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Zearalenone, an estrogenic component, in bovine milk, amount and detection method; A systematic review and meta-analysis

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ABSTRACT

Zearalenone (ZEN) and its metabolites are a potent component with estrogenic potential that can enter milk. ZEN and its metabolites have the ability to disturb the function of endocrine glands. The aim of this systematic review was to estimate the level of ZEN and its metabolites in milk. This study was performed with these keywords; zearalenone, ZEN, bovine milk, ruminant milk, milk, dairy products, and milk product in various databases. 946 manuscripts were collected from databases and at the end, 17 manuscripts were reviewed according to the inclusion criteria. ZEN was identified in 59 % of studies. The most common methods of analysis were UHPLC, HPLC and ELISA. Meta-analysis was performed with CMA (Comprehensive Meta-Analysis) software. No publication bias was observed in meta- analysis. But, heterogeneity was recorded between studies. The measurement method was identified as one of the sources of heterogeneity through meta-regression tests and subgroup analysis. Furthermore, in meta- analysis test, the total estimate of milk contamination with this mycotoxin was 0.036 ±0.017 μg/L. So far, the permissible limit for this compound in milk has not been announced, but these compounds have the ability to disturb the endocrine glands in low amounts. Therefore, it is necessary to regularly measure and control this mycotoxin and its metabolite in milk with valid methods.

1. Introduction

Zearalenone (ZEN) is one of the mycotoxins that contaminates corn and animal feed in abundance. It is produced by Fusarium species. ZEN is found in animal feed more than other mycotoxins [15]. Contamination of cereals with this mycotoxin has been observed both before and after harvest [13]. In previous studies, ZEN was detected in 17 % of silage samples and 28 % of feed samples [15]. In another study, 68 % of maize silage samples were contaminated with zearalenone [40]. Exposure to ZEN is commonly found in milk of animals that have consumed large amounts of contaminated silage at once or in animals that have used low-contaminated animal feed over a long period of time [50]. This mycotoxin is stable in livestock feed and does not decompose under physical and thermal processes [7].

ZEN has lipophilic properties and accumulates in foods of animal origin [28,49]. In a study, the level of ZEN intake per cow was estimated at 0.5 mg per day. In this study, 17–38 % of silage samples, compound feed and feed used by cows were contaminated with this mycotoxin [15].

This mycotoxin is a nonsteroidal estrogenic mycotoxin [2]. In terms of chemical structure (Fig. 1), it is similar to natural estrogen such as 17 β-estradiol [1,17,31]. ZEN has the ability to bind to human estrogen receptors (ER-α and ER-β) [31]. Therefore, it has the greatest effect of interference in reproductive systems [17].

These components are able to bind to estrogen receptors and produce estrogenic activities in the body [47]. It is α- estrogen receptor agonists. ZEN affects the synthesis and secretion of many sex hormones in the body [67]. Estrogenic property of this mycotoxin has been seen even in

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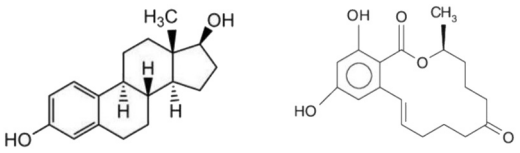

17β-estradiol Zearalenone

Fig. 1. Chemical structure of Estrogen (17 β- estradion) and Zearalenone.

small amounts. It affects the reproductive system and leads to a decrease in the number of sperm in males [67]. In the oral exposure of laboratory animals, infertility and changes in the level of progesterone have been seen in low doses [45].

It is converted into active metabolites by the protozoa of the digestive system in the ruminant [50]. It is metabolized into two compound in ruminants, α-zearalenol (α-ZEL) and β- zearalenol (β-ZEL) [66]. These components have a cumulative property in the body [57,58]. The α-zearalenol has more estrogenic properties than others [41,49]. ZEN and its metabolites are an endocrine disturb components in animals [74]. These components lead to reproductive and infertility problems in farm animals [1]. This mycotoxin causes damage to oocyte maturation, embryolethal resorption and reduces sperm motility, thus leading to economic losses in farm animals [16,24,75]. It also reduces milk production in animals [27]. It leads to liver cell death and liver damage. A possible mechanism in this process is oxidative stress. This mycotoxin leads to oxidative damage in the liver and biochemical changes in the liver [59]. Glutathione depletion and oxidative stress damage were observed in liver cells exposed to ZEN [32]. Oxidative damage and apoptosis were observed in the intestines of juvenile grass carp exposed to ZEN for 10 weeks [61]. Furthermore, this compound is an immunotoxin [43]. It has been observed in carp that exposure to ZEN for four weeks led to the damage to their cellular immune functions [61]. It should be noted that the detoxification process of this mycotoxin is complex. This is because it has enterohepatic recirculation before metabolism and subsequent elimination [7].

Milk is a nutritious food for humans and babies. Breast milk is the ideal food for babies [29]. Feeding babies with mother's milk is not always available, and in some cases, babies are fed with cow's or goat's milk [37]. Babies are more sensitive to the toxic effects of mycotoxins due to their lower body weight and lower ability to detoxify toxic compounds [5]. Therefore, due to the fact that milk may be dominant in the diet of some babies and children, this point should be paid attention to and the product's safety guaranteed. Exposure of children to ZEN leads to premature puberty [14].

83 % of the milk produced and consumed in the world is supplied by cows' milk [8]. Although the rumen of cows is an important barrier against mycotoxins, many research studies have proven the presence of mycotoxins in milk [21]. The occurrence of ZEN in milk is not as high as Aflatoxin M1, but several reports have been recorded of its presence in raw milk [8]. In experimental studies, it has been observed that ZEN and its metabolites in the amount of 1.3 ppm in the milk of cows that received food rations containing 25 ppm of ZEN [52]. In a study in Ecuador, most of the milk samples were contaminated with ZEN, although the amount was lower than the legal limit [57,58]. In another study, ZEN was detected in 98.8 % of milk samples [25]. Therefore, in this systematic review, all the studies available in the databases on the identification methods and level of ZEN and its metabolites in milk were surveyed. Furthermore, a general estimate was also made with meta-analysis regarding the level of this mycotoxin in cow's milk.

2. Method

2.1. Search strategy

To do this systematic review, keywords (zearalenone or ZEN) and ("bovine milk" or "ruminant milk"or milk or "dairy products" or "milk product") were selected. Scopus, Web of science and PubMed databases were considered without limitation in publication time. Databases were searched in April 2024 with selected keywords. This step was done by 2 authors (P.S and A.A).

2.2. Inclusion and exclusion criteria

The manuscripts that measured the level of ZEN and its metabolites by common analytical methods were included in this systematic review. The food considered was cow milk. Other food and other dairy products as well as human milk were excluded. Several studies have been conducted on the validation method and were not conducted on real samples. In these studies, this mycotoxin was spiked on the samples and then its level was measured. Therefore, these manuscripts were excluded from the systematic review. Some manuscripts also researched the level of mycotoxin in the feed used by animals, which was excluded due to the fact that it was not in the scope of this systematic review.

2.3. Meta-analysis and meta - regression

Statistical analysis was done for the data that had the mean, standard deviation and the number of samples. CMA (Comprehensive Meta-Analysis) software was used for this purpose. First, a meta-analysis was performed. In the next step, subgroup analysis and meta-regression was done. Meta-regression grouping was done based on analytical methods. Heterogeneity and publication bias were also performed. These tests were done by two different authors (K.G and P.S) with CMA software.

3. Results

3.1. The search process and quality assessment

The search was performed in valid databases. 946 articles were obtained. Duplicate articles were removed in endnote software. According to the exclusion criteria, 573 articles were excluded. Related studies were selected according to the questions and assumptions in the protocol. 75 articles were selected for full text review. The full text evaluation was based on 5 points. These 5 factors included: appropriate sample size, use of valid methods to measure ZEN in milk, announcement of details and validating factors in the measurement method such as percent recovery, LOD, and LOQ, considering confounding factors, and announcing the results as numbers. The papers that received three or more were included in the study. In the end, 17 articles were selected (Fig. 2).

3.2. The data extracted for table

Most of the studies were validation methods or experimental studies, and few studies have been conducted on actual samples. First author, year of publication, country of research, the type and level of analyte, analytical method, recovery percent, and level and sample size were extracted from the articles and displayed in the Table 1. Studies were divided into two categories based on whether only ZEN was measured or ZEN with its metabolite. Most studies measured only zearalenone.

Fig. 3 shows the distribution of the level of ZEN in milk based on the year of research. The graph was drawn based on the highest detected value in manuscripts. All amounts with different units were converted into ng/mL. The highest level detected was in 1988. Furthermore, Table 1 also shows the geographical distribution of the research

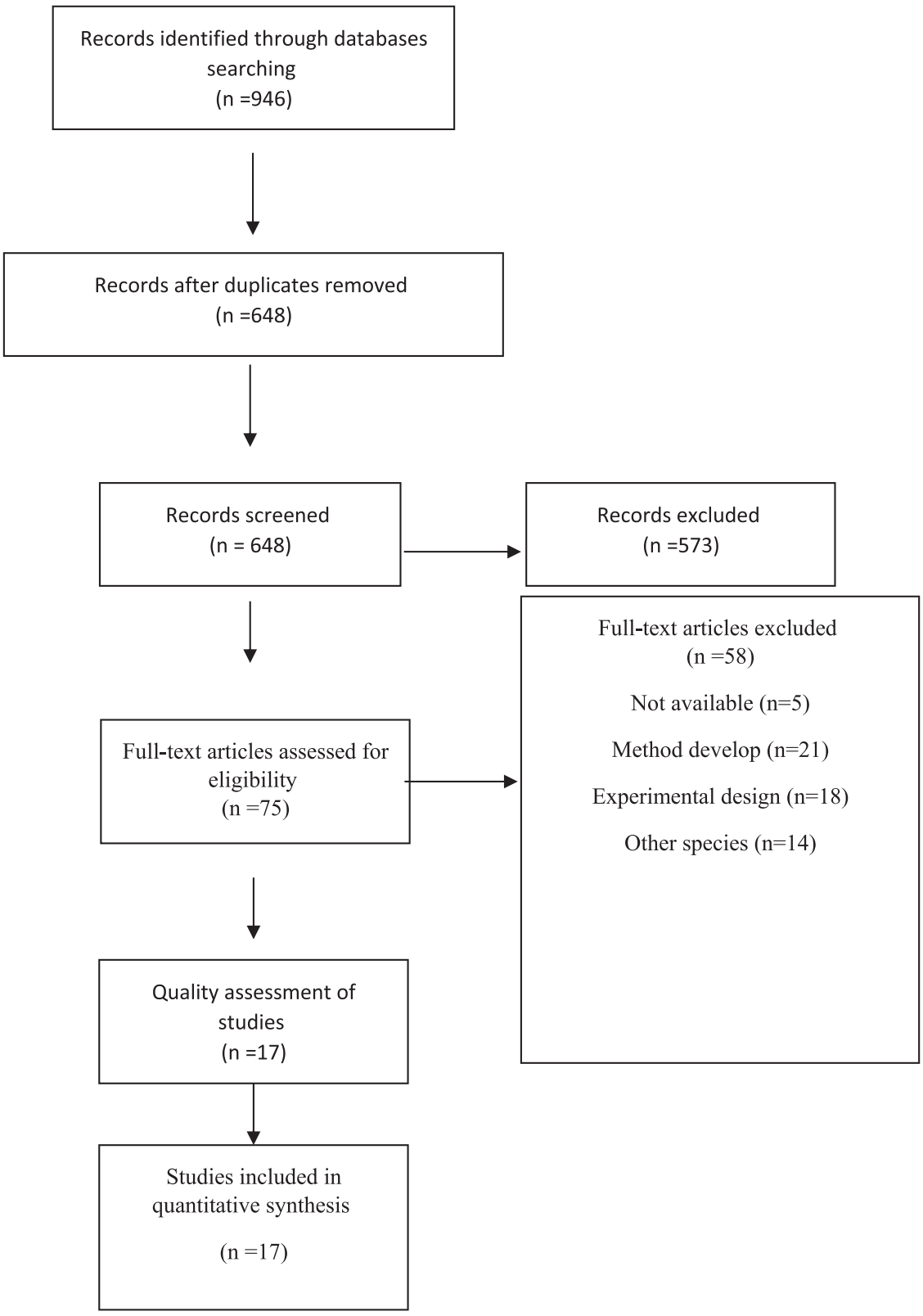

Fig. 2. The diagram of study.

conducted regarding the measurement of ZEN in milk. Zearalenone -producing fungi are found in all geographical environments, especially in temperate and warm zones [36,53]. Therefore, it has a worldwide distribution [55]. The extracted data shows the limitations of the studies. According to the climate changes in recent years, more evaluation is necessary. The planet is undergoing climate change. Due to the use of fossil fuels, the temperature of the earth is increasing [36]. The production of greenhouse gases, including carbon dioxide, is increasing. The risk food contamination by fungi is increasing [31]. In experimental observations, it has been observed that the production of ZEN from fungi

Table 1
The data extracted of selected manuscripts according to inclusion criteria.

Author/ Date	Country	The type of Analyte	Analytical method	Recovery Percent	Sample size	Level and the type of milk
Abdallah/2019 [1]	Egypt	Zearalenone	HPLC–fluorescence detector (FLD)	-	20	Raw cow milk ND
Azcona/1990 [6]	USA	Zearalenone	ELISA	92–107 %	12	Commercial milk ND
Flores-Flores/2018 [19,20]	Spain	Zearalenone	LC–MS/MS	87.2–101.5 %	30	ND
Ben Hassouna/2023 [9]	Tunisia	Zearalenone	HPLC (fluorescence detection)	96.1–97.9 %	150	Raw cow milk ND
Leite/2023 [42]	Portugal	Zearalenone	UHPLC-MS/MS	94.4–119.8	20	Raw cow milk ND
Morais/2023 [54]	Brazil	Zearalenone	HPLC (fluorescence detection)	93–112 %	30	Skimmed UHT milk <LOD
Wu/2022 [74]	China	Zearalenone α-Zearalanol	HPLC (fluorescence detection)	Zearalenone: 75–92.3 α-Zearalanol: 70.2–91.8	-	Raw cow milk ND
Frey/2021 [22]	Brazil	α- Zearalanol β- Zearalanol	LC–MS	-	32	UHT Milk α-ZEL: in the range 334–936 (ng/ L) β-ZEL: in the range (332–1949 (ng/L)
Gonzalez-Salamo/ 2017 [28]	Spain	Zearalenone, α-Zearalanol b-Zearalanol α-Zearalenol b-Zearalenol	LC–MS	70–120 %	9	Whole and skimmed cow milk In the range 0.21–4.77 (μg/L)
Huang/2014 [33,34]	China	Zearalenone α-Zearalenol	UHPLC–ESI–MS/MS	76–106 %	Raw milk=30 Liquid Milk=12 Milk power=8	Raw milk (ng/ kg) Zearalenone: 14.9 ± 6.0 a-Zearalenol: 24.3 ± 16.1 Liquid milk (ng/ kg) Zearalenone: 20.5 ± 11.1 a-Zearalenol: 36.7 ± 7.9 Milk power (ng/ kg) Zearalenone: 11.6 ± 1.1 α-Zearalenol: 43.1 ± 18.5
Mahmoudi/2014 [46]	Iran	Zearalenone	ELISA	-	70	Raw milk 1.34±1.42 (ng/mL)
Mao/2017 [49]	China	Zearalanon, α-Zeralanol β-Zeralanol α-Zeralenol β-Zeralenol	UHPLC	Mean:84 %	250	Raw milk β-Zeralanol: 0.038–0.53 β-Zeralenol:0.0099 α-zeralanol:0.098 α-zeralenol:0.016 (μg/kg)
Pleadin/2017 [56]	Croatia	Zearalenone	ELISA	87.6–94.3 %	105	Raw milk 5.5±30.5 (μg/L)
Puga-Torres/2021 [57,58]	Ecuador	Zearalenone	ELISA	-	209	Raw milk 0–0.0102 (mg/L)
Rocchetti/2021 [60]	Italy	α-Zearalenol	UHPLC	-	45	Raw milk In the range <0.1–5.25 (ng/mL)
Scott/1988 [63,64]	Canada	Zearalenone	HPLC (fluorescence detection)	74–100 %	5	Raw milk 62.5 ± 3.7 Boiled milk: 58.0 ± 5.0 (ng/mL)
Winkler/2015 [73]	Germany	Zearalanon, ∑ZEN	LC-MS/MS	94–117 %	10	Raw milk ZEN: 0–0.0075 ∑ZEN:0–0.0075 (ng/ mL)

Note: ∑ZEN: ZEN, α-ZEL, and β-ZEL

increases with the increase of carbon dioxide gas [11].

3.3. Result of meta – analysis and meta- regression

The studies that had mean, standard deviation and sample size were selected from Table 1. A overall estimate was made with a random effect model. This value was calculated as 0.036±0.017 μg/L. To determine heterogeneity, I^2-square, P-value and Q-value tests were performed. The results of all three showed highly heterogenicity. The P-value was 0 and the I^2-square was calculated as 99 %. The publication bias was also calculated. For this purpose, the Begg and Egger test was chosen. The P-value in the Begg and egger tests were 0.25 and 0.16 respectively. Therefore, there was no publication bias. In order to find the cause of heterogeneity, subgroup analysis was performed. Subgroup analysis is used to assess heterogeneity to determine differences between studies. Studies were divided into two groups based on the measured device. The result of p value of this test was 0. Therefore, the type of analytical method has an effect on heterogeneity. The p-value of meta-regression

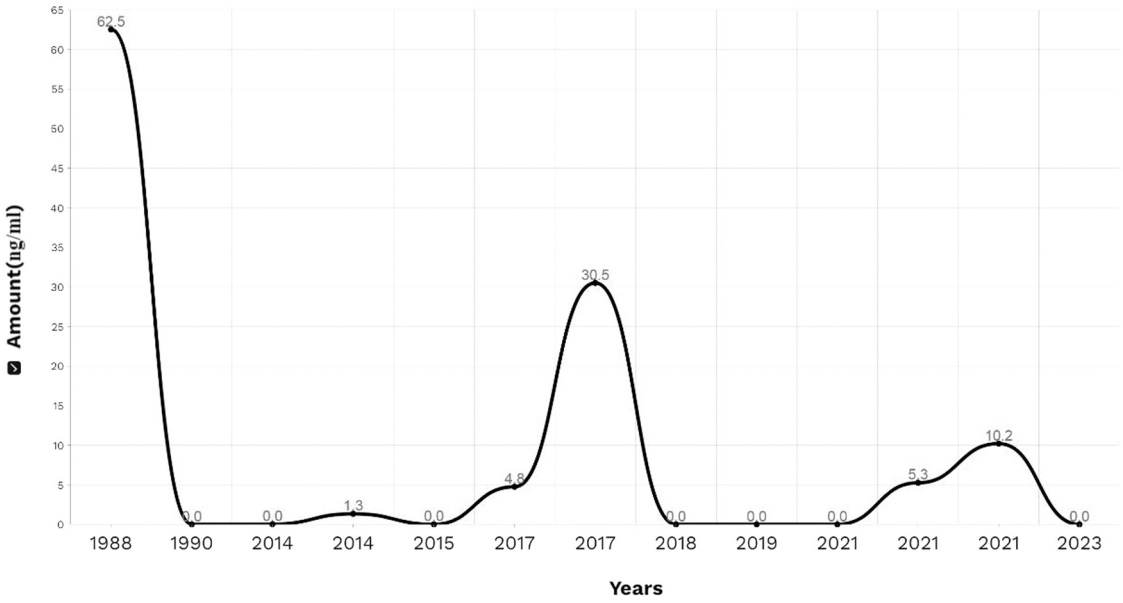

Fig. 3. The level of ZEN in milk samples based on the year.

was also 0 and it was determined that the type of device has an effect on the difference between studies. With the analysis sensitivity test, it was found that one of the studies [46] pulled the results towards itself.

4. Discussion

This systematic review focused on ZEN and its metabolites in milk. In addition to its adverse health effects, this mycotoxin causes severe economic losses to the livestock industry [18–20,50]. Therefore, its evaluation is important both economically and healthily.

Isolating ZEN can be difficult because the milk matrix is high in protein and fat [73]. Although several articles have been published on the development of the various separation methods, few studies have been conducted on real samples. Therefore, a limited number of studies were selected for data extraction (Fig. 1). ZEN was not detected in milk samples in 41 % of studies (Table 1). In 58 % of studies, only ZEN itself was measured and metabolites were not considered.

Based on the extracted data, the majority of studies focused on raw milk and less processed milk was evaluated. Of course, it is worth mentioning that processing did not play a role in reducing this mycotoxin. The stability of ZEN was examined in Scott's study. No significant difference was observed between raw milk and boiled milk [63,64]. Furthermore, other studies have confirmed that zearalenone is heat stable [31,72]. Temperatures above 180 °C lead to a significant reduction of this mycotoxin [69]. Heating food at this temperature will usually reduce its nutritional value. In the Azcona study, which evaluated ZEN with the ELISA, it was not identified in commercial milk samples [6]. The ELISA method is one of the methods widely used in food industry and laboratories to identify mycotoxins. In the studies, the level of LOD was determined by the ELISA method to be 1.75 μg kg−1 in food samples [65]. But, the LOD for this mycotoxin was determined to be 0.25 μg kg−1 by LC-FLD method [44]. The sensitivity of the ELISA method is lower in complex matrices [35]. However, the results of ELISA were confirmed by Immunoaffinity chromatography in this study [6].

In Mahmoudi's study, the level of ZEN in raw milk and liver of bovine was measured. It was lower in raw milk than in the liver [46]. This result is predictable. The liver is the site of metabolism of this mycotoxin. Another study detected significant levels of zearalenone in milk [56]. Metabolites were not measured in this study. Significant amounts of ZEN were detected (Table 1). Moreover, in study by Puga-Torres et al., only ZEN was measured in milk [57,58]. In a study conducted in Egypt, ZEN was detected in feed samples but not in milk. In this study, the amount of ZEN in animal feed was determined from 1 to 11.9 μg/kg [1]. It has been observed in vitro experimental studies that ZEN is converted into its metabolites in the environment of rumen fluids [30]. In the study by Huang et al. [33,34], both ZEN and its metabolites were measured in raw milk (Table 1). It was observed that the amount of metabolites is more than that of ZEN itself [33,34]. This result emphasizes the measurement of metabolites in milk is essential. In particular, the estrogenic power of the α-zearalenol metabolite is much higher than that of the parent [8,56]. The estrogenic activity of α-zearalenol is three to four times that of its parent [8].

Common methods for identifying ZEN and its metabolites are chromatography, and ELISA in the extracted data [70]. It has been reported that the sensitivity of the ELISA method is lower in complex matrices [33,34]. In this review, it was determined that the most commonly used chromatography methods were UHPLC and HPLC. In HPLC, isocratic conditions and reversed-phase are used [53]. The detectors used were both fluorescence and mass. These methods are valid, selective, reliable and sensitive for different matrices [39,48]. In none of the selected studies, the GC-MS method was used to determine this mycotoxin in milk. This method requires derivation [12].

Meta-analysis summarizes the results of individual studies and gives a single result. This result is useful for determining the direction for future studies. With the meta- analysis test, the overall average contamination of milk with ZEN was estimated as 0.036±0.017 μg/L. The permissible limit of ZEN in various food products is in the range of 0.03–1 mg/L [57,58]. The Food Commission of the European Union has announced the limit for this mycotoxin in different foods between 20 and 400 μg/kg (1881/2006/EC)[67]. Therefore, the level calculated for ZEN was within this range. Of course, there is no permissible value limit under regional or international standards for the amount of ZEN in milk.

The source of heterogeneity can be identified from Meta – regression and subgroup analysis. Studies were grouped based on the type of measurement method. For this purpose, two chromatography and ELISA groups were considered. After these tests, a significant difference was seen. Therefore, the measurement method is effective in the heterogeneity of studies. The highest value reported in Fig. 3 was 62.5 ng/mL, which is very different from the values of the rest of the studies. This value was measured by ELISA. As the meta-regression results showed that the method of measurement plays a role in the heterogeneity of studies, it is possible that this difference is caused by the method of

measurement. Moreover, with the sensitivity test, the results of the Mahmoudi [46], study were different from the results of other studies. In this study, the measurement method was ELISA. On the other hand, the measurement method had an effect on the heterogeneity of the studies. It is recommended to use the ELISA measurement method for screening. This method is economical and fast, and sample preparation is also simple [71]. HPLC and LC/MS are precise methods for mycotoxins and their derivatives [68]. Due to the fact that mycotoxins show natural fluorescence, the HPLC/FLD method is also used to identify and determine the amount of these compounds [9]. Both of these methods are capable of detecting low concentrations of mycotoxins in complex matrices [71]. In a study, the results obtained from the measurement of ZEN in food with two LC-MS/MS and HPLC-FLD methods were the same [74].

Table 1 shows that comprehensive information on the level of this mycotoxin in milk in all geographical regions is not yet available. Therefore, for the accuracy of this overall estimate, it is necessary to carry out more research. It is also necessary to take measures to prevent the contamination of livestock food. Temperature and humidity are two important factors for fungus growth and toxin production [62]. The fungi that produce mycotoxin grow in temperature conditions of about 28°C and humidity of 13–18 % [10]. Therefore, it is necessary to control the factors that play a role in the production of mycotoxins, such as temperature, pH, humidity, and physical damage to the plant body [3]. These conditions should not be provided for fungus growth. One of the most effective ways to reduce this mycotoxin in milk is to train farmers [4]. Good agricultural practices, proper management of crops before harvest and after harvest should be educational items [26]. Another strategy that can be proposed is the need to set international laws regarding the correct transportation of livestock feed during their trade. One of the factors in the occurrence and severity of food contamination by mycotoxins is the transportation conditions [51]. The animals feed is usually transported by ship. Poor hygiene practices during transportation, such as storing the product at high temperatures or under heavy rainfall, lead to contamination of the product with mycotoxins [38]. In rainy conditions, loading is usually delayed, so the product is damaged by moisture [76]. It is necessary to control the moisture level in products sensitive to mycotoxins during their storage and transportation [23].

5. Conclusion and future researches

The results of this systematic review showed that a higher percentage of studies focused on ZEN itself and did not evaluate its metabolites. The estrogenic properties of its metabolites are more than the ZEN. Therefore, it is recommended to evaluate the level of mycotoxin and its metabolites in real samples. The methods based on high-performance chromatography for the evaluation of this mycotoxin in food have good accuracy and sensitivity. The permissible level for ZEN is not set in milk. Due to the high per capita consumption of milk, it is necessary to regulate it specifically. Furthermore, considering that the origin of this mycotoxin is in animal feed milk, it is recommended to carry out a systematic review in this regard for future research.

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Ethics approval

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CRediT authorship contribution statement

Amirhossein Abedini: Writing – original draft. **Parisa Sadighara:** Writing – review & editing, Writing – original draft. **Behrouz Akbari-adergani:** Writing – review & editing. **Nader Akbari:** Writing – review & editing. **Kiandokht Ghanati:** Investigation. **Burhan Basaran:** Investigation.

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.

Data availability

The data that has been used is confidential.

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