



## Lentil allergens identification and quantification: An update from omics perspective

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### ABSTRACT

Among legumes, the lentil (*Lens culinaris*) is a major dietary component in many Mediterranean and Asian countries due to its high nutritional value, especially protein. However, allergic reactions triggered by lentil consumption have also been documented in many countries. Complete allergens profiling is critical for better management of lentil food allergies. Earlier studies suggested Len c 1, a 47 kDa vicilin, Len c 2, a seed-specific-biotinylated 66-kDa protein, and Len c 3, low molecular weight lipid transfer proteins (LTPs) were major allergenic proteins in lentils. Recently, mass-spectrometry-based proteomic platforms successfully identified proteins from lentil samples homologous to known plant allergens. Furthermore, *in silico* analysis using 337 protein sequences revealed lentil allergens that have not previously been identified as potential allergens in lentil. Herein, we discuss the feasibility of omics platforms utilized for lentil allergens profiling and quantification. In addition, we propose some future strategies that might be beneficial for profiling and development of precise assays for lentil allergens and could facilitate identification of the low allergen-containing lentil cultivars.

### 1. Introduction

Lentil (*Lens culinaris*) is a legume and a rich source of protein (22–26% dry weight; Longobardi et al., 2017). Lentil is also a good source of vitamins, dietary fiber, and minerals (Ibanez, Martinez, Sanchez, & Fernández-Caldas, 2003; Longobardi et al., 2017); fat content and *trans*-fatty acids contents are lower than other legumes, such as chickpeas (*Cicer arietinum*), cowpeas (*Vigna unguiculata*), lupins (*Lupinus albus*), faba bean (*Vicia faba*), and pigeon bean (*Cajanus cajan*) (Ene-Obong & Carnovale, 1992; FAO, 2019; Urbano, Porres, Frías, & Vidal-Valverde, 2007). In 2019, around 5.7 million tons of lentils were produced around the world, wherein China, India, Nepal, Bangladesh, Canada, and USA (Fig. 1A and B) were the major lentil producers (FAO, 2019; Rawal & Navarro, 2019).

Although lentil is a common component of the diet in many

countries, allergic reactions due to lentil consumption have been reported by many research groups (Leonardi et al., 2014; Sackesen et al., 2020; San Ireneo, Ibáñez, Sánchez, Carnés, & Fernández-Caldas, 2008). Strong IgE-mediated hypersensitivity and immune cross-reactivities have been reported in many populations, especially in Mediterranean and Asian countries (Crespo, Pascual, Burks, Helm, & Esteban, 1995). LTPs are well-known plant allergen proteins, have also been identified in lentils (Akkerdaas et al., 2012; Asero, 2011; E. Finkina et al., 2007; Shaheen et al., 2019). However, it is important to note that allergic reactions vary with race, age, and other factors (Dalal, Binson, Reifen, Ballin, & Somekh, 2002; Mahdavinia et al., 2021; Sasaki et al., 2020; Wollmann et al., 2015).

To date, numerous efforts have been made to identify and quantify lentil allergens. A search on the NCBI website using the keywords “legumes”, “allergen/allergy”, and “proteomics” revealed a total of 3663

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like proteins as well as the methods utilized for identification and quantification. Additionally, we performed *in silico* analysis using 337 lentil protein sequences, which provided a list of potential allergens. We also considered potential research strategies that would help to elucidate and validate detailed information for the lentil allergome as well as help to develop strategies for lentil allergy management through modifications in food systems.

## 2. Known lentil allergens or allergen-like proteins

According to the plant allergen database (<https://www.allergome.org>; <https://www.allergenonline.org>), a total of ten lentil allergen proteins, namely Len c, Len c 1, Len c 1.0101, Len c 1.0102, Len c 1.0103, Len c 2, Len c 2.0101, Len c 3, Len c 3.0101, and Len c Agglutinin were identified and verified (Akkerdaas et al., 2012; Sánchez-Monge et al., 2000). A recent update (February 2021) of the online allergen database (<https://www.allergenonline.org/>) also reports three allergen protein groups from lentil along with 951 sequences. However, it is important to note that different databases may be highly redundant, and/or missing many of the recently identified allergens. The majority of known allergens in lentil seeds belong to the cupin, tryp alpha amyl, and lectin legB family (Punta et al., 2012). However, recent studies identified a number of allergens or allergen-like proteins from lentil samples (Table 1).

Based on the available information, lentil allergens can be grouped into four major protein families, namely convicilin, vicilin, LTP, and lectin. As expected, phylogenetic analysis with the same protein group homologs from different members of the *Fabaceae* family reveals close evolutionary relationships (Supplementary Fig. 1). This result is in agreement with earlier studies that showed plant allergen homologs shared highly conserved sequences (Arora, Kumar, Singh, & Singh, 2020; Verma, Kumar, Das, & Dwivedi, 2013).

Among the lentil allergens, Len c 1 (Len c 1.01), the earliest discovery as a major allergen, is a 50 kDa  $\gamma$ -vicilin chain from the cupin superfamily (López-Torrejón et al., 2003). Vicilin, also known as 7S globulins, are among the major storage proteins of legumes (Finkina et al., 2017). A total of three genetic isoforms namely Len c 1.0101, Len c 1.0102, and Len c 1.0103 have been identified. Len c 1.02 is a 12- to 16-kd protein of beta-subunit of lentil vicilin protein that may be produced by the posttranslational modification of the precursor Len c 1.01 (López-Torrejón et al., 2003). On the other hand, Len c 2 protein is an IgE-binding component of a 66 kDa seed-specific biotinylated protein. It is isolated from boiled lentils (Bouakkadia et al., 2015; Sánchez-Monge et al., 2000). Len c 3, a lipid transfer protein (LTPs) is a small molecular weight protein (around 9 kDa) that belongs to the superfamily “prolamin”. A subfamily of six lentil LTP isomers designated as Lc- LTP1-6 has been identified and verified as immunologically potential allergens using immunoblot analysis and mass spectrometry-based platforms (Akkerdaas et al., 2012; Asero, 2011; Bogdanov et al., 2015; E. I. Finkina et al., 2020; Shaheen et al., 2019). LTPs are considered as one of the main plant allergens that are highly cross-reactive. They can easily bind to different types of lipid molecules such as fatty acids, phospholipids, sterols, galactolipids, and lignins (Shenkarev et al., 2017). However, LTP showed a lower binding affinity to IgE proteins isolated from sera of patients with lentil allergy by heating and digestion processes (E. I. Finkina et al., 2020). Besides Len c, agglutinin (lectin) has been reported in several studies (Bouakkadia et al., 2015).

In our recent proteomic-based discovery study, we have identified a total of 44 allergens and allergen-like proteins from lentil, including Len c 1, Len c 2, Len c 3, vicilin, convicilin, legumin, lectins along with their isomers (Shaheen et al., 2019). Label-free quantitative analysis of these allergen proteins revealed that Len c 1, Len c 2, and vicilin are the most abundant allergens, whereas convicilin and Len c 3 are less abundant (Shaheen et al., 2019).

**Table 1**

List of Lentil allergen and allergen-like proteins identified and quantified by various studies.

Name of the allergen/allergens like proteins	Identification Method	Quant Method	References
Albumin 2, Allergen Len c 1.0101 partial, Allergen Len C 1.0102 partial, Beta-lathyrin 2 partial, Chain D lentil lectin, Convicilin partial, Convicilin Precursor, CVC partial, Endochitinase, Lectin, Legumin, Legumin A, Legumin A precursor, Legumin A2, Legumin B, Legumin K, Legumin type B alpha chain precursor, LTP 2, LTP 6, Pollen specific pectin methylest, Provicilin, Seed albumin 2 partial, Storage protein, Vicilin, Vicilin 14 kDa component, Vicilin 47 K, Vicilin partial, Vicilin precursor, Vicilin type C partial,	LC-MS/MS	Label-Free	(Shaheen et al., 2019)
Len c 3	Immunoblot, MALDI-TOF	–	(Akkerdaas et al., 2012)
Len c 1, Len c 2	Immunoblot	–	(Sánchez-Monge et al., 2000)
Len c 1.0101, Len c 1.0102, Len c 1.0103, Len c 1.02	Immunoblot, ELISA	–	(López-Torrejón et al., 2003)
Len c 1	Peptide microarray immunoassay	–	(Vereda et al., 2010)
Len c 1	Peptide microarray immunoassay	–	(Sackesen et al., 2020)
Len c 1	IgE Immunoblotting	–	(Armentia et al., 2006)
LTP1, LTP2, LTP3, LTP4, LTP5, LTP6, LTP7, LTP8 LTP-1, LTP-3	Biochemical assay	–	(E. Finkina et al., 2007)
Len c 1, Len c 2, Len c 3, Len c Agglutinin	ELISA	–	(Bogdanov et al., 2015)
Proviciline, Viciline, 14 kDa component, F-box protein, ATP-dependent Clp protease, Pyruvate phosphate dikinase regulatory protein 1, Glutathione S-transferase	1D, 2-DE Immunoblot, LC-MS/MS	–	(Bouakkadia et al., 2015)
Len c 3	Immunoglobulin Binding Assay	–	(E. I. Finkina et al., 2020)
Convicilin, Len c 1.0101, Len c 1.0102, Lectin	LC-MS/MS	–	(Bautista-Expósito et al., 2018)
Len c 1.0101, Len c 1.0102	MALDI-TOF, LC-ESI-LIT-MS/MS	–	(Scippa et al., 2010)

## 3. Methodologies for identification and quantification of lentil allergen and allergen-like proteins

Identification and characterization of allergens and allergen-like proteins from food samples is one of the key interests in the food industry. Therefore, a wide range of methodologies has been developed to characterize the allergen proteins from food samples. These methodologies are mainly established methods including DNA-based polymerase chain reaction (PCR), immunology-based approach, and mass spectrometry-based platforms (Table 1). Herein, we briefly discuss the methods that have been utilized to identify and characterize the legumes and/or lentil allergen proteins.

### 3.1. PCR-based methodologies

DNA-based methods such as polymerase chain reaction (PCR), transcriptomic, microarray assay, and single nucleotide polymorphisms (SNPs) have been used for the identification and quantification of allergenic epitopes (Besler, 2001; Monaci & Visconti, 2010; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010). Identification of allergens using a gene-based platform is an indirect method but fast and reliable (Wang et al., 2011). SNPs are also used for identifying the mutations in genes (Shaheen et al., 2019; Soo, Walczyk, & Smith, 2012). The microarray-based genetic approach has not only been used to detect the legume epitope proteins, but also determine and monitor the disease profile of allergens and to establish multi-allergen tests (Flinterman et al., 2008; Harwanegg et al., 2003; Shreffler, Lencer, Bardina, & Sampson, 2005). Additionally, electrophoresis after the PCR assay is proposed by several authors to evaluate the amplicons (Sena-Torralba, Pallás-Tamarit, Morais, & Maquieira, 2020).

### 3.2. Conventional Immunology-based approach

The immunological-based approach is the basic technique for searching novel and known allergen proteins from biological samples (González-Buitrago et al., 2007). This platform is often combined with different technologies with one common thread using antibodies that recognize allergen proteins or part of these proteins (epitopes). The combination of immunochemical identification and gel electrophoresis provides a wide range of information about the level of interrogated protein, molecular weight of the protein, and its dispensation between the cellular fragments (Litovchick, 2020). Most of the known lentil allergen proteins such as Len c 1 (López-Torrejón et al., 2003), Len c 2 (Sanchez-Monge et al., 2004), and LTPs (Akkerdaas et al., 2012) have been successfully identified by the immunoblot-based approach. Currently, numerous commercial allergen strips are available as a low-cost test, which provides rapid results, ideal for quick diagnosis of many known allergen proteins (Hamid, Elfedawy, Mohamed, & Mosaad, 2009).

Similarly, ELISA is one of the most popular immunological methods where proteins are directly detected from the allergenic source (Immer & Lacorn, 2015). A recent study successfully utilized the ELISA-based method to demonstrate the allergenicity and allergen compositions of 53 Chinese peanut cultivars (Wu et al., 2016). However, the efficacy of multiple ELISA kits to detect allergen proteins from a wide range of lupines and legumes showed variable sensitivity among the samples, which relates to the cross-reactivity of the sample species (Koeberl, Clarke, & Lopata, 2014). A number of factors can determine the performance of the ELISA methods to detect food allergens (Abbott, 2010), wherein the major challenge of this method is the selection of an ideal buffer for the extraction of proteins from the food samples (Parker, 2015). Nevertheless, the application of polyclonal and/or monoclonal antibodies for detection, identification, and quantification of precise allergen proteins is still divisive (Ascoli & Aggeler, 2018).

### 3.3. Peptide microarray-based immunoassay

Peptide-based microarrays are similar to protein microarrays, but they have been constructed using thousands of pre-synthesized peptides on a microarray surface (Li et al., 2021). Compared to proteins, synthesized peptides are more stable, easy to produce and modify, and more importantly, less expensive. Recently, peptide-based microarrays have been successfully applied for detection and mapping of the IgE epitopes of several food allergens (Flinterman et al., 2008; Han et al., 2016; Kühne et al., 2015; Martínez-Botas & de la Hoz, 2016) including lentils (Sackesen et al., 2020; Vereda et al., 2010). Using lentil allergic patients' serum samples, for the first time, Vereda and coworkers identified several IgE-binding sequential epitopes of Len c 1 which are in the C-terminal region (Vereda et al., 2010). Similarly, a recent work by

Sackesen and coworkers also used microarray-based immunoassays to detect antibodies such as IgE and IgG4 binding to Len c 1 epitopes in the patients' sera. The study also revealed that IgE and IgG4 binding to epitopes were significantly higher in the reactive patient than the tolerant one. However, IgE epitopes binding to Len c 1 were not detectable for all lentil allergic patients as it was not the only allergen in lentils (Sackesen et al., 2020). The peptide-based microarray technique required a low amount of human serum sample and showed higher sensitivity compared to the conventional methods. Therefore, it could be adopted as a cost-effective screening method for the identification of other lentil allergen epitopes (Kühne et al., 2015; Li et al., 2021).

### 3.4. Mass spectrometric-based proteomic methodologies

Recently, liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomic platforms have been successfully utilized and are considered a reliable method to identify and quantify plant allergen proteins (Ahsan, Rao, Gruppuso, Ramratnam, & Salomon, 2016; Gavage et al., 2020; López-Pedrouso et al., 2020; Nakamura & Teshima, 2013; Righetti & Boschetti, 2016; Stevenson, Houston, & Thelen, 2010; Thelen, 2009). In this section, we discuss LC-MS/MS-based proteomics platforms that have been used to identify and quantify lentil allergen proteins.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) is a well-established and widely used mass spectrometry-based method in food chemistry. MALDI-TOF has been shown to be suitable for the identification of known food contamination and allergen proteins composition during industrial production (Calvano, Bianco, Losito, & Cataldi, 2021). Earlier studies showed successful identification of known lentil allergen proteins such as Len c1 and Len c3 using immunoblot followed by MALDI-TOF analysis (Akkerdaas et al., 2012; Scippa et al., 2010). However, recent advancement of LC-MS/MS-based methods mainly known as shotgun proteomics or discovery proteomics can be essential for this purpose. This platform provides highly accurate identification and quantitation of many known and unknown plant allergens and allergen-like proteins with their posttranslational modified forms (Rahman et al., 2021; Rost, Muralidharan, & Lee, 2020; Spiric et al., 2017). Only a handful of gel-based and/or shotgun proteomic analyses have been performed to catalogue the total proteome and allergen proteins from lentil seed samples. Thus far, only a couple hundred lentil seed proteins have been profiled using the gel-based proteomic approach (Akkerdaas et al., 2012; Bouakkadia et al., 2015; Scippa et al., 2010; Shaheen et al., 2019). Most of the earlier proteomic studies successfully identified major lentil allergen proteins such as Len c1, Len c2, Len c3, proviciline, viciline, convicilin, and lectin (Akkerdaas et al., 2012; Bouakkadia et al., 2015; Scippa et al., 2010). However, for the first time, in a recent study, we have identified and sequenced the isoforms of LTPs, legumins, and many other allergen-like proteins such as beta-lathyrin 2, albumin 2, endochitinase, pollen-specific pectin methylester from lentil seed using nano-LC systems coupled with high-resolution mass spectrometry (Shaheen et al., 2019). Taken together, these earlier studies suggest that optimization of protein extraction and sample preparation methodologies coupled with an advanced LC-MS/MS pipeline could significantly improve the sequencing depth of lentil seed proteome, thus providing identification and confirmation of many novel allergen proteins.

Targeted proteomics can be performed using technical methodologies including selected reaction monitoring (SRM), multiple reaction monitoring (MRM) and parallel reaction monitoring (PRM), with the latter being one of the new concepts and application of mass spectrometry-based proteomics invented in the last decade (Gillet et al., 2012; Picotti & Aebersold, 2012; Picotti, Bodenmiller, Mueller, Domon, & Aebersold, 2009; Picotti et al., 2010). Among targeted proteomic platforms, absolute quantification (AQUA)-MRM is considered one of the most reliable for accurate protein quantitation from any biological sample (Ahsan, Wilson, & Thelen, 2018; Picotti et al., 2009). The great



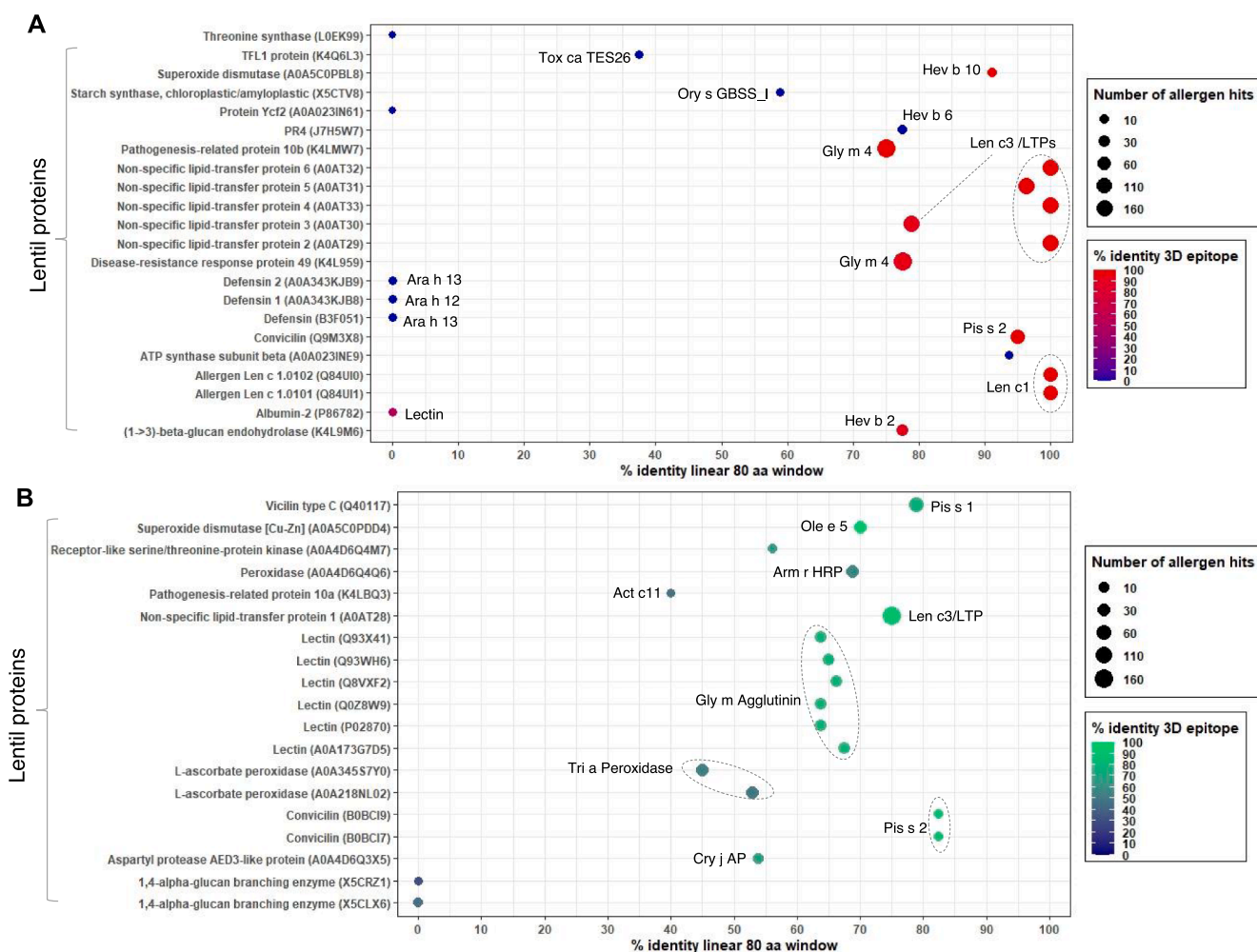
advantage of using the targeted proteomic platform is the specific identification, and quantification of a target set of peptides and/or proteins in an MS analysis with high sensitivity, reproducibility, and quantitative accuracy. Therefore, the application of targeted proteomic pipelines has rapidly increased in the last five years particularly in the field of food industry (Ahsan et al., 2016; Gavage et al., 2020). Targeted proteomic-based assays has been successfully utilized for identification and quantification of many well-known legume allergen proteins (Gavage et al., 2020; Stevenson et al., 2010). Recently, we have established the ideal peptide list of three lentil allergen proteins namely Len C1.0101, Len c 1.0102, and LTP2/4/5/6 for MRM-based assay (Shaheen et al., 2019). One of the main criteria of developing a successful MRM-based assay depends on the reproducibility of the targeted peptides in the actual biological sample (Ahsan et al., 2016). Due to the lack of a large-scale discovery proteomics dataset, the lentil seed proteome data is still fragmentary. Therefore, a great deal of discovery proteomic analysis remains to be done to develop an efficient and precise MRM-based quantitation assay for lentil allergome.

#### 4. *In silico* analysis for potential lentil allergens

*In silico* or computational methods are widely used to determine or predict physicochemical properties such as structures and domains of a protein. The probability of a protein being allergenic can also be calculated *in silico* by using different algorithms and comparing the

structures that are known to have allergenic properties (Garino et al., 2016; Maurer-Stroh et al., 2019). Although, *in silico* methodologies are incapable of distinguishing between the sensitization and elicitation phases of allergies, the FAO/WHO and the European Food Safety Authority (EFSA) guidelines state that a query protein is potentially allergenic if it has a sequence identity of >35% identical over a frame of 80 amino acids or matched exactly with 6 to 8 amino acids with an allergen (Maurer-Stroh et al., 2019).

Although, *in silico* analysis has been successfully employed to determine the potential allergenicity of many plant proteins (Garino et al., 2016; Kulkarni, Ananthanarayan, & Raman, 2013), to the best of our knowledge, no large-scale *in silico* analysis has been conducted using the lentil protein database. Therefore, it is imperative to investigate a genome/proteome-based search for potential allergen-like proteins. While the complete lentil genome sequence is not publicly available, we extracted a total of 337 lentil protein sequences from the UniProt database (Taxonomic ID: 3864). These protein sequences were further subjected to AllerCatPro, an online-based pipeline to determine the allergenicity potential (Maurer-Stroh et al., 2019). Out of 337 lentil proteins analyzed, a total of 22 and 19 proteins were categorized as strong and weak allergens, respectively (Fig. 2, Supplementary Table 3). As expected, well-characterized lentil allergens such as LTPs and Len c1 were identified with strong evidence (Fig. 2A). Two pathogenesis-related (PR) proteins, namely PR4 and PR-10b, and a disease-resistance response protein 49 (DRR49) were also identified as



**Fig. 2.** *In silico* identification of potential lentil allergens. A total of 337 lentil protein sequences obtained from UniProt database were subjected to AllerCatPro (<https://allercatpro.bii.a-star.edu.sg/>). A-B, scatterplots showed 22 proteins were identified with strong evidence (A) and 19 lentil proteins were identified with weak evidence (B) as potential allergens, respectively. Detailed information can be found in supplementary table 3.



- Systemic proteomic screening of thousands of lentil cultivars may identify lentil cultivars with low allergenicity.
- Most of the plant allergen proteins are seed storage and defense-related proteins, therefore, the effect of geographical variation and cultural practices on lentil allergome should also be investigated.
- Processing steps could significantly influence the allergenicity of many legume seed proteins including lentils (Beyer et al., 2001; Cabanillas, Jappe, & Novak, 2018; Cuadrado et al., 2009; Mondoulet et al., 2005). Therefore, analyses of complete lentil allergome profiles in response to cooking methods (boiling, frying etc.) would be a beneficial strategy to minimize the allergic reaction to the sensitive population.

## 6. Conclusion

The *in silico* analysis revealed that the complete profiling of lentil allergome is still in the juvenile stage due to the lack of a publicly available genome sequence. Precise detection, quantification, and meaningful labeling of lentil allergen proteins could significantly mitigate the allergenic reaction of these sensitive populations and thereby, could offer better consumer safety. Thus, various omics platforms such as *in silico* analysis, peptide-based microarray, and mass spectrometry-based proteomics could significantly contribute to the identification and quantification of potential lentil allergens.

## Author contributions

Conceptualization, N.A; data analysis and bioinformatics, N.A, F.Z. N, A.W, O.H; writing, review, and editing; N.A, O.H, N.S, S.G, A.I.C, F.Z. N, S.B.F. All authors have read and agreed to the published version of the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochms.2022.100109>.

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