

## Original Research Article

### **Rational Design of a Multi-Epitope Vaccine for Yak Milk Allergy by Immuno-informatics approach**

#### **ABSTRACT**

Milk is a wholesome nutrient source, but one of the problems associated with milk consumption is the allergy observed in humans due to major milk proteins. Yak is one of the milch animal which is well adapted to high altitude and serve as source of nutritious milk for inhabiting human population. However, yak and cow milk are similar in composition, and are known to elicit hyperimmune reaction especially in infants and children. The several therapeutic attempts have already been made to counter cow's milk allergic response but their response is temporary in nature. So, the following study is an attempt to overcome Yak's milk allergic response by *in-silico* multi-epitope vaccine construction. For this, the suitable epitopes and adjuvants were coupled for increased vaccine efficacy and immune stimulation. The vaccine construct was found to be antigenic and free from allergens and toxins. As per computational modelling, the proposed multiepitope vaccine is physically stable and may induce immune responses in allergic conditions.

**Keywords:** *Yak milk allergy, Multi-epitope, Vaccine, in-silico*

#### **1. INTRODUCTION**

India is the major milk producer in the world, with 230.58 million tonnes (MT) produced in 2022-2023, making the dairy industry a significant contributor to global nutrition (BAHS, 2023). Cattle produce the majority of milk worldwide, with the remainder coming from buffaloes, goats and sheep, followed by camels, horses, donkeys, Mithun and yak have a minor dairy contribution (BAHS, 2023). The yak (*Bos grunniens*) is a long-haired mammal from the Bovidae family. They are one of the world's few domestic animals with an extremely limited geographical distribution (Yang *et al.*, 2023). Furthermore, Yaks are well adapted to high altitude living, with thick fur to protect against cold and low-oxygen environments. Arunachal Pradesh, Sikkim, Uttar Pradesh, Himachal Pradesh, and Jammu and Kashmir are the Indian states that raise yaks. According to the most recent livestock census in 2019, India has 57570 yaks, and undergone a decrease of 25% from the previous livestock census in 2012 (BAHS, 2023). The decline in the yak population could be attributed to lower remuneration, as younger generations are hesitant to continue nomadic yak rearing. The Food Safety and Standard Authority of India (FSSAI) recently approved the Himalayan Yak as a 'food animal' in November 2022. In addition to meat, yaks are also used for their milk, which is high in fat and protein, and their wool, which is used to make clothing and other textiles (Freeman and Bradley, 2007). Yak milk is unique in that it contains a high concentration of nutrients, making it a valuable raw material for producing meals for newborns, the elderly, and specific groups of people (Park and Haenlein, 2006). Aside from its nutritional value, yak milk and its products may provide functional benefits such as immune support, inflammation reduction, and improved heart health (Guo *et al.*, 2014). In comparison to dairy

cows, the average daily milk yield of yaks is 1.5 kg after calving, and in the lactation period, yaks produce 150 to 500 kg. The milk-producing ability of yaks depends on breed, age, parity, and body condition; pasture growth; pasture quality; raising areas; milking time; milking methods; and other environmental factors (Dong *et al.*, 2007).

Cow's milk allergy (CMA) or cow's milk intolerance (CMI) is a growing public health concern across the globe, with an increase in prevalence attributed to environmental and genetic factors. CMA is one of the most common food allergies in the developed world, affecting up to 2-7% of children during their early childhood. CMA are divided into two categories: IgE-mediated reactions (usually occur within minutes of exposure) and non-IgE-mediated reactions (occur hours after ingestion), sometimes mixed (IgE and non-IgE mediated) form also observed in few individuals (Dhesi *et al.*, 2020). There are nearly 20 different protein fractions found in cow milk; out of them, casein protein ( $\alpha$ s1,  $\alpha$ s2,  $\beta$  and  $\kappa$  casein) and whey protein (alpha-lactalbumin and beta-lactoglobulin) are the main allergens (Wood *et al.*, 2013). Yak milk proteins, like most other mammalian milks, are primarily composed of four individual caseins (alpha-s1-, alpha-s2-, beta-, and kappa-casein) and the major whey proteins (alpha-lactalbumin, beta-lactoglobulin, serum albumin, lactoferrin, and immunoglobulins) (Sheng *et al.*, 2008). The total casein concentration in yak milk (40.2 g/L on average) is 1.5 times that of cow milk and 11 times that of human milk (Malacarne *et al.*, 2002; Li *et al.*, 2010).

Urticaria, angioedema (usually of the eyes and lips), and gastrointestinal symptoms (diarrhoea and vomiting) can all be symptoms of IgE-mediated reactions. In cases of non-IgE-mediated CMA, gastro-esophageal reflux disease (GORD), altered bowel habits, atopic eczema, chronic constipation, and colic are observable signs (Dhesi *et al.*, 2020). In light of advances in immunoinformatics, a new discipline for designing multi-epitope vaccines has recently emerged. This discipline has allowed us to gain a better understanding of the host immune response, which has significantly speed up vaccine development. If we are looking for a long-term cure for milk allergies, then there is need to aim for developing breeds that produce allergy-free milk by identifying advantageous polymorphisms in immunogenicity-related genes. Immunotherapy, a non-vaccine treatment, employs interferons, cytokines such as Granulocyte-macrophage colony-stimulating factor, and other growth factors to alter the immune response (Dalglish *et al.*, 2015). Immunotherapy, which is typically a passive method of managing food allergies, can thus be transformed into vaccination, an active method. Immunotherapy vis-a-vis vaccination forms a two-pronged approach for tackling the issue of allergy due to milk consumption. Additionally, prioritising proteins to find B and T cell epitopes is also required for the development of vaccines that are directed either against a pathogen that causes disease or against a food allergy. This study encompasses to identify epitopes and incorporate them into the *in-silico* vaccine development.

## **2. METHODS**

### **2.1 Retrieval of protein sequences from data bank**

Total five milk protein sequence viz.  $\alpha$ -lactalbumin (Uniprot Id: Q9TSR4),  $\alpha$ -S1-casein (Uniprot Id: A0A344X7B7),  $\alpha$ -S2-casein (Uniprot Id: A0A344X7B8),  $\beta$ -casein (Uniprot Id: A0A6B0RXU8) and  $\kappa$ -casein (Uniprot Id: L8IIT8) retrieved from Uniprot data bank (<https://www.uniprot.org/uniprotkb>) then saved in FASTA format for further antigenic properties analysis. For analysis of the protein's antigenicity, we use VaxiJen 2.0 (<http://ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>), a popular server capable of calculating antigenic proteins with over 80% accuracy (Doytchinova and Flower, 2007). The VaxiJen results for the top antigenic proteins were then selected for further analysis.

**Comment [H1]:** Access date?

**Comment [H2]:** What were the adjusted condition for analysis on VaxiJen? I am mention selected organism, threshold level etc. I don't think VaxiJen is suitable for Milk protein analyses as it is designed for viruses, bacteria and tumor proteins.

**Comment [H3]:** Access date?  
Please add access dates to all the mentioned tools

## 2.2 Physicochemical characterization and allergenicity of the selected proteins

ProtParam, a popular tool on the Expasyserver (<http://exp.asy.org/cgi-bin/protpraram>), was used to determine the functional physicochemical parameters of the chosen antigenic protein (Wilkins *et al.*, 1999). Protein allergenicity was assessed using AllerTOPv2.0 (Dimitrov *et al.*, 2014).

## 2.3 Cytotoxic T cell (CTL) epitopes prediction

NetCTL 1.2 (<http://www.cbs.dtu.dk/services/NetCTL/>) was used to identify CTL epitopes from the selected milk protein (Larsen *et al.*, 2007).

**Comment [H4]:** Access date?

## 2.4 Helper T cell epitopes (HTL) prediction

The milk protein's HTL were identified using the IEDB webserver (<http://tools.iedb.org/mhcii/>), a well-known MHC II binding prediction tool.

**Comment [H5]:** Access date?

## 2.5 Prediction of B cell epitopes

Linear B cell lymphocyte (BCL) epitopes of the selected milk protein were identified from the ABCpred web server (<http://ailab.ist.psu.edu/ABCpred/>).

**Comment [H6]:** Access date? And adjusted conditions?

## 2.6 Multi-epitope vaccine construction, structural properties and refinement

The criteria for selecting epitopes for the multi-epitope vaccine construction for milk protein allergy were: (a) promiscuousness, (b) overlapping CTL and HTL epitopes, (c) immunogenicity, (d) population coverage, (e) high affinity towards HLA alleles (predicted by docking analysis), and (f) no overlap with any human gene (to prevent autoimmunity). To construct a multiprotein, multi-epitope vaccine (MPVC), vaccine sequence was created by selecting high-scoring CTLs, HTLs with high affinity, and B-cell epitopes. The epitopes were linked using three linkers: CTL (AAY), HTL (GPGPG), and B epitope (KK). The vaccine design included four adjuvants via linkers: defensin, universal memory T-cell helper peptide (TpD), Pan HLA-DR reactive epitope (PADRE), and an M-cell ligand. To increase immunogenicity, defensin was added to the N terminal, M-cell ligand to the C terminal, and HHHHHH to facilitate vaccine purification in future studies.

**Comment [H7]:** Only the immunogenicity and docking tools are mentioned in the following methodology, where are tools/servers for population coverage analysis, HLA alleles affinity, Homology analysis with human gene promiscuousness, CTL and HTL overlapping analysis?

**Comment [H8]:** Add the UniPort/PDB/NCBI ID of the adjuvant

## 2.7 Antigenicity, Allergenicity and Physio-chemical assessment of vaccine candidate

We prioritized identifying non-allergenic, and highly antigenic epitopes for use as vaccine candidates in humans. Thus, the VaxiJen (<http://www.ddg-pharmfac.net/vaxijen/>), AllerTOPv.2.0 (<https://www.ddg-pharmfac.net/AllerTOP/>) and ProtParam tool (<http://exp.asy.org/cgi-bin/protpraram>) of the ExPASy were respectively used for antigenicity,

**Comment [H9]:** Access date?

allergenicity and physio-chemical assessment of constructed vaccine. Only nontoxic and non-allergenic epitopes were filtered based on assessment scores in the mentioned web-servers.

## 2.8 Structure prediction, validation, visualization, and analysis

The vaccine construct's tertiary structure was predicted using the Phyre2 server, while the secondary structure was predicted using the PSIPred 4.0 Protein Sequence Analysis Workbench (Geourjon and Deleage, 1995).

## 2.9 Molecular docking

For an effective immune response, vaccines must interact with the host's immunological receptors. The ClusPro 2.0 (<https://cluspro.bu.edu/login.php>) server was used for protein-protein docking. We used that server to predict the interaction of multi-epitope vaccine with immune receptors, TLR2 and TLR4.

## 2.10 Immune response simulation

The vaccine's sequence was submitted to C-ImmSim (<https://kraken.iac.rm.cnr.it/C-IMMSIM/>) for immune response analysis. C-ImmSim models a mammalian immune system's response to vaccines, both humoral and cellular (Rapinet *et al.*, 2010; Dey *et al.*, 2022).

## 3. Results and Discussion

### 3.1 Physicochemical Properties of Proteins used for Vaccine Construction

Among all major proteins found in Yak milk, Alpha-s2-caesin has largest molecular weight while Alpha lactalbumin has lowest molecular weight. Alpha lactalbumin was the only protein found to be stable and additionally all proteins are found to be allergen when predicted by AllerTOPv2.0 webtool. Beta casein, Alpha – s1-caesin and Alpha-s2-caesin was found to possess antigenicity and crossed threshold of 0.4 set by VaxiJen server.

**Table 1: Physicochemical properties of proteins used for vaccine designing**

	Alpha lactalbumin	Beta caesin	Kappa caesin	Alpha – s1-caesin	Alpha-s2-caesin
<b>Number of amino acids</b>	142	224	194	214	222
<b>Molecular weight (KDa)</b>	16247.59	25107.33	21609.73	24465.84	25989.65
<b>Asp + Glu</b>	21	23	16	31	28
<b>Arg + Lys</b>	13	16	15	20	30
<b>Instability index</b>	stable	unstable	unstable	unstable	unstable
<b>Aliphatic index</b>	91.27	97.37	80.98	85.19	75.05
<b>Grand av. of hydrophobicity (GRAVY)</b>	-0.169	-0.154	-0.275	-0.464	-0.668
<b>Allergy</b>	allergen	allergen	allergen	allergen	Allergen

**Comment [H10]:** If your designing vaccine for allergic reaction please add the Toxicity evaluation of the epitopes using ToxinPred or any other tool. In last sentence you have mentioned only non-toxic and non-allergenic epitopes were selected but you haven't listed the toxicity prediction tool.

**Comment [H11]:** Mentioned two tools but only one reference? Please check the citations

**Comment [H12]:** Please mention the reasons to use these receptors?

**Comment [H13]:** Why you set 0.4 threshold as far I know this level is used for viral proteins, please mentioned the citation/article reference from which you are using this threshold for milk proteins

Antigenicity	Non-antigen	Probable antigen	Non-antigen	Probable antigen	Probable Antigen
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**Comment [H14]:** Please add the VaxiJen predicted scores in the result section

### 3.2 Prediction of T Cell and B cell epitopes

For construction of vaccine, the prediction of CTL epitopes was done through NetCTL1.2 server and antigenicity were observed by the VaxiJen server. A total of 134 probable CTL epitopes were predicted for Alpha lactalbumin, out of which 6 epitopes crossed the threshold limit. Four epitopes were above threshold limit for Beta casein and seven epitopes were identified as significant for kappa-caesin. For Alpha – s1-caesin and Alpha-s2-caesin, 2 epitopes each were found to exceed threshold limit. For effective reduction in vaccine construct size, only two CTL epitopes were considered for further use (Table 2). HTL epitopes form a crucial component of immune response and it were predicted using IEDB MHC II server which were predicted in Table 3. The web server ABCpred was used to predict B-cell epitopes and are mentioned in Table 4. Antigenicity, allergenicity, and toxicity assessments were performed on the projected linear B-cell epitopes and filtered on the basis of highest scores for MPVC construct.

**Table 2: Prediction of CTL epitopes for vaccine construction**

Protein	Epitopes	C terminal Amino acid number (cle)	NetCTL Score
Alpha-lactalbumin	ILDKVGINY	0.9740	2.5805
	CTTFHTSGY	0.2724	2.5163
Beta-casein	SSEESITRI	0.8141	0.7509
	FTESQSLTL	0.9151	1.9709
Kappa-casein	TTEAVESTV	0.9186	1.0043
	LINNQFLPY	0.8891	1.7526
Alpha-S1-casein	YPELFRQFY	0.9714	0.7987
	AYPSGAWYY	0.9441	0.7928
Alpha-S2-casein	ATEEVKITV	0.9065	0.9002
	STSEENSKK	0.9597	0.7842

**Table 3: Prediction of HTL epitopes for vaccine construct**

Protein	Start	End	Peptide	Adjusted Rank	Epitope core
Alpha-lactalbumin	10	24	VGILFHATQAEQLTK	0.03	FHATQAEQL
Beta-casein	130	144	PVEPFTESQSLTLTD	0.06	FTESQSLTL
Kappa-	49	63	IQYVLSRYPYGLNY	0.03	LSRYPYGL

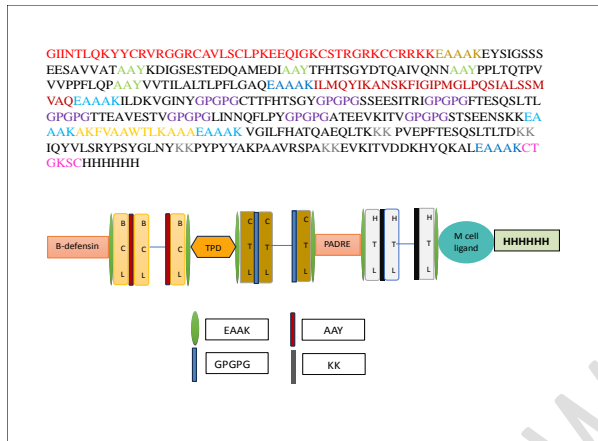
casein					
Alpha-S1-casein	78	92	PYPYYAKPAAVRSPA	0.08	YYAKPAAVR
Alpha-S2-casein	83	97	EVKITVDDKHYQKAL	0.02	ITVDDKHYQ

**Table 4: Prediction of B cell epitopes for different target proteins**

Protein	Rank	Sequence	Start position	Score
Alpha-lactalbumin	3	TFHTSGYDTQAIVQNN	49	0.82
Kappa casein	6	VVTILALTLPLGAQE	8	0.81
Beta casein	2	PPLTQTPVVVPPFLQP	90	0.86
Alpha-S1-casein	6	KDIGSESTEDQAMEDI	57	0.80
Alpha-S2-casein	2	EYSIGSSSEESAVVAT	66	0.90

### 3.3 Designing of Multi-Protein Multi-Epitope Vaccine Construct

For construction of MPVC, six HTL and twelve CTL epitopes with greatest binding for HLA alleles and six B-cell epitopes with immunogenic properties, free from allergens and toxins were considered. For vaccine construction, there is an array of adjuvants can be used. A group of proteins i.e. Defensins ( $\alpha$ -defensins and  $\beta$ -defensins) have known to have enhanced immune response in humans and animals (Kohlgraf *et al.*, 2010). Therefore,  $\beta$ -defensin was used as adjuvant with B cell epitope at N terminal with EAAAK linker. AAY, GPGPG, and KK linkers were used to connect B cell epitopes, CTL epitopes, and B HTL epitopes respectively. PADRE is known to induce effective amount of CD4<sup>+</sup> T cell responses and is capable to increase vaccine potency (Hung *et al.*, 2007). Universal memory T-cell helper peptide (TpD) is able to induce a long-lasting memory recall response in peripheral blood mononuclear cells in humans (Fraser *et al.*, 2014). M cell ligand have also known to have role in increasing antigen delivery and overall systemic immunity (Wang *et al.*, 2014). Therefore, adjuvants i.e. PADRE, TpD and M cell ligand were utilized in vaccine construct using EAAAK linkers. Further, HHHHHH and EAAAK linker were coupled at C-terminal for easier purification of vaccine. To make it simple to purify the vaccine, HHHHHH and EAAAK linker were connected at the C terminal (Fig. 1). The designed MPVC was tested positive for high antigenicity along with non-allergic and non-toxic properties.



**Figure 1: Multiprotein multi-epitope vaccine construct. With color coding for different adjuvants and epitopes**

### 3.4 Physicochemical properties, antigenicity, and allergenicity of multi-epitope vaccine construct

Designed MPVC was tested for different physicochemical properties by the ProtParam tool. The molecular weight of vaccine was around 43.7kDa with 412 amino acid residues. Aliphatic index value was calculated to be 73.06 and Grand average of hydropathicity (GRAVY) was found to be -0.312. The calculated value of the theoretical isoelectric point (PI) was 8.97. The vaccine construct was shown to have high antigenicity. The vaccine was non-allergenic with nearest protein accession number UniProtKB accession number Q6DQL1.

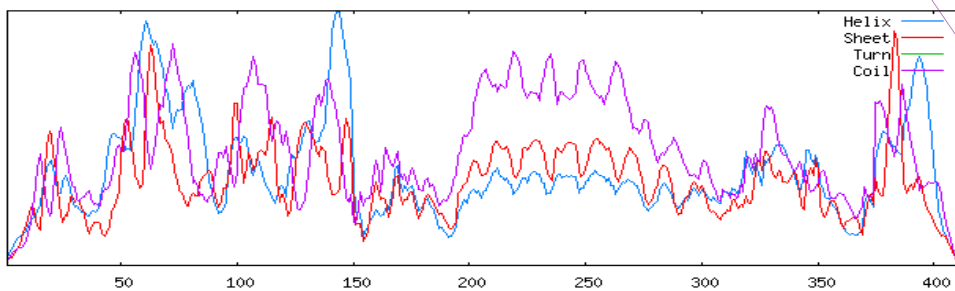
**Comment [H15]:** Vaxijen score?

### 3.5 Secondary and tertiary structure prediction and validation

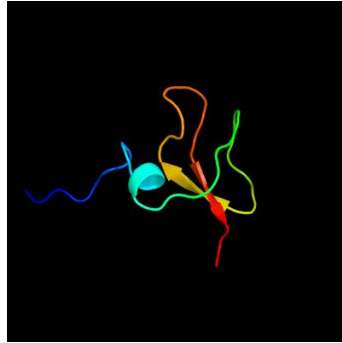
Secondary structure of vaccine plays an important role in stabilization of peptide subunit vaccine. The PSIPred and SOPMA server was used to analyse the secondary structure of vaccine construct, which showed that the secondary structure consisted of 22.82% (94 amino acid residues) helix, 7.28 % (30 amino acid residues) extended strand and 69.94% (288) random coil (Fig. 2). The Phyre2 webtool and Galaxy Refine was utilized to enhance the MPVC's tertiary structure (Fig. 3).

**Comment [H16]:** Not mentioned in the methodology, in methodology you have mentioned the PsiPred for secondary structure analysis not the SOPMA.

**Comment [H17]:** Please provide the GalaxyRefine score for initial and refined model and also add the Ramachandran plot analysis for the 3D model validations



**Figure 2: Secondary structure of vaccine construct using SOPMA server**



**Figure 3: Tertiary structure of vaccine construct using Phyre2 server**

### 3.6 Docking of multi-epitope vaccine construct with receptors

Docking is usually performed to ascertain the interaction of in-silico vaccine with its immunological cell receptors in order to elicit immune response (Ramirez *et al.*, 2023). For docking purposes, the 3-dimensional structure of human TLR genes were obtained from protein repository (PDB ID: 2A0Z and 3FXI). Docking was performed on the whole vaccine construct using ClusPro software (Kozakov *et al.*, 2017). Models with largest number of clusters and lowest energy was chosen as the best-docked complex among these models.

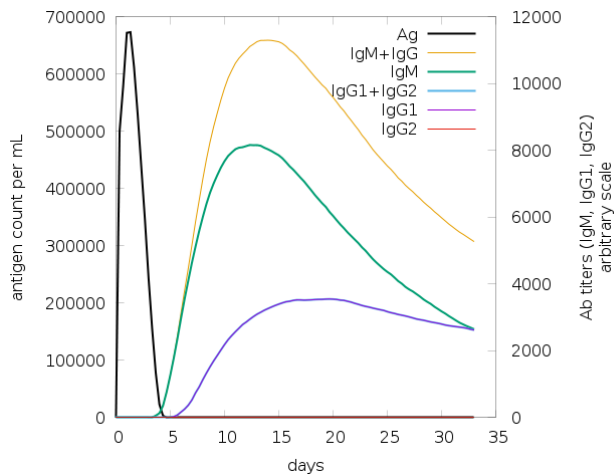
### 3.7 Immune simulations of vaccine construct

Efficacy of vaccine depends on both long-time stimulation of innate and adaptive immunity which are pillars of immunity (Clem, 2011). There are several tools for computer simulation of immune response of in-silico vaccine construct including MiStImm (Kerepesi *et al.*, 2019), the C-ImmSim simulator (Castiglione and Bernaschi, 2004). Constructed vaccine is tested for its immunological response using C-ImmSim simulator. The whole process of immune simulation involves binding of B-cell epitopes, HLA Class I and II epitopes and binding of TCR which ultimately elicits immune response. The resultant immune response was illustrated through activation and increase of IgM antibodies level as primary response. It was followed by secondary immune response by subsequent rise of IgM + IgG rather than IgM. The various immunoglobins rise in response to antigen as depicted in Figure 4. IgM and IgG antibodies are known to produce immune response in presence of foreign antigen (Gong *et al.*, 2020). There are allergen specific IgG antibodies known to counter the effects of IgE in multiple ways, thereby reducing allergic response (Kanagaratham *et al.*, 2020). This technique of immunotherapy for food allergies has been previously illustrated. The oral vaccine has been previously found to be effective in food allergies such as egg (Martorell *et al.*, 2011). Therefore, the effective MPVC construct can be used for designing and use for milk allergy.

**Comment [H18]:** Mention the visualization tool used for taking this image

**Comment [H19]:** Where are the calculated energies, images and score obtained from the docking analysis? Nothing is mention or shown here please improve the docking results as this mentioned results giving nothing about the docking analysis

**Comment [H20]:** Where is the cellular response result? Please add that in supplementary data or adjust in the manuscript



**Fig. 4: Vaccine response in inducing immunoglobulins response**

#### 4. Conclusion

Allergy due to yak milk can be tackled through multi-pronged approach including use of MPVC vaccine. The inclusion of all essential epitopes *i.e.* CTL, HTL, and B-cell epitopes which are directly involved in immune response increases the efficacy of construct in milk allergy condition. The vaccine construct was found to be highly antigenic, non-allergenic, and non-toxic in *in-silico* analysis. Designed vaccine can provide better direction to tackle the issues of milk allergy along with its consumption for allergic human population in near future. Moreover, Multi-epitope vaccine paves away ahead for interventions in the field of immunoinformatics to tackle food allergy.

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