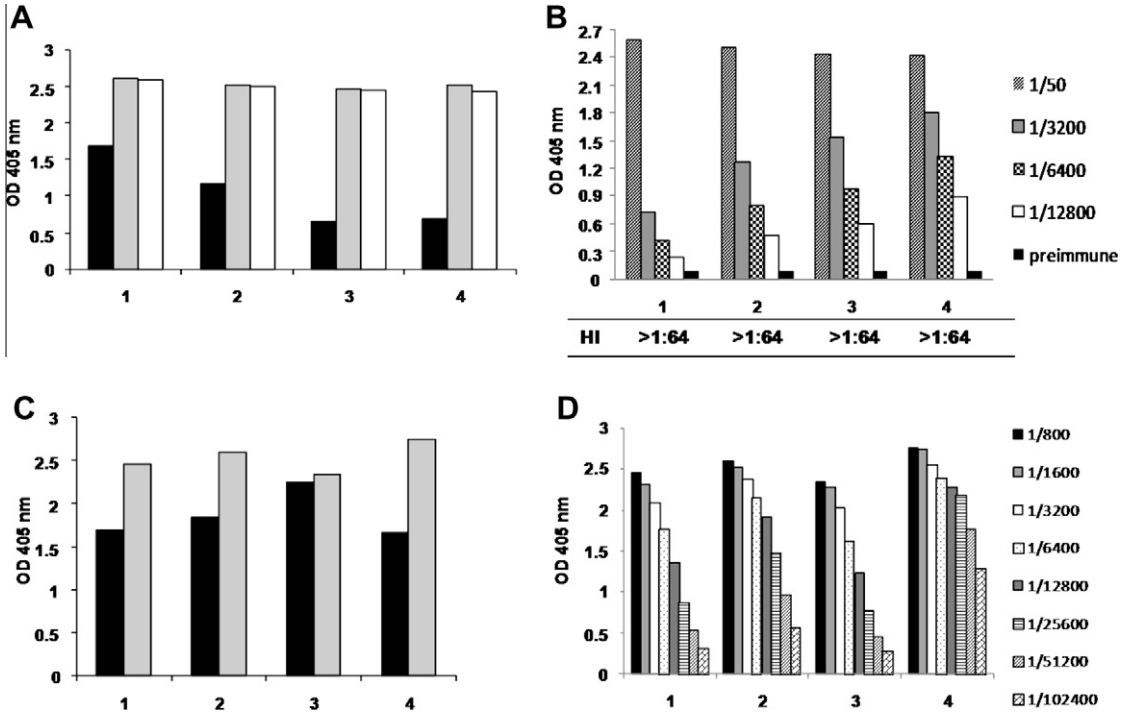


**Fig. 6.** IgG antibody immune response in mice immunized with TSNDPs extracted from larvae containing the HAhisKDEL protein (19 μg/dose) or purified HAhisKDEL protein (10 μg/dose). Specific antibodies were measured by ELISA. Panel A: HA-specific IgG antibodies detected in sera from mice immunized with crude preparations (sera tested at 1:50 dilution) 15 days after the first immunization dose (black bars), 15 days after the second immunization dose (grey bars) and 15 days after the third immunization dose (white bars). Panel B: HA-specific antibody titers in sera from mice ($n = 4$) 51 days after the first immunization dose with TSNDPs extracted from larvae containing HAhisKDEL protein. The titers obtained for each serum in the hemagglutination inhibition (HI) test are also detailed in this panel. Panel C: HA-specific IgG antibodies detected in sera from immunized mice with the purified HAhisKDEL protein (sera tested at 1:800 dilution) 15 days after the second immunization dose (black bars) and 15 days after the third immunization dose (gray bars). Panel D: HA-specific antibody titers in sera from mice ($n = 4$) 45 days after the first immunization dose with purified, larvae-derived HAhisKDEL protein.

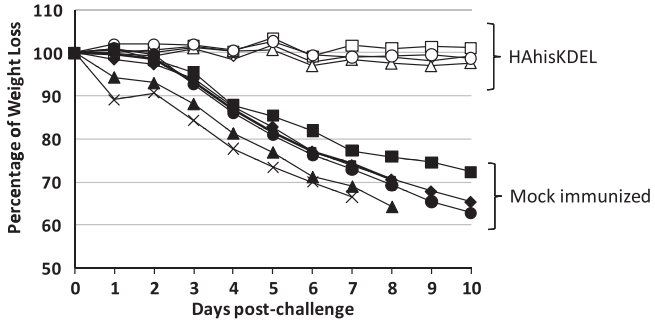


**Fig. 7.** Protective immune response against a virulent H1N1 influenza virus in mice immunized with purified HAhisKDEL protein. Control, mock-immunized mice ($n = 6$; black symbols) and purified, larvae-derived HA-immunized mice ($n = 4$; white symbols) were challenged intranasally with $4 \times LD_{50}$ of the H1N1 A/PR/8/34/1 virus strain. Mice were monitored individually for 15 days after the challenge, analyzing clinical symptoms of influenza disease. The figure indicates the weight loss observed in both groups of immunized mice until all control mice died or were euthanized (10 days). All mice immunized with larvae-derived HA were fully protected and did not suffer any weight loss.

We demonstrated that the use of KDEL mediated greater recombinant HA accumulation than did the Mel signal peptide (198% in larva and 298% in Sf21 cells). These differences in expression levels were statistically significant. Additionally, in the baculovirus-specific infection conditions used in this work, we have shown that *T. ni* larvae generated between 400% and 600% more HA than Sf21 cells per biomass unit. The KDEL retention signal has been shown to increase recombinant protein production in different expression systems such as plants, bacteria, yeast or insect cells [41–45]. However, this is the first time to be used in insects as living biofactories.

The KDEL retention signal has also been used for properly protein folding, enzymatically active, human telomerase (hTERT). The recombinant hTERT-KDEL protein was directed to the endoplasmic reticulum (ER), which is rich in chaperones. This increases the expression of soluble recombinant hTERT, promoting proper folding using intrinsic ER chaperone proteins [45].

Although non-Sf21 insect cells, such as High 5 cells, may express higher quantities of HA [7,54], *T. ni* insect larvae represent a more efficient alternative for producing this protein. Taking into consideration only the HA protein recovered from the soluble fraction of insect extracts, we obtained approximately 451 μg of HAhisKDEL from 1 g of insect biomass. To obtain the same quantity of this protein in cultured Sf21 cells, we would need approximately 3.96 g of cells (approximately $4.8 \times 10^8$ cells). Previous expression studies of HA from the A/New Caledonia (H1N1) virus have been conducted in bioreactors of 15 l by infecting Sf21 cells, giving rise to 12 mg of recombinant HA after extraction of soluble protein from 6.4 g of cellular pellet [6]. This result indicates that 1.87 mg of non-purified HA is present per gram of cellular pellet. With the protein extraction method described in this work, we obtained, as previously mentioned, 0.451 mg of soluble HAhisKDEL per gram of fresh larvae. Therefore, using the production method described here, 26.61 g of larval biomass (approximately 106 larvae) are comparable in productivity to a 15-l bioreactor. Further studies to improve HA protein extraction from larvae should be undertaken because a portion of the HA produced in larvae was not efficiently solubilized by the extraction method used in this work (data not shown). Although higher expression levels in cultured insect cells have been reported [7], the HA versions expressed in that

study corresponded only to one globular domain of the protein (HA1) with a lower molecular weight. In this study, we expressed a more complex protein (the $HA0_{18-529}$ version of the protein), which contains more antigenic epitopes and regularly presents more problems for its expression as a recombinant protein, as described previously [7,55,56].

Other groups have explored the use of insect larvae (silkworm and *Heliothis virescens*) as living factories to produce the influenza HA protein. An HA derived from an equine influenza was expressed in silkworms by a recombinant silkworm baculovirus but was recovered in quantities as low as 0.4–4 μg/larva [57]. Another HA molecule from an H7N1 virus was expressed in *Heliothis virescens*, with production yields of 0.3 mg HA/100 mg of total soluble proteins from larvae [21]. In our hands, *T. ni* larvae expressed greater quantities of recombinant HA protein per biomass unit (1.03 mg HAhisKDEL/100 mg of total soluble protein).

To our knowledge, there are not comparative studies of recombinant production yields obtained after baculovirus infection of larvae per os vs. injection. It is possible to use pre-occluded virus or polyhedrin positive virus to infect larvae, but it is difficult to establish a system in which all larvae receive the same virus dose. In contrast, by injection we may administrate exactly the same virus dose in each insect. It has two main advantages, the yield of production reproducibility among infected larvae, and the time after infection at which larvae show maximum recombinant expression levels (approximately 48 earlier than when infected per os). Although inoculation of larvae by injection has to be done one by one, it takes only a few seconds, less time than inoculation of embryonated eggs, the classical system to produce influenza vaccines, and the number of potential vaccines doses that could be obtained in a single larva is much higher than the single dose which is obtained from an inoculated egg.

In the present work, the functionality and immunogenicity of larvae-derived HA has been evaluated. The HA protein was glycosylated in larvae, and non-purified HA elicited potent humoral immune responses with specific anti-HA antibody titers that were, in some immunized animals, higher than 1/12,800 after three immunization doses. Antibody titers in immunized mice remained very high for at least one year after the last immunization dose. Additionally, the larvae-derived HA induced antibodies presenting hemagglutination inhibition titers of at least 1:64 in mice, which is considered sufficient for protection against influenza infection [47–50].

An aspect regarding protein expression in insects is a described modification of the core glycan on N-glycosylated proteins and sometimes results in addition of a fucose in an alpha-1,3-linkage that can provoke an allergic response in mammals (ref reviewed in Harrison & Jarvis, Advances in Virus Research 68: 159–191, 2006). This work is a first attempt to develop technology that increase the production of the hemagglutinin protein, which is considered a difficult to express protein, at least at the levels required to be used in global vaccination campaigns. Mice vaccinated with crude larvae antigens or purified larva-derived HA exhibited no adverse side effects, indicating that, even after several vaccination doses with non-purified antigens, no anaphylactic reaction was produced in immunized animals. To be used in humans the larva-derived HA, the level of purification required will be very high and second rounds of purification will be required. Further experiments in different animal species as well as extensive characterization of the larvae-derived HA have to be done to ascertain its safety. However, our results suggest that a crude extract from larvae bearing the HA protein could be used to vaccinate farm animals as a low-cost vaccine formulation.

To demonstrate the protective effect of larvae-derived HA against influenza in mice, purified HAhisKDEL protein was used to immunize mice, inducing full protection against a lethal dose of PR/8 virulent virus challenge in all vaccinated animals. With the dose used in this vaccination experiment (10 μg/dose), we obtained approximately 40 vaccine doses from 1 g of insect biomass (approximately four infected larvae). Further studies to increase the efficiency of HA recovery from infected insects could increase the number of vaccine doses obtained because the total amount of HA expressed could reach more than 3 mg/g of insect biomass (data not shown).

We have found that *T. ni* larvae, in combination with the baculovirus vector AcNPV, could represent one of the most efficient systems for producing recombinant HA-based vaccines in a cost-effective manner. The low production costs of insect biomass and the easy production scale-up could facilitate the supply of all vaccine doses required for seasonal or pandemic situations. In 9 weeks, it is possible to generate more than 7 tons of insect biomass from a single female butterfly [25]. The larvae-derived HA protein was easily purified with no problems beyond those of the insect cell-derived HA protein. Additionally, larvae-derived products are relatively safe in terms of contamination with adventitious agents because this expression system uses no animal compounds (i.e., animal sera) in the production process.

In conclusion, the use of *T. ni* larvae in an improved system for recombinant HA production may be an important step toward meeting seasonal and pandemic demands with minimal investment in manufacturing infrastructure, especially when addressing the demands of developing countries, for which a vaccine's cost is critical to its application.

## Competing interests

The authors have no competing interests to declare.

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