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Introducing a Novel Composite Polymer for Food Packaging

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ABSTRACT

Introduction: Safety and quality control of foods have attracted significant global attentions. Intelligent and active packaging materials are emerging areas of food technology, which is attracting a lot of attention in the food industry. The present study with the aims of incorporating *Rosa damascena* extract (RDE) into the chitosan-gum Arabic (CH-GA) through the casting method and investigating its antimicrobial property as potential application in the food packaging were conducted.

Methods: Preparation of films based on CH-GA containing RDE was conducted via casting method. The antimicrobial activity of the designated films was investigated by the disk diffusion assay. The morphology of the fabricated films was determined under the field emission scanning electron microscopy.

Results: The prepared films had antimicrobial activity against *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*, and *Bacillus cereus* ranging from 5.50 mm to 9.33 mm inhibition zone diameter. The film containing the RDE has a rough surface and the pure film exhibited a smooth, dense surface, a uniform structure, and no cracks.

Conclusion: This result showed that the CH-GA film containing RDE can be used as an active packaging material in the food industry for enhancing the freshness of the protein-rich foods.

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Introduction

Global concerns, including environmental and climatic changes are forced researches and policies on sustainable economic cultures (1, 2). Numerous methods have been recommended as a part of the sustainable development to keep natural resources, decrease pollution, and inhibit global waste accumulation (3). Biodegradable based packaging materials can be used for reducing this concerns owing to their biocompatibility, biodegradability, non-toxicity, antioxidant, and antibacterial properties (4). In the last decades, the biodegradable films/nanofibers are produced from the wastewaters and by-products of numerous food sectors, suggesting an alternative approach to natural food packaging materials (5). Chitosan (CH) and gum Arabic (GA) has been extensively applied in the food packaging applications among the numerous natural polymers due to its easy film-forming capacity, biodegradability, appropriate oxygen and water vapor barrier ability, and excellent mechanical characteristic (6). These are utilized in many different applications, including food and medical formulations, chemistry, and other biological properties. However, the low antioxidant and antibacterial potentials of CH and GA films, which are critical parameters for an active food packaging film, limit their potential application in the food packaging (7, 8).

Moreover, important attention has been paid to the incorporation of natural extracts and essential oils into the polymeric nanofibers/films in the food industries. Essential oils (EOs) and extracts have always been regarded as trustworthy and safe food preservatives in the food based products, which can limit lipid oxidation and delay the spoilage microbial growth (9). *Rosa damascena* Mill. (Damask rose, oil-bearing rose, and pink rose), is a native aromatic plant of the Middle East that is extensively utilized in a wide variety of food products, especially Iranian yogurt-based drink and pickles regarding its flavoring and high levels of phenolic compounds and anthocyanins (6). It has broad antimicrobial activity against molds/yeasts and Gram-negative and Gram-positive bacteria without adverse effect on sensory attributes of food products (10). The leaves of *R. damascena* belonging to the *Rosaceae* family, has traditional applications owing to its

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biological properties, such as antidiabetic, antiatherosclerotic, anti-inflammatory, anticonvulsant, carminative, digestive, and analgesic effects (11). The food safety (GRAS-Generally Recognized as Safe) of plant essential oils and extracts has been completely confirmed through the Food and Drug Administration (FDA) of the USA (12). Based on our findings, there is no information regarding the possibility of incorporation of *R. damascena* extract (RDE) into the CH-GA based films. Therefore, the present study with the aim of incorporating RDE through the casting method and investigating its antimicrobial property were conducted.

Materials and Methods

R. Damascena Extract Preparation

Fresh Damask rose (*R. damascena* Mill.) flowers were supplied from a local market in Kermanshah, Iran. The obtained plants were identified at the Faculty of Agriculture, Razi University (Kermanshah, Iran). The preparation of the extract was conducted by 20 ml extraction solution (ethanol: water, 20:80 volume/volume) mixture and concentrating using a rotary evaporator (Heidolph, Germany) (6). Moreover, all culture media and chemicals were purchased from the Merck, Germany.

Fabrication of CH-GA based film containing R. damascena extract

CH (medium molecular weight = 250 KDa, 75-85% deacetylated) and GA spray dried from acacia tree were purchased from Sigma-Aldrich (UK) and Merck (Germany), respectively. In order to prepare film based on CH-GA, 2 g of CH was dissolved in 100 ml of 1% acetic acid solution. To achieve proper distribution of CH, the CH solution was stirred for 3 h at room temperature (24 ± 1 °C) on a heater stirrer (IKA, Germany). Then, glycerol at the rate of 0.75 ml/g was added as a plasticizer to the CH solution and stirred for another 30 min (13). The amount of 14 g of GA was mixed in 100 ml of distilled water and stirred for 5 h at 50 ± 1 °C. Then, glycerol was added at the rate of 0.75 ml/g as a plasticizer and stirred for 30 min. CH and GA were mixed in a ratio of 40:60 and stirred for 30 minutes at room temperature (14). Then, 5% RDE was added and mixed for another 30 min at room temperature (24 ± 1 °C). Finally, the final solution was homogenized with a homogenizer at a speed of 12600 × g for 1 min. After evaporating the solvent at room temperature for 48 h, the prepared films were separated from glass molds (diameter = 12 cm) and used for other experiments in this study.

Antimicrobial activity of CH-GA based film containing R. damascena extract

Antimicrobial property of the prepared films against *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (ATCC 19118), *Bacillus subtilis* (ATCC 6633), and *Bacillus cereus* (ATCC 11774) were investigated by the disk diffusion method (15). The preparation of the mentioned bacteria has been conducted by activating in tryptic soy broth plus 0.6% yeast extract for 24 h at 37 ± 1 °C (16). The prepared films were cut into 6 mm diameter and placed on Mueller Hinton agar culture medium containing 7 log CFU/ml of bacteria. After incubating at 37 ± 1 °C for 24 h, the diameter of the growth inhibition zone was measured (17).

Scanning electron microscopy of CH-GA based film

The morphology of the fabricated films was determined under TeScan MIRA3 field emission scanning electron microscopy (FE-SEM) after affixing to sample stubs and sputter-coating with a thin layer of gold at an accelerating potential of 15 kV with a working distance of 10 mm (18).

The release of R. damascena extract from CH-GA based film

The release rate of RDE from designed films was investigated by dissolving 0.1 mg of the sample in 100 ml of 85% ethanol-water solution and stirring at 100 rpm for 84 h under dark conditions at 4 ± 1, 25 ± 1, and 37 ± 1 ºC. At any specified time, 1 ml of the solution was taken and its concentration was measured at a wavelength of 530 nm using a UV-visible spectrophotometer based on the method of Guo et al., (2020) (19).

Statistical Analysis

The experiment was repeated three times. Data analysis was conducted using the SPSS program (version 21 for Windows, Chicago, IL, USA). The results were presented as mean ± standard deviation. A statistical significance was considered as $P < 0.05$.

Results and Discussion

The release of R. damascena extract from CH-GA based film

The amount of RDE cumulative release from CH-GA film at 4, 25 and 37 ºC is shown in Fig. 1. Based on the findings of this study, the amount of cumulative release of RDE from CH-GA film increased significantly with increasing

temperature ($P < 0.05$), which can be due to the increased mobility of macromolecule chains (20). Moreover, 100% of the RDE was released from the CH-GA film at 37 °C after 72 h. Similar findings have been found by Wu et al., (2015) who evaluated the diffusion of cinnamon essential oil in nanoliposome incorporated into the gelatin film (21). Ghadetaj et al. (2018) also indicated a same effect of nanoemulsion formation on the release kinetics of *Grammosciadium ptrocarpum* Bioss. essential oil from whey protein isolate film (22). Same findings are also in agreement with those reported by Aziz1 & Almasi, (2018) for releasing *Thymus vulgaris* L. extract from whey protein isolate film (2). Li et al., (2021) also found a similar abrupt release (about 25% at 24 h) for encapsulated eugenol from gelatin nanofibers (23). Maroufi et al., (2021), indicated that that the thyme essential release curve from poly (lactic acid) nanofibers displayed a 62 h-plateau period (24).

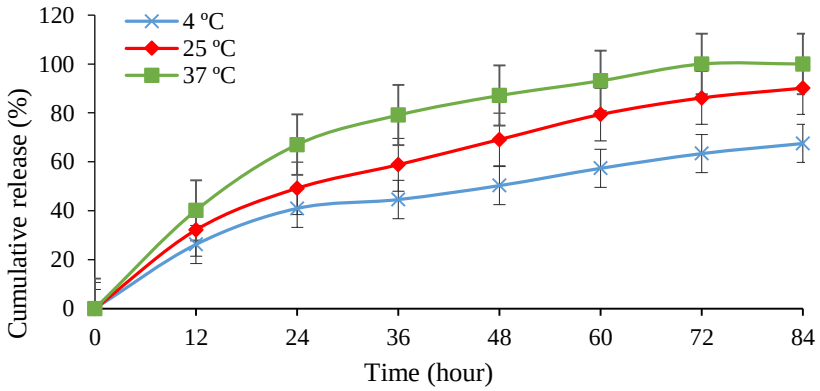

Figure. 1. The release of *R. damascena* extract from CH-GA based film.

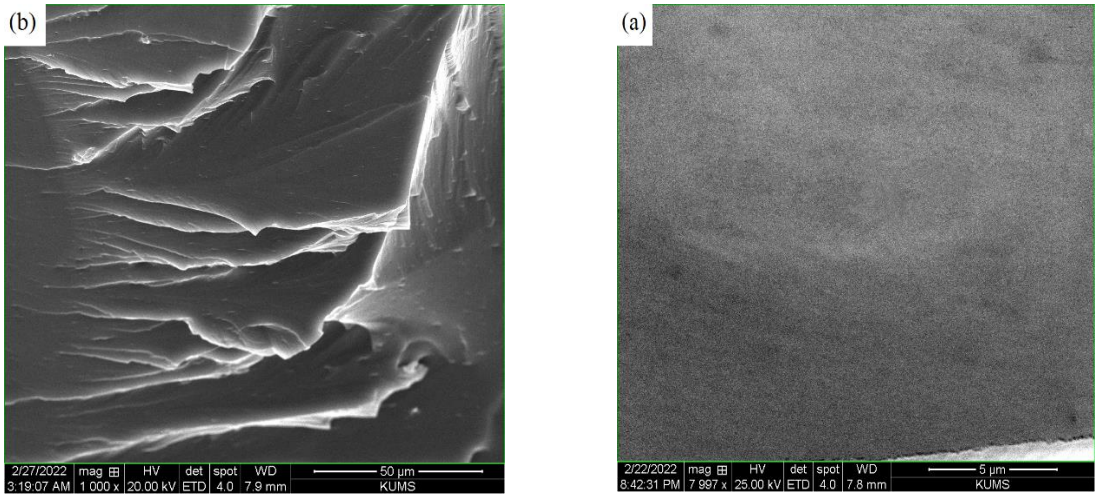

Figure. 2. SEM micrograph of pure film (a) and film + RDE (b).

Scanning electron microscopy of CH-GA based film

The FE-SEM micrographs of pure films and those containing RDE are presented in Fig. 2a and 2b, respectively. As shown in Fig. 2a, the pure film had a smooth, dense surface, a uniform structure, and no cracks. The film containing the RDE has a rough surface probably due to the protruded structures mediated by the various chemical constituents of the RDE compounds (18). The results of the present study exhibited a homogeneous and dense microstructure without phase separation, and this suggested that there was good biocompatibility between the extract and CH-GA matrix (25).

Antimicrobial activity of CH-GA based film containing R. damascena extract

The antimicrobial property of the prepared film against *S. aureus*, *L. monocytogenes*, *B. subtilis*,

and *B. cereus* are shown in Table 1. After removing the straight CH-GA polymer from the agar plate, the growth of bacteria did not find and a growth inhibition zone did not observe. This antimicrobial activity is in agreement with the findings of other researchers regarding CH-methyl cellulose nanofiber (26), CH nanoparticles (27), and CH-gelatin film (28). In the present study, the film containing RDE had antimicrobial properties against all investigated microorganisms (Table 1). It is to be noted that RDE components may lead to membrane disintegration in bacteria, hydrolysis of membrane components, and leakage of intracellular electrolytes and proteinaceous constituents, and therefore RDE had the potential to be used to control spoilage and pathogenic microorganism's growth for extending shelf-life of perishable food (12, 29). Previous studies also have indicated that RDE could inhibit the growth of pathogenic bacteria, such as *B. cereus*, *E. coli*, *Salmonella* Typhi, and *S. aureus* (10, 30).

Table 1. Antibacterial activities (inhibition zone diameter; mm) of chitosan-gum Arabic film containing *Rosa damascena* extract (RDE).
ND: not detected

Formulation	*S. aureus*	*L. monocytogenes*	*B. subtilis*	*B. cereus*
Pure chitosan-gum Arabic	ND	ND	ND	ND
Chitosan-gum Arabic + RDE 5%	8.49 ± 0.06	9.33 ± 0.08	6.78 ± 0.02	5.50 ± 0.01

Conclusion

In the current study, we considered the fabrication of antimicrobial packaging with RDE anthocyanins and CH-GA film. The results of the present study showed that RDE successfully incorporated into the CH-GA film via casting method, as the SEM micrograph confirmed this phenomenon, to prepare an antimicrobial film. This result showed that the CH-GA film containing RDE can be used as an active packaging material in the food industry for enhancing the freshness of the protein-rich foods.

Conflict of Interest

The authors declare no conflict of interest.

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The Environmental Factors Determining the Physical Activity of Children: A Narrative Review

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ABSTRACT

The levels of physical activity (PA) is an important for the health of children and environmental factors play a vital role in shaping children's attitudes, behavior and physical-mental development. Hence the identification of the environmental factors that may contribute to children`s health is important. The relevant literature between 2015-2020 was reviewed, and the factors classified according to three principal environments of home, neighborhood and school. Findings highlight the need for more studies, especially into contextual factors and design-related characteristics of the environments. Increasing child`s PA opportunities including active play and commute vs. sedentary behavior (SB) in all of the three environments were suggested through: 1) proper presence and availability of PA supportive places (either indoors or outdoors), routes (sidewalks, cycling routes) and equipment, 2) consideration of practical threshold for walking/cycling time and distance to schools and neighborhood destinations, 3) provision of neighborhood with more traffic/social safety, 4) limitation of child`s sedentary time (ST), SB supportive devices number and accessibility, 5) emphasizing the importance of teachers, child care providers and family role (role modeling, support, attitude, rules, socioeconomic status (SES), perceptions, concerns, priorities and physical-mental health). Implementation of policies and measures targeted at enhancement of the environments PA supportive qualities simultaneous with promotion of knowledge of planners, designers, teachers, child care providers and families about children`s PA importance is needed.

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Introduction

Importance of Physical Activity for Children

PA is considered to be an important determinant of children`s physical and mental health. The PA of children can affect their health and development including cognitive function, scholastic achievement, movement skills or executive function (1-4); measures of adiposity; musculoskeletal health, psychological and cardiometabolic health (5). In spite of some disparities in such PA associations with physical-mental health or academic achievement revealed by some studies (6, 7), higher levels of total physical activity (TPA) including both light physical activity (LPA) and moderate-to-vigorous (MVPA) integrated with lower levels of SB is considered to be crucial to children`s development and health promotion.

Importance of environment

The environment and its components including all involved objects, people and events, shape and affect child`s health and development conditions such as their developing brain structure and function (8), early childhood value structure (9, 10), long-term attitudes and behavior (11). Hence, some researchers are of the opinion that family should be considered as child`s educational setting (12), and cultivation of suitable and healthy environments is absolutely essential whether at home or school as well as in the community (8).

Environmental Factors of Child`s Physical Activity

Understanding the environmental factors that affect children`s PA within the three mentioned environments appears to be important to developing a strategy to increasing children`s

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physical activity level (PAL) and consequently providing them with better physical and mental health and development as one of their certain rights. Accordingly, many researchers in different areas have focused their efforts on the identification of these factors in different locations and environments of home, neighborhood and school. It makes it possible to compare the environments and their relevant factors role in relation to child`s TPA through a clearer overview or identify and assess advantages and disadvantages of every factor and environment at the end. Literature on the subject published in the years 2015-2017 in different mentioned areas (including art and humanities, psychology especially environmental phycology and behavioral psychology, environmental science, social science and medicine) have been sought. The evidence pieces were reviewed considering both preschoolers and school-age children categories while mentioned separately in the context.

According to the relevant evidence published before 2015, some built environment characteristics such as the availability of recreational places (playgrounds, parks or sidewalk), local streets connectivity, diverse land-use integrated with walkable destinations, public transportation access have positive associations with child`s daily PA and active commute. Whereas some other features including commute distance, traffic volume, crime rate and parental safety-related concerns resulting in higher transportation, less outdoor play and neighborhood independent mobility decrease have negative relationships with it (13-28).

The current review aimed to identify the environmental factors affecting child`s PA classified according to their environments of home, neighborhood and school among the more recent pieces of evidence. These environmental factors are categorized into two groups of physical and socioeconomic.

Home, Neighborhood and School Environmental Factors of Child`s Physical Activity

It is widely reported that children`s TPA and outdoor time/activities are decreasing, whether due to interesting activities within the home (29, 30) or as a result of other causes such as parental concerns and priorities (31-38). Researchers findings indicate that children`s total time engaged in MVPA is continuing to decline over time in different environments indoors and outdoors as their indoor proportion of daily time is increasing year after year vs. the outdoor (39). It has also been demonstrated that children`s play are changing from unstructured free kinds outdoors to structured supervised indoors with less frequency and shorter duration (31, 32, 40, 41). Meanwhile, according to some evidence, children`s lowest proportion of overall MVPA occurs at home, while their highest MVPA proportion is provided out of home, either at school (42) or in the neighborhood environment; so that it seems that the most percentile of total time in the environments that is designated to MVPA belongs to the neighborhood environment in compare to home and school environments (39, 42, 43). On the other hand, in some researchers` opinion, household/family and school environments have more significant influences on child`s PA participation rather than community environments (44).

According to some investigations, a low proportion of children population adhere to the PA and dietary guidelines (29, 45, 46). It has been shown that racial/ethnic minorities, rural areas residents and Latinos have less PA levels in comparison with main groups, residents of suburban and urban areas and non-Latinos (13).

Home Environmental Factors of Child`s PA

Home-related child`s PA predictors found consist mainly of home physical and socioeconomic factors from availability of PA healthy/non-healthy equipment and resources to family members role through theirs socioeconomic status (SES), role modeling, supportive attitude and behavior, perceptions and physical-mental well-being conditions.

Home Physical Factors

Some investigators take the view that being of high-risk or low-risk kind, environment of child`s home plays a vital role in providing their different levels of health factors. As shown by some research, preschool children living at home with higher-risk food or activity environment not only have a higher level consumption of energy-dense snacks and sweetened drinks than those in lower-risk home environments, but also are less active, spend more time watching TV and eat less fruit and vegetables in compare to their peers (47). Associations between home physical environment and boys` after-school PA and ST

have been also reported by a study. Actually, availability of home PA resources seems to have positive association with boys` afterschool TPA and negative relation with their afterschool ST. Home physical environment elements had been assessed through some relevant items:

a) Home PA resources (e.g. play space indoors, cardio equipment, jumping ropes, balls) availability whether at home or in the yard

b) The number of TVs, video game consoles and computers at home (48)

There is also some evidence indicating that specific PA behavior at home has associations with child`s PA, and home availability of PA areas and equipment has mediational role for parents-child PA (49). Additionally, bedroom electronic presence and absence of parental supervision are considered as significant predictors of child`s SB (50) and screen time (SCT) (51). Based on another study findings, electronic devices (except to music devices) ownership has an inverse relationship with socioeconomic status (SES) (parents` education) and fewer devices of such kind especially in child`s bedroom leads to their less SCT. The study showed that both children's MVPA and active play equipment (except to bicycle) possession had positive association with SES (household income) while no relationship with each other (52).

Table 1. Domestic Factors determing Children`s PA

	Home Physical Factors of Child`s PA	Affected Item	References Title	Authors	Date	Participants	Location
1	Home environment activity risk level	Child`s Activity/SCT	(47)	Schrempft S et al	2015	1096 preschoolers Aged 4	Gemini
2	Home physical environment: PA/SB resources accessibility at home (Play space, cardio equipment, jumping role, balls, number of TVs, video game consoles)	Boys` Afterschool TPA/ST	(48)	Lau EY et al	2015	671 schoolchildren 6th grade	South Carolina
3	Electronic devices presence	Child`s SB	(50)	Roberts JD et al	2017	144 schoolchildren aged 9-10	Washington DC
4	Electronic devices presence	Child`s SCT	(52)	Dumuid D et al	2016	427 schoolchildren aged 9-11	Australia

Abbreviations: PA: physical activity; SB: sedentary behavior; SCT: screen time.

Home Socioeconomic Factors

Parental attitude, behavior, perception, support, age, SES (education, income) and physical-mental health have been shown that may affect differently children`s PAL and SB including their recommended PA and ST achievement or non-achievement. Even child`s perceived family environment is said to be positively associated with their leisure time physical activity (LTPA) (53).

Family SES

Family members SES including their income, age and education is believed to be responsible for children`s PA partly, even though, there are some disparities demonstrated in evaluated relationships between the mentioned factors and child`s PA/SB. For example, while according to some findings medium or high levels of it can strongly lead to lower screen-based SB in school-age children (54), some inverse relationships between higher SES of child`s family and their PA have been also declared. An experiment in Southern Brazil on 2604 children aged 6 showed a negative association between SES of family and maternal schooling with children`s PALs, without any associations between early life biological factors and children`s PA behavior after a 6-years follow up visit (55). Again, children who belong to lower-income families within rural setting are reported to be engaged in more PA weekly. Actually, parents of lower-income families encourage activity among their children by utilizing immediate environment and using it for play. They are also more likely to be directly involved in PA with their children, whereas, parents of more affluent families focus on organized opportunities more often than their peers who belong to lower-income families (56). Further, a study has indicated that preschoolers with middle-aged parents were more likely to have PA less than needed (57).

On the other hand, as children`s increasing SCT is being reported even among 1-3-years-old toddlers, in some researchers` view, there is a

positive relation between parents` education and less increase in SCT of children under three (58).

Parents` Behavior: Support and Role Modeling

In evaluating factors related to toddlers` SCT change conducted on 1827 children in Finland, they found that higher SCT of mothers has an effect on children`s larger SCT increase (58). Again, assessing parents SCT as the strongest predictor of child SCT in another study supports the idea of the influence of parental role modeling and behavior on children`s ST. Parents attitude is another factor which affects child`s ST (51). Additionally, children`s TV time being partially mediated by parental TV time had also been observed in an ENERGY-project done in seven European countries on 5729 children and their parents (59). Moreover, children from physically inactive families have been showed that are 3.5 times more inactive compared with their peers with physically active both parents (22). Again, parental PA role modeling at age of 11-12 observed by some researchers as one of the three strongest predictors of lower levels of children`s screen-based SB at age 13-14 also emphasizes the vital importance of parental role modeling in shaping children`s PA/SB behavior (54). Petersen et al., assessed 39 studies in a systematic review and reported that there was a weak positive association between child and parent PA regardless of child age, parent-child dyad gender, and type of PA (60).

In spite of such clear effects of parental behavior and role modeling for their children`s PA, they are disparately assessed yet. For example, even though, there appear to be some evidence of significant positive relationships between fathers-children weekday and weekend vigorous physical activity (VPA) (61), fathers influence on children`s PA has been evaluated modest while positive in some other documents (62).

Parental support of children`s PA may occur through their participation, supervision, transportation or encouragement. It has been shown that parents` supportive role for school-age children aged 11-13 can strongly predict children`s PA, MVPA and screen-based SB at age 13-14 (54). Furthermore, associations between home social environment and girls` after-school PA and ST have been observed in a study due to parental support effects on girl`s afterschool TPA, MVPA and ST. Home social environment elements had been assessed by 3 subscales:

a) Parental LTPA and sports participation (evaluated through items about two sports most frequently-played, leisure time TV watching, walking, and biking)

b) Parental support for children`s PA (either tangible support such as transportation, PA participation with children or supervision or intangible support such as encouragement)

c) Family rules to monitor the time children spent on watching TV and playing video/computer games (48).

Seeking more parents-related factors of children`s PA, some researchers` view is that lack of parental cooperation and negative interactions between child and parents might act as barriers to PA of children. In fact, family interplay can serve as a barrier to moderating child`s PA (63). Again, based on the study done on more than five thousand children in several countries in Europe, children`s TV time can be partially mediated by modeling effect of parents` sport participation (59).

Parental Physical and Mental Well-being

The physical and mental well-being of parents not only has associations with their own PA and obesity (64, 65), but also influences children`s PA and SB. As a study shown, parents` perceived work-life stress negatively affects family interplay which is considered as a notable link between child`s PA habits at home and parental stress (63). Data on 56 women who had Roux-en-Y Gastric Bypass (RYGB) surgery at 5 Swedish hospitals and objective PA measurements of their 75 children aged 7 to 14 between 3months before and 9 months after maternal RYGB showed an increase in children`s SB and a significant decrease in their MVPA without any observed difference for women or their spouses (66). Consequently, maternal depressive symptoms association with higher risk for preschoolers` obesity and even scarcely measured link between mother anxiety and dissatisfaction with it are samples supporting the idea (67). Findings of this kind can alarm us that how much vulnerable our children could be.

Neighborhood Environmental Factors of Child`s PA

It has been proposed that spending time outdoors can result in many positive outcomes for children`s physical and mental health from decrease in their anxiety, stress or asthma to increase in well-being feeling of them (68-70). Consequently, a limited daily time spent

outdoors among children is a global concern. According to some investigations, it is only 4 to 7 minutes for average children (71, 72). Anyway, identification of neighborhood child`s PA factors seems to be helpful for decision makers, practitioners, designers, families and all of those who seek procedures [to provide children with higher daily PAL.

Table 2. Socioeconomic Factors determining Children`s PA

Home Socioeconomic Factors of Child`s PA			Affected Item	reference studies				
				Title	Authors	Date	Participants	Location
Family SES	1	Parent`s SES (Household income)	Child`s MVPA	(52)	Dumuid D et al	2016	427 schoolchildren aged 9-11	Australia
	2	Parents` SES, Maternal Schooling	Child`s PA	(55)	Knuth AG et al	2017	2604 preschoolers Aged 6	Brazil
	3	SES (family income)	Child`s PA	(56)	Cottrell L et al	2015	566 schoolchildren and preschoolers aged 5-15	Rural West Virginia
	4	Parental Age	Child`s PAL	(57)	Botey AP et al	2016,	631 schoolchildren and preschoolers aged 2-13	Canada
	5	Parents` Education	Toddlers` SCT	(58)	Matarma T et al	2016	1827 preschoolers Aged 1-3	Finland
Parents` Role Modeling and Supportive Behavior	6	Maternal SCT	Toddlers` SCT	(58)	Matarma T et al	2016	1827 preschoolers Aged 1-3	Finland
	7	Family active/inactive mode	Child`s activity	(22)	Zaltauskee V et al	2016	3802 schoolchildren aged 7-8	Lithuania
	8	Home social environment: family rules, Parents` LTPA and supportive behavior (participation, supervision, transportation, encouragement)	Girls` afterschool PA/ST	(48)	Lau EY et al	2015,	671 schoolchildren 6th grade	South Carolina
	9	Family rules	Child`s SB	(50)	Roberts JD et al	2017	144 schoolchildren aged 9-10	Washington DC

Abbreviations: PA: physical activity; SES: socioeconomic status; MVPA: moderate-to-vigorous; SCT: screen time; LTPA: leisure time physical activity; ST: sedentary time; SB: sedentary behavior.

Presence, accessibility, usage frequency and different characteristics of neighborhood built environment features (such as recreational facilities and public green/open spaces, sports/play grounds, sidewalks, cycling paths and local streets) and their PA supportive equipment, neighborhood commute mode, parent/child concerns, perceptions and priorities, family SES, and neighborhood social disparities are involved in neighborhood-related environmental factors of child`s PA and SB.

Neighborhood Physical Factors of Child`s PA

Neighborhood PA Supportive Destinations Presence and Accessibility

It has been proposed that the presence of and access to neighborhood destinations and local services related to child development have positive association with early childhood physical health and well-being (73). There are reports indicating that close proximity to recreation places influences on preschoolers` PA (74). As shown by a case study, shorter experienced or perceived walking distance (equal to or less than 10 minutes) to neighborhood destinations e.g. outdoor swimming pool, skiing or other kinds of winter recreation areas, relative`s or friend`s home, biking/hiking/walking trails or paths and public open spaces had been significantly reported by more parents of active children who had fulfilled American daily PA recommendations (60min/day). Similarly, statistics have showed

lower relative odds of those children when their parents perceived more walking distance to nearest bus or metro train stations from their home (75).

Additionally, according to some other examinations, distance and use of unstructured public open spaces/parks are correlated with girls` LTPA, and greater distance to them leads in decreasing their use for LTPA by children (53). Moreover, higher frequency of PA among both preschoolers and school-age children aged 1-12 and longer duration of PA as well as less SCT for school-age children aged 7-12 have been observed in the case of closeness of urban green spaces (76). Some researchers hold an opinion of other neighborhood built environment factors like play equipment access as notable predictors of child`s recommended daily PA (60min/day) fulfilling. Actually, greater existence of active play supportive facilities and amenities in their neighborhood built environment are reported by parents of more active children (75). Researchers take the view that neighborhood open/green spaces distance from child home affect their mental health too. Data from a study on 3586 children aged 5.9 from Scotland has showed that children whose home is more than 20 minutes (walking distance) far from open/green spaces, not only display over 2 hours more weekly TV time than their peers with less than 5 minutes walking distance from neighborhood green/open spaces, but also have worse mental health (77).

In spite of the fact that public open spaces serve as key elements of built environment of neighborhoods supporting wide ranges of PA, some inconsistent and mixed associations between their various features and PA have been reported (78). For instance, the association between girl`s playgrounds availability and their BMI appear to become moderated in some cases by family SES and race or ethnicity status. For example, it has been demonstrated that higher availability of playgrounds is associated with White and high-SES girls` lower BMI percentile but higher BMI percentile among girls from African-American and low-SES families. While, for boys, SES is reported to moderate the association between their availability of parks and BMI (79). Meantime, a NET-Works study on 534 low-income parents of preschoolers revealed surprisingly no significant association between frequency of park use and children`s LPA or MVPA while showed that it is inversely related to children`s less ST and positively associated with parental more MPA and less ST. Moreover, according to this study, park use frequency was significantly positively associated with parent-reported supportive behaviors for children`s PA (80).

Neighborhood Features Characteristics

Both ways (sidewalks, cycling paths, local and main streets) and relevant destinations of neighborhood such as recreational public places are involved in this part of child`s PA factors study.

Neighborhood features and characteristics, in some researchers` view, can influence child`s PA/SB and other factors of their physical-mental health. Associations between some neighborhood attributes like it`s walkability and more incivilities in the home-surrounding immediate block with more park use frequency have been observed (80). So are neighborhood environment safety-related issues (e.g. traffic safety, social safety) effects mentioned through different researches results as child`s PAL predictors, whether perceived by parents (73-75) or children (81). Some researchers have conducted investigations into clusters of neighborhood attributes that influence child`s PA/SB. For example, a longitudinal cross-sectional study on children aged 5-6 and 10-12 showed that a distinct cluster with characteristics of mixed land use, many playgrounds and sport places had contributed to children`s less TV viewing on weekends 3 years later (82). Further, results from a national cross-sectional study on more than 3800 Lithuanian children aged 7.3 years indicated that family urban living area and recreational facilities and playgrounds availability were significantly associated with more likelihood of children sufficient daily PA (22).

As a result of a successful intervention in relation to neighborhood PA, Richard Krajicek Foundation seeking provision of safe public playgrounds stimulating daily PA of children living in deprived neighborhoods has reached to some satisfying results. Fortunately, the idea implementation has been showed statistically to lead in more usage and PA intensity among children in Netherlands. In fact, data revealed that children in addition to their higher energy-expenditure (EE) were involved in MVPA on tailored Krajicek playgrounds 3% more than on

the ten control playgrounds (13% vs. 10%). Moreover, Krajicek playgrounds were found significantly less often empty (83).

Streets are also involved in neighborhood built environment factors related to child`s PA. For example, results of a built environment and play study in Washington DC area showed that streets without cul-de-sac had been related to children`s higher SB (50). Further, a study in Indonesia explored influence of streets aspects on children`s activities and eventually classified them according to their scores (from the highest-score to the lowest-score determinant aspects that respectively had encouraged children to engage in more active to more passive activities in different local or main streets of their neighborhood): Traffic calming, size, layout, green space, play space, accessibility, quality of equipment and materials (81).

Table 3. Neighborhood Physical Factors of Child`s PA (Neighborhood PA Supportive Destinations Presence and Accessibility)

	Neighborhood PA Supportive Destinations Presence and Accessibility	Affected/Non-affected* Item	References				
			Title	Authors	Date	Participants	Location
1	unstructured parks/open spaces distance unstructured parks distance and use	their use by children for LTPA girls` LTPA	(53)	Fueyo JL et al	2016	1777 schoolchildren aged 9-11	Cordoba city
2	play equipment access, greater existence of active play supportive facilities and amenities, shorter walking distance to neighborhood destinations (up to 10 minutes)	child`s daily recommended PA achievement	(75)	Robert J D et al	2016	144 school-age children aged 7-12	Washington DC
3	closeness of urban green spaces	frequency of children`s PA (aged 1-12), PA duration and ST of children (aged 7-12), girl`s ST duration	(76)	Akpinar A	2017	422 parents of children aged 1-18	Turkey
4	longer open/green spaces distances	weekly TV time and mental health	(77)	Aggio D et al	2015	3586 preschoolers aged 5.9	Scotland
5	park use frequency park use frequency	children ST , parents ST/MPA child`s LPA/MVPA *	(78)	French SA et al	2017	534 low-income parents of preschoolers	USA

Abbreviations: PA: physical activity; MVPA: moderate-to-vigorous physical activity; LTPA: leisure time physical activity; ST: sedentary time; LPA: light physical activity; MPA: moderate physical activity.

Since many today-parents prefer to transport their children to relative neighborhood destinations, some suggest that turning child`s school or other destinations commute into an active transport can help to graft children`s PA onto their daily life (84). Hence, some studies have focused on children`s school commute mode. For instance, based on an study findings, children and their parents had preferred streets with three common characteristics including low traffic speed (30km/h vs. 50 or 70 km/h), separation of cycling path with a hedge (rather than a curb or nothing) and path evenness (vs. very uneven or moderately even) (84).

Again, another study conducted on 988 9-12-years-old children in Toronto revealed that about 40% of children including around half of those transported by motorized vehicle had shown preference to bike home-to-school distance. In addition, children`s lower BMI (among those participated in the spring, for the morning school trip) and higher PA (among those participated for the afternoon trip) were associated with their preference to cycle the home-to-school way (85). Additionally, an exploration into multi-level factors of school travel mode shift (from sedentary to active) led to identifying some required school commute environments changes encouraging such a kind of travel mode switch: shorter home-to-school distance, better safety, less cycle paths/lanes availability, and greater programs related to both safety and walking promotion. Moreover, the research showed that children with more outdoor places use after school transfer were more likely to change their school commute

mode to an active type (86). However, perceived walking time to school (PWTS) appears to act as the most important negative predictor of children`s active school commute when biking to or from school is considered unusual. Meanwhile, public transport accessibility, public school attendance, school service access and walking preconditions of designs and context have been shown in association with PWTS as well as public transport accessibility and school service access have been declared to be related to children`s active school commute (87).

Anyway, some intervention kinds seem to have worked at least partly. Some societies have already experienced different kinds of interventions in neighborhood built environments aiming at stimulating children`s more PA duration or frequency. For example, what has been implemented as Play Streets in San Francisco as is the case in seven other sites by closing neighborhood streets to be used by children for recreational activities in order to increase their PA, not only has strengthened the residents community, but also has led to children`s and youth`s increased engagement in vigorous PA (VPA) (88).

Table 4. Neighborhood Physical Factors of Child`s PA (Neighborhood Features Characteristics: Destinations and Routes)

Neighborhood Features Characteristic`s (Destinations and Routes)			Affected/Non-Affected* Item	References				
				No.	Author	Date	Participants	Location
Destinations Characteristics	1	walkability, safety, active play areas and esthetics importance to parents	Child`s Daily recommended PA Achievement	(75)	Robert J D et al	2016	144 school-age children aged 7-12,	Washington DC
	2	family urban living area and recreational facilities availability and playgrounds	children daily PA	(22)	Žaltauskė V et al	2016	3802 schoolchildren 7-8 years old	Lithuania
Streets, Sidewalks and Cycling Roads Characteristics	3	streets without cul-de-sac	Children`s SB	(50)	Roberts JD et al	2017	144 schoolchildren aged 9.7	Washington DC
	4	PWTS threshold, public transport accessibility and school service access public transport accessibility, public school attendance, school service access and walking preconditions of designs and context	child`s school active commute PWTS	(87)	Mehdizadeh M et al	2017	735 schoolchildren aged 7-9	Iran
	5	home-to-school distance reduction, walking-promotion programs provision and improved safety	school commute mode shift (sedentary-to-active)	(86)	Lee C et al	2017	165 primary-school-age children	Texas
	6	streets with cycle path separated with a hedge plus the path evenness and street low traffic speed	route preferences for child`s cycling alone	(84)	Ghekiere A et al	2015	305 schoolchildren from 5th and 6th grade	Belgium

Abbreviations: PA: physical activity; PWTS: perceived walking time to school.

Neighborhood Socioeconomic Factors

Parents` Perceptions, Concerns, Priorities and SES

Undoubtedly, parents play a vital role in children`s PA-related behavior in different environments. Nevertheless, parental perceived neighborhood built environment are discussed diversely as a considerable PA factor of children. For example, while some researches demonstrate that parents perceived neighborhood environment is not associated with child`s LTPA and BMI (53), there is evidence indicating importance of parental perception of neighborhood as children`s PA predictor. Exploring relationship between parents perception of built environment and children`s active play, a diverse population of 144 children aged 7-12 and their parents were observed in Washington DC. Findings revealed that walkability and safety, active play areas and esthetics of its built environment were 4

neighborhood-related factors considered important by parents of active children (75).
Some researchers have declared that parents` perception of neighborhood safety is positively related to early childhood general health and both social and emotional development (73). Moreover, it has been shown that among different races, parental perceived barriers can act as negative predictors decreasing the number of days children engage in 60min/day of PA or more in a week. Among white parents, concern over derivers excess of neighborhood speed limits, and among minority-race parents, perceived neighborhood crime rate had acted as positive predictors of children`s SB in a study (89).
Furthermore, it has been stated that parental preferences for and perceptions about home-to-school commute environment which are probably different from children`s can affect child`s school travel active or inactive mode. Apparently, children had considered cycle path evenness plus local speed limit while degree of cycle path separation from vehicle road and speed limit seemed to be associated significantly with the street parents had chosen and preferred for their children cycling along (84).
In addition, parental SES has its own effects on child`s PA in neighborhood environment. A study showed that their income and higher age had been in association with PWTS as well as more cars in family possessions, mothers` higher education and driving license had been related to children`s active school commute (87). Again, children`s greater independent mobility and at least one parent with part-time job (among those participated for the afternoon trip) have been reported to be associated with their preference to cycle the home-to-school way (85).

Neighborhood Social Disparities

Some scientists share the view that child`s psychological stress caused by multi-level environments with social disparities and injustice can have influences on their PA/weight status. Actually, interaction of these two recently mentioned may be affected by child`s psychological stress (44).

Table 5. Neighborhood Socioeconomic Factors of Child`s PA (Parents and Neighborhood Community)

	Neighborhood Community and Parents` Perceptions, Priorities and Concerns	Affected/Non-Affected* Item	References				
			Title	Authors	Date	Participants	Location
1	Parental Perceived Neighborhood	Child`s LTPA/BMI *	(53)	Fueyo JL et al	2016	1777 school-age children aged 9-11	Cordoba city
2	-parental perception of neighborhood walkability, safety, active play areas and esthetics importance -parental shorter perceived walking distance(up to 10 minutes) to neighborhood destinations	Child`s Daily Recommended PA Achievement	(75)	Robert J D et al	2016	144 school-age children aged 7-12	Washington DC
3	psychological stress caused by neighborhood social disparities	interactions of child`s PA and multi-level environments	(44)	Li Y et al	2016	65 schoolchildren aged 8-13	Eastern Alabama
4	SES and race/ethnicity status SES	association between girls` park/playground availability and BMI association between boys` park/playground availability and BMI	(79)	Hughey SM et al	2017	13469 school-age children 3rd-5th grade	US

Abbreviations: PA: physical activity; LTPA: leisure time physical activity; SES: socioeconomic status; BMI: body mass index.

School Environmental Factors of Child`s PA

Places out of home where children are taught or cared for in their parents` absence such as childcare center, family child care home (FCCH), preschool and school included in this part of the review. Totally, curriculum, equipment, material, staff`s knowledge, behavior, and role modeling in these different facilities can influence child`s PA whether directly or indirectly. Undoubtedly, due to great proportion of children`s early years

spent in such environments while their basic health habits are being shaped gradually as physical-mental growth and development are in process, it is necessary to focus on their relative environmental factors affecting on children`s PA.

School Physical Factors of Child`s PA

Based on young children`s PA recommendations (90), childcare PA of preschoolers should consist of at least 60 min structured PA engagement, at least 60 min unstructured activity involvement and less than 60 min SB per day unless they are sleeping. Nevertheless, according to some evidence, children`s PA in preschool seems to contribute poorly to their daily recommended PA fulfilment (91). On the other hand, there are reports indicating that procedures like children`s full-day kindergarten (FDK) enrolment may help them make up this disappointing situation partly. As shown by a study, FDK enrolled preschoolers` outdoor play time probability and PA participation likelihood are more than those of part-day kindergarten (PDK) enrolled children. In addition, it has been shown that the first group had less likelihood of TV watching on the weekdays compared to their PDK enrolled peers (92). Moreover, some researchers are of the opinion that children`s day care attendance results in smaller increase in children`s SCT (58). Totally, environmental factors associated with children`s PA in preschools have been explored through studies conducted in different countries. Some researchers in the U.S have declared children`s outdoor play time, indoor play space suitability and indoor play teacher encouragement but not participation as the strongest 3-5-years-old preschoolers` MVPA environmental predictors (93). On the other hand, researchers in Brazil have described indoor recreation room and parks/playgrounds as 4-years-olds` protective factors against their highly SB (allowing consistent motor activities and games which stimulate children not to remain still). They have also stated indoor recreation room as a factor inversely associated by 5-years-olds` PA and demonstrated indoor recreation room, playground/park and recess as predictors which increase the likelihood of 6-years-olds` more activity (91).

Meanwhile, investigators in the U.K. found no significant association between childcare environmental factors (fixed/portable equipment, active/sedentary opportunities, time allowed outside, time children seated, time reported spent in gross motor play) with preschoolers` LPA/MVPA. Nevertheless, they found that both active opportunities and play in snow outside had been positively associated with children`s SB. Consequently, they have suggested that childcare policies encouraging child-driven plays letting children have freedom of movement indoors and outdoors in childcare environment might be more effective in stimulating preschoolers` childcare PA. They also came to the conclusion that childcare environment had a limited effect upon preschoolers` in-care PA so that other environments or communities like parent-child groups might be more helpful in preschoolers` PA facilitating or stimulating (94). Again, in Australia, results from a cross-sectional study on 68 toddlers aged 1.0-2.9 and 233 preschoolers aged 3.0-5.9 revealed differences in interactions with their childcare environment between these two groups. Actually, children`s less SB was in relationship with sedentary places for toddlers vs. portable play equipment for preschoolers (95).

Surprisingly, explorations in Ohio resulted in no association between indoor play environment, outdoor playground, fixed/portable play equipment and weather/clothes policies of childcare environments with children`s PA there. Researchers found that children with at least 60 min/day outdoor time spent in childcare centers had more MVPA in both childcare time and during the rest of their day(96).

A systematic review article published in 2015 supports the just mentioned idea by evaluating overall influence of outdoor time on 3-12-years-olds` more PA and less SB positively. In fact, they have provided evidence indicating that children had 2.2 to 3.3 times higher PA outdoors compared with indoors (29).

For older children, school environment may provide poor or satisfying PA opportunities. It is said that nearly half, in Strong`s opinion (97) or up to 40% (98) of the time needed for children`s daily PA (60min) can be provided through their school recess. As shown by a study, schoolyard characteristics and recess are affective considerably on school-age children`s PA. An 8-week intervention on elementary school children during 2013 showed that procedures such as pavement painting or providing proper playground equipment could raise the school-age children`s PA during the recess (99). Further, results of a behavior examination on 316

students in 5 schoolyard types suggested that the highest children`s MVPA had occurred in grass areas and playgrounds while solid surface areas had been related to their highest ST proportion. In addition, the examination revealed that girls had spent more ST in comparison with boys in all types of schoolyard areas (98). Again, through a playground environment assessment aiming at identifying areas promoting schoolchildren`s MVPA in two different urban schools one of which had offered a Jog and Walk Stars (JAWS) program, researchers observed that the most populated area of schoolyard differed from the general blacktop areas on non-JAWS days with approximately 50% of children sedentary there to the JAWS track with 99% of children participating in MVPA (100). Seeking procedures effective in promoting children`s PA at school environment, other interventions have been also examined. Guided plays implementation aiming at taking advantages of both child`s natural abilities to learn through play experience expressing their autonomy and prepared environments in addition to adult scaffolding simultaneously has been suggested as a rather successful pedagogical approach (101).

Child`s PA increasing school-based opportunities such as short breaks designated to PA indoors/outdoors may be effective given their complete reach and low costs per child. After-school programs, in spite of their apparently lower reach, could lead in some socioeconomic benefits such as parental participation that could partly make up their higher costs.

Table 6. School Physical Factors of Child`s PA

School Physical Factors of Child`s PA		Affected/Non-Affected* Item	References				
			No.	Author	Date	Participants	Location
1	outdoor time	children`s PA and SB	(29)	Gray C et al	2015	children aged 3-12	Different
2	painting on schoolyard pavement and providing proper playground equipment	children`s school PA during recess	(99)	Grant V et al	2015	approximately 150 school-age children: 3rd-6th grade	American Indian reservation
3	child`s day care attendance	SCT	(58)	Matarma T et al	2016	1827 preschoolers Aged 1-3	Finland
4	indoor recreation room and playground indoor recreation room indoor recreation room, recess, playground	4-years-olds` SB 5-years-olds` PA 6-years-olds` activity likelihood	(91)	Barbosa SC et al	2016	370 preschoolers aged 4-6	Brazil
5	outdoor play time, indoor play space suitability	Children`s MVPA	(93)	Henderson KE et al	2015	389 preschoolers aged 3-5	U.S
6	childcare environmental factors active opportunities and outside play in snow	preschoolers` LPA/MVPA * Children`s SB	(94)	Hesketh KR et al	2016	201 preschoolers aged 3-4	U.K
7	sedentary places for toddlers portable play equipment for preschoolers	children`s SB behavior	(95)	Peden ME et al	2017	68 toddlers aged 1.0-2.9, 233 preschoolers aged 3.0-5.9	Australia
8	indoor play environment, outdoor playground, foxed/portable play equipment, weather/clothes policies outdoor time in childcare centers	children`s PA * MVPA	(96)	Copeland KA et al	2016	388 preschoolers	Ohio

Abbreviations: PA: physical activity; MVPA: moderate-to-vigorous; SCT: screen time; SB: sedentary behavior; LPA:light physical activity.

School Socioeconomic Factors of Child`s PA

Teachers, child care providers and staff in addition to parents can play a crucial role in shaping school PA behavior and levels of both preschoolers and school age children. Actually, they are reported to have both positive and negative effects on children`s PA. Although, some disparities are also reported. For example, child care providers are said to influence on child`s health behavior through their role modeling for

both children and parents (102, 103). Accordingly, it can be a matter of concern in view of the fact that an exploration on 166 family child care home (FCCH) providers in North Carolina revealed that nearly 90% of them were obese while about half of them fulfilled neither PA nor fruit and vegetable guidelines. Additionally, more than half of the providers had reported high stress (103). Further, FCCHs weaker PA and nutrition regulations related to children in comparison with preschools (104, 105) are another problem.

It is necessary to mention that the accuracy of participants` PA levels classification could be questioned in the case of studies based on self-reported data, and so are the objectively measured cases in accelerometer-based studies due to the device limitations and bias (106).

On the other hand, U.S researchers have demonstrated children`s indoor play teacher encouragement but not participation as the strongest 3-5-years-old preschoolers` MVPA environmental predictors (93).

Meanwhile, U.K. investigators studies have not found any significant associations between interpersonal factors such as class composition, children per staff member, government funded places, staff behavior/mean age in years/mean years in childcare/mean years at setting with preschoolers` LPA/MVPA (94). Similarly, explorations in Ohio resulted in no association between PA training of staff and with children`s PA there (96).

Table 7. School Socioeconomic Factors of Child`s PA

	School Physical Factors of Child`s PA	Affected/Non-Affected* Item	References No.	Author	Date	Participants	Location
1	U.K childcare interpersonal factors	preschoolers` LPA/MVPA*	(94)	Hesketh KR et al	2016	201 preschoolers aged 3-4	U.K
2	indoor play teacher encouragement but not participation	preschoolers` MVPA	(93)	Henderson KE et al	2015	389 preschoolers aged 3-5	U.S
3	FCCH providers` role modeling for children and parents	preschoolers` PA habits and outcomes	(103)	Tovar A et al	2017	166 FCCH providers	North Carolina
4	PA training of staff	children`s PA	(96)	Copeland KA et al	2016	389 preschoolers	Ohio

Abbreviations: PA: physical activity; MVPA: moderate-to-vigorous; LPA: light physical activity; FCCH: family child care home.

Study limitations

Despite a major strength of the present study was its wide range of studied locations, the explored literature was limited to those published in English.

Conclusion

The review emphasizes the importance of the parental role and proper presence, availability and safety of PA supportive spaces, routes and equipment as the most frequently considered physical predictors of child`s PA. Family-based education policies addressing promotion of parents` knowledge about relevant topics should be enforced. Consideration of the mentioned characteristics by planners and designers may stimulate more active commute and active plays among children in all of the three environments. More attention to parents` involvement in school PA supportive programs may be effective as well. After all, play opportunities (eitherer structured and supervised or free and unstructured), proper material, active play fixed/portable equipment and interventions should be applied based on settings, locations climatic conditions, context and design characteristics, children`s age, gender and requirements.

Moreover, the season's effects on children's PA and environments have not been properly considered in previous studies due to their largely short duration Explored factors in the reviewed studies rarely or limitedly involved cultural and economic factors or some of those related to the environment architecture or context (such as climate, light, color, dimensions, form, natural/artificial ventilation or the environments spaces view). In addition, there were some disparities in evaluated associations between child`s PA factors and outcomes that might have been caused by some neglected items such as cultural, racial or ethnic differences, child`s age, gender or family conditions. Physical factors were explored in a wider range in the case of neighborhood environment. Socioeconomic factors were more discussed than physical factors in the case of home environment while they were rarely studied in relation to school environment. Therefore, additional studies considering these items are warranted.

Conflict of Interests

The authors confirm no conflict of interests.

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JOURNAL OF NUTRITION FASTING AND HEALTH

Epidemiological Evaluation of Water- and Foodborne Outbreaks in the United States and Europe

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ABSTRACT

Introduction: Water- and foodborne illnesses are of major public health concerns. However, the significance of foodborne diseases are generally underestimated. Therefore, in this study we aimed to emphasize on the importance of control of foodborne illnesses trough highlighting data about outbreaks, hospitalizations and deaths caused by contaminated food in the developed countries from 2015 to 2020.

Method: In this descriptive-analytical study, 105 and 152 cases of water- and foodborne illnesses were reported in the United States (CDC) and Europe (ECDC) in 2015-2020.

Results: The most reported causative agents were *Salmonella* spp*, Cyclospora, Escherichia coliBacillus cereus, Clostridium perfringens* spp, and *Listeria monocytogenes* were in the US and *Salmonella,* Norovirus*,* Calicivirus*, Campylobacter, B. cereus,* and *C. perfringens* in the EU.

Conclusion(s): According to the results, CDC and ECDC analyses could provide insights into the most critical pathogens and food sources help the authorities to control foodborne illnesses.

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Introduction

Many pathogens can infect humans through food consumption (1). According to a CDC report, an outbreak of foodborne illness occurs when two or more people become infected from the same food or drink (2). Foodborne diseases range from mild illnesses to severe problems, which sometimes endanger people's health and lead to their hospitalization and death (3).

Contamination can occur at any stage of the food supply chain (production to distribution) (4). Factors such as cross-contamination, mixing raw and cooked food, undercooking, poor personal hygiene, and favorable conditions for microbial growth may cause food contamination. Foodborne illnesses occur through consuming contaminated food (5). There is also evidence that raw foods may be contaminated by wildlife, soil, air, irrigation water, and fertilizer (6). The fecal-oral route can also transmit pathogenic microorganisms from one person to another (6). The transmission of diseases through contaminated water and food is primarily biological in nature (7-9). More than 250 types of foodborne diseases (FBDs) have been identified worldwide. Bacteria and their toxins, followed by viruses and parasites, are the most common biological agents (10). Foodborne illnesses are primarily caused by viruses and bacteria (11). The high prevalence of some causes of water- and foodborne diseases such as *Shigella* put them in the group of bioterrorist agents (12). Foodborne diseases can also occur by consuming chemicals such as heavy metals or toxins from plants and animals (3). Food processing environments can also be considered a source of contamination. Therefore, food safety requires inspection of the processing environment, especially when cleaning and disinfection methods fail (13). However, testing the final products is not sufficient to ensure safety, and a negative result does not mean that there are no microbes in the entire product (13). Nowadays, the number of people infected by foodborne pathogens has also been increased due to increasing the number of centers for food preparation and distribution such as restaurants and snack shops and using foods such as fast foods without requiring long cooking and high heat (12).

Additionally, foodborne illnesses are becoming a significant challenge due to emerging microorganisms and toxins and increasing antibiotic resistance and food contamination from new production methods (14).

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Water- and food-borne illnesses are of major causes of death that threaten socioeconomic development worldwide (15). According to World Health Organization (WHO), about 600 million people worldwide become ill each year from contaminated food, of which 420,000 die, and 33 million lose their healthy lives (16). Consequently, a foodborne disease monitoring system is needed to identify, monitor, and warn about FBDs. Such system helps identify the cause and burden of FBDs, and thus, it help reduce FBDs and their harm in the society (15).

Foodborne illness prevalence is monitored by the Centers for Disease Control and Prevention (CDC) in the United States and the European Centers for Disease Control and Prevention (ECDC) in the EU Member States (17).

Each year, the CDC summarizes foodborne diseases in its annual monitoring report and publishes the data through the NORS[1] dashboard. These statistics and epidemiologic data assist policy-making and decision-making.

ECDC is an EU agency, which aims to strengthen the European defense system against infectious diseases. The main tasks cover a broad spectrum of activities, including scientific advice, microbiology, public health training, health communication, international relations, surveillance, epidemic intelligence, response, preparedness, and publishing the scientific journal of Eurosurveillance (18). Water- and foodborne diseases and common human and animal diseases are among the issues addressed by the ECDC (18,19).

The two monitoring systems provide information such as the patient's location, cause, prevalence, number, severity, as well as the foods associated with reported FBDs and outbreaks (20).

Based on our review, a comparative data collection has been provided by gathering information about the prevalence of water- and foodborne illnesses published by the CDC (in the United States) and ECDC (in Europe). Thus, food safety hazards can be detected faster, warned earlier, and prevented and controlled if we have a clue (21). Providing scientific evidence to authorities may also make it easier for them to develop the most efficient strategies to prevent and control foodborne illnesses.

Materials and Methods

This descriptive-analytical study reviewed reports of foodborne outbreaks in the US and EU during a five-year period from 2015 to 2020, which were available on the CDC and ECDC websites (9,22). Generally, the criteria for diagnosing foodborne diseases were based on epidemiological findings, incubation period, and clinical findings in patients, while laboratory diagnosis relies on determining the cause of outbreaks (23).

Table 1. Reported cases of water- and foodborne outbreaks in the United State of America (CDC)

Year	Type of contaminated food	Contaminating microorganisms	Number of patients	States	Number of hospitalizations	Number of deaths	Recall[2]
2015	Raw Sprouted Nut Butter Spreads	*Salmonella* Paratyphi *B* variant L(+) tartrate(+)	13	10	0	0	✓
	Rotisserie Chicken Salad	*E. coli* O157:H7	19	7	5	0	✓
	Mexican Style Restaurant Chain	*E. coli* O26	55	11	21	0	-[3]
			5	3	1	0	-
	Soft Cheeses	*Listeria monocytogenes*	30	10	28	3	✓
	Cucumbers	*Salmonella* Poona	907	40	204	6	✓
	Pork	*Salmonella I* 4,[5],12:i:- and *Salmonella* Infantis	192	5	30	0	✓
	Unknown	*Cyclospora*	546	31	21	0	✗
	Raw, Frozen, Stuffed Chicken Entrees	*Salmonella* Enteritidis	5	1	2	0	✓
	Raw, Frozen, Stuffed Chicken Entrees	*Salmonella* Enteritidis	15	7	4	0	✓

1. National Outbreak Reporting System

2 . If you have recalled products, don't eat them. Throw them away.

3 . There was no report

Year	Type of contaminated food	Contaminating microorganisms	Number of patients	States	Number of hospitalizations	Number of deaths	Recall[2]
	Frozen Raw Tuna	*Salmonella* Paratyphi B variant L(+) tartrate(+) and *Salmonella* Weltevreden	65	11	11	0	✓
	Ice Cream	*Listeria monocytogenes*	10	4	10	3	✓
2016	Shell Eggs	*Salmonella* Oranienburg	8	3	2	0	✓
	Unknown	*Cyclospora*	384	-	-	-	-
	Beef Products	*E. coli* O157:H7	11	5	7	0	✓
	Frozen Strawberries	Hepatitis A	143	9	56	0	✓
	Frozen Scallops	Hepatitis A	-	-	-	-	✓
	Alfalfa Sprouts	*Salmonella* Reading and *Salmonella* Abony	36	9	7	0	✓
	Flour	*E. coli* O121 and O26	63	24	17	0	✓
	Frozen Vegetables	*Listeria monocytogenes*	9	4	9	3	✓
	Raw Milk	*Listeria monocytogenes*	2	2	2	1	✗
	Pistachios	*Salmonella* Montevideo and *Salmonella* Senftenberg	11	9	2	0	✓
	Alfalfa Sprouts	*E. coli* O157	11	2	2	0	✓
	Alfalfa Sprouts	*Salmonella* Muenchen and *Salmonella* Kentucky	26	12	8	0	✗
	Organic Shake & Meal Products	*Salmonella* Virchow	33	23	6	0	✓
	Packaged Salads	*Listeria monocytogenes*	19	9	19	1	✓
2017	Leafy Greens	*E. coli* O157:H7	25	15	9	1	✗
	Unknown	*Cyclospora*	1065	-	-	-	-
	Maradol Papayas	*Salmonella* Urbana	7	3	4	0	✗
	Maradol Papayas	*Salmonella* Newport and *Salmonella* Infantis	4	4	2	0	✗
	Maradol Papayas	*Salmonella* Anatum	20	3	5	1	✓
	Maradol Papayas	*Salmonella* Infections	220	23	68	1	✓
	Vulto Creamery Soft Raw Milk Cheese	*Listeria monocytogenes*	8	4	8	2	✓
	I.M. Healthy SoyNut Butter	*E. coli* O157:H7	32	12	12	0	✓
2018	Tahini Produced by Achdut Ltd.	*Salmonella* Concord	8	4	0	0	✓
	-	*Salmonella* Agbeni	7	5	0	0	✓
	Pork Products	*Listeria monocytogenes*	4	4	4	0	✓
	Romaine Lettuce	*E. coli* O157:H7	62	16	25	0	✓
	Raw Chicken Products	*Salmonella* Infantis	129	32	25	1	-
	Ground Beef	*Salmonella* Newport	403	30	117	0	✓
	Deli Ham	*Listeria monocytogenes*	4	2	4	1	✓
	Ground Beef	*E. coli* O26	18	4	6	1	✓
	Gravel Ridge Farms Shell Eggs	*Salmonella* Enteritidis	44	11	12	0	✓
	Chicken	*Salmonella I* 4,[5],12:i:-	25	6	11	1	-
	Raw Turkey Products	*Salmonella* Reading	358	42	133	1	✓
	Hy-vee Spring Pasta Salad	*Salmonella* Sandiego	101	10	25	0	✓

Year	Type of contaminated food	Contaminating microorganisms	Number of patients	States	Number of hospitalizations	Number of deaths	Recall[2]
	Fresh Express Salad Mix Sold at McDonald's Restaurants	*Cyclospora*	511	16	24	0	✗
	Del Monte Fresh Produce Vegetable Trays	*Cyclospora*	250	4	8	0	✓
	Imported Fresh Crab Meat	*Vibrio parahaemolyticus*	26	8	9	0	✗
	Kellogg's Honey Smacks Cereal	*Salmonella* Mbandaka	135	36	34	0	✓
	Pre-Cut Melon	*Salmonella* Adelaide	77	9	36	0	✓
	Shell Eggs	*Salmonella* Braenderup	45	10	11	0	✓
	Romaine Lettuce	*E. coli* O157:H7	210	36	96	5	✗
	Dried Coconut	*Salmonella* Typhimurium	14	8	3	0	✓
	Chicken Salad	*Salmonella* Typhimurium	265	8	94	1	✓
	Kratom	*Salmonella I* 4,[5],12:b:-	199	41	50	0	✓
	Raw Sprouts	*Salmonella* Montevideo	10	3	0	0	✗
	Frozen Shredded Coconut	*Salmonella I* 4,[5],12:b:- and *Salmonella* Newport	27	9	6	0	✓
2019	Hard-boiled Eggs	*Listeria monocytogenes*	8	5	5	1	✓
	Cut Fruit	*Salmonella* Javiana	165	14	73	0	✓
	Fresh Express Sunflower Crisp Chopped Salad Kits	*E. coli* O157:H7	10	5	4	0	✗
	Romaine Lettuce	*E. coli* O157:H7	167	27	85	0	✓
	Ground Beef	*Salmonella Dublin*	13	8	9	1	✓
	-	*Listeria monocytogenes*	24	13	22	2	-
	Fresh Basil from Siga Logistics de RL de CV of Morelos, Mexico	*Cyclospora*	241	11	6	0	✓
	Northfork Bison	*E. coli* O103 and O121	33	8	18	0	✓
	Papayas	*Salmonella* Uganda	81	9	27	0	✓
	Flour	*E. coli* O26	21	9	3	0	✗
	Karawan Brand Tahini	*Salmonella* Concord	6	3	1	0	✓
	Raw Oysters	Multiple Pathogens	16	5	2	0	✓
	Deli-Sliced Meats and Cheeses	*Listeria monocytogenes*	10	5	10	1	-
	Frozen Raw Tuna	*Salmonella* Newport	15	8	2	0	✓
	Pre-Cut Melon	*Salmonella* Carrau	137	10	38	0	✓
	Ground Beef	*E. coli* O103	209	10	29	0	✓
	Butterball Brand Ground Turkey	*Salmonella* Schwarzengrund	7	3	1	0	✓
2020	Unknown Source 3	*E. coli* O157:H7	18	9	6	0	✓
	Leafy Greens	*E. coli* O157:H7	40	19	20	0	✗
	Unknown Source 1	*E. coli* O157:H7	32	12	15	1	-
	Deli Meats	*Listeria monocytogenes*	12	4	12	1	-
	Wood Ear Mushrooms	*Salmonella* Stanley	55	12	6	0	✓
	Peaches	*Salmonella* Enteritidis	101	17	28	0	✓
	Onions	*Salmonella* Newport	1127	48	167	0	✓
	Bagged Salad Mix	*Cyclospora*	701	14	38	0	✓

Year	Type of contaminated food	Contaminating microorganisms	Number of patients	States	Number of hospitalizations	Number of deaths	Recall[2]
	Enoki Mushrooms	*Listeria monocytogenes*	36	17	31	4	✓
	Clover Sprouts	*E. coli* O103	51	10	3	0	✓

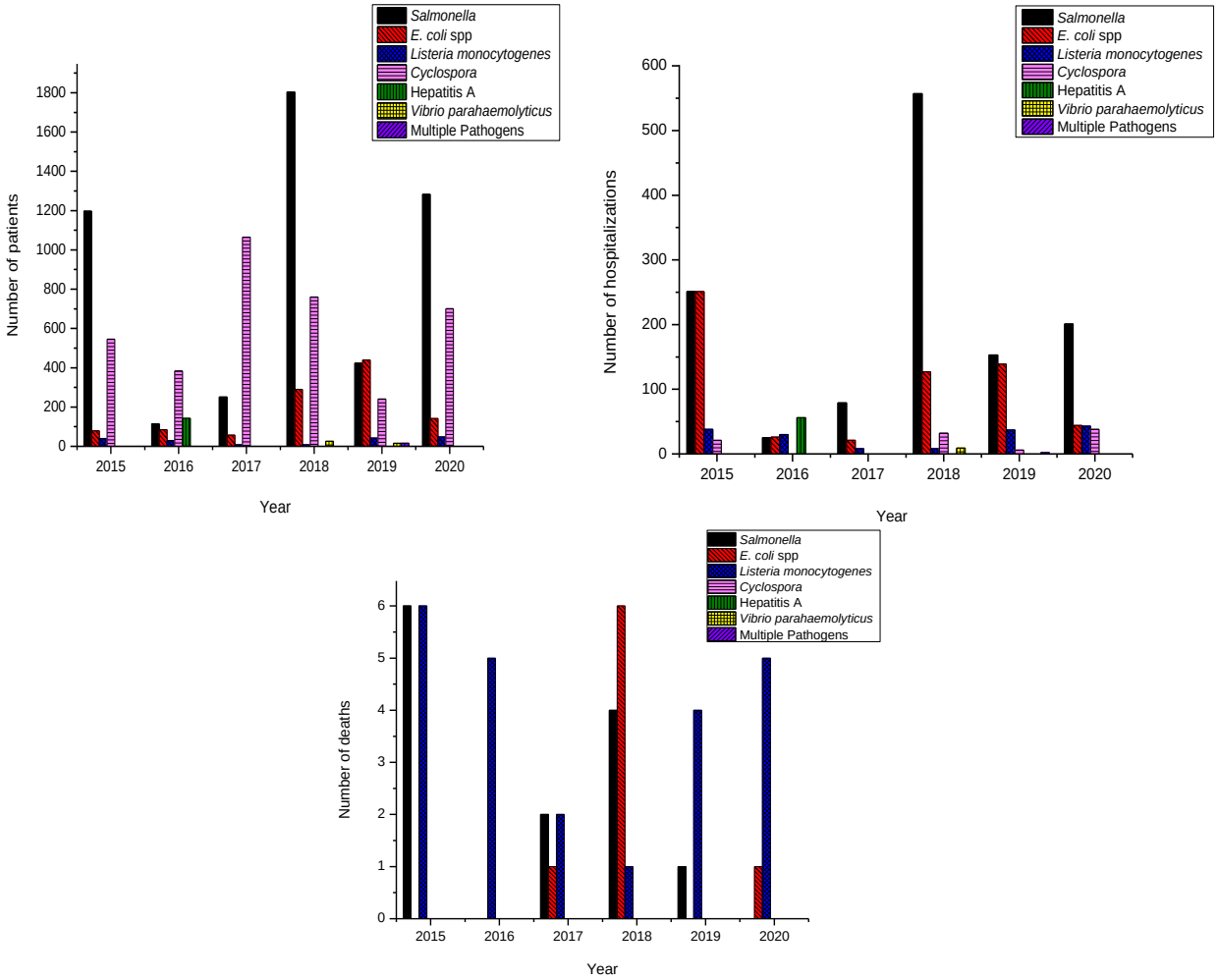


Figure 1. Reported cases of water- and foodborne outbreaks in the United State of America (CDC)

Results

CDC reported a total of many water- and foodborne pathogens were transmitted from water and food during 2015-2020. According to these reports, the lowest bacterial foodborne cases occurred in 2017 (8 cases), and the highest prevalence was in 2018 (24 cases). A total of 11 foodborne and waterborne illnesses occurred in the US in 2015, with cucumbers being the most common source and Salmonella Poona as the most common responsible pathogen. In 2016, a total of 14 water- and foodborne occurred and frozen strawberries were the most common source of FBDs, while cyclospora and hepatitis A were the most common causative pathogens. There were 8 cases of water- and food-borne illness in 2017. Maradol papayas were the most common source of FBDs, and Cyclospora and Salmonella Thompson were the most common causative pathogens. A total of 24 water- and foodborne illnesses were reported in 2018. Salad Mix Sold at McDonald's Restaurants and ground beef were the most common source of FBDs. In this regard, the most common causative pathogens were Cyclospora and Salmonella Newport. There were 17 water- and food-borne

outbreaks in 2019. Eating fresh basil from Siga Logistics de RL de CV from Morelos, Mexico, and ground beef were the most common source of FBDs. In addition, Cyclospora and E. coli O103 were the most common causative pathogens. There were a total of 10 water- and foodborne illnesses in 2020. Onions and bagged Salad Mix were the most common source of FBDs, and Salmonella Newport and Cyclospora were the most common causative pathogens. Table 1 presents the reported cases of water- and foodborne outbreaks in the United States (CDC).Table 1. Reported cases of water- and foodborne outbreaks in the United State of America (CDC)Figure 1. Reported cases of water- and foodborne outbreaks in the United State of America (CDC)According to ECDC, several pathogens were transmitted from water and food during 2015-2020 in Europe. The highest number of FBDs (35 cases) were reported in 2018 and the lowest number (17 cases) in 2016. There were 18 waterborne and foodborne illnesses reported in 2015 in Europe. Vegetables, fruits, cereals, sprouts, herbs, spices, and products thereof, Fish, shellfish, mollusks, crustaceans, eggs, and egg-based products, as well as meat and meat--based products were the most common source of FBDs, and Salmonella was the most common causative pathogen. There were 17 incidences of water- and food-borne diseases in 2016 that occurred. Vegetables, fruits, cereals, sprouts, herbs, spices and products, mixed food, Buffet meals, eggs, and egg-based products were the most common source of FBDs in these reports, and Salmonella was the most common causative pathogen. There were 21 cases of water- and foodborne diseases in 2017. Water, eggs, and egg-based products were the most common source of FBDs, and Salmonella was the most common causative pathogen. There were 35 water- and foodborne diseases reported in 2018. Water, eggs, and egg-based products were the most common source of FBDs in these outbreaks, and Salmonella was the most common causative pathogen. A total of 32 water- and foodborne diseases were reported in 2019. Water, eggs, and egg-based products were the most common source of FBDs, and Salmonella and Norovirus were the most common causative pathogens. The number of water-borne and foodborne diseases in 2020 reached 29. Fish and fishery products, water and other beverages, eggs, egg-based products, meat and meat-based products, were the most common source of FBDs, and Salmonella was the most common causative pathogen. Table 2 represents the reported cases of water- and foodborne outbreaks in Europe (ECDC).Table 2. Reported cases of water- and foodborne outbreaks in Europe (ECDC)Figure 2. Reported cases of water- and foodborne outbreaks in Europe (ECDC).

Table 2. Reported cases of water- and foodborne outbreaks in Europe (ECDC)

Year	Type of contaminated food	Contaminating microorganisms	Total outbreaks	Human cases	Hospitalized	Deaths
2015	Eggs, and egg products Meat, and meat products	*Salmonella*	953	6616	1719	3
	Milk, cheeses, and dairy	*Campylobacter*	387	1440	129	1
	Milk, cheeses, and dairy Mixed food, and Buffet meals Other foods	Shiga toxin-producing *E. coli* (STEC)	69	674	62	0
	Mixed food, and Buffet meals	*Listeria*	14	230	25	4
	Meat, and meat products	*Yersinia*	13	54	9	0
	-	*Vibrio*	4	29	0	0
	-	*Brucella*	1	2	1	0
	Eggs, and egg products Meat, and meat products	Other bacterial agents[4]	29	337	23	0
	Meat, and meat products Other foods	*C. botulinum*	24	60	43	0
	Milk, cheeses, and dairy Mixed food, and Buffet meals	Other bacterial toxins[5]	825	8787	454	3

4 . 'Other bacterial agents' include *Francisella, Shigella,* pathogenic *E. coli* other than Shiga toxin-producing *E. coli*, and other unspecified bacteria.

5 . 'Other bacterial toxins' include toxins produced by *Bacillus, Clostridium other than Clostridium botulinum, staphylococcal* toxins, and other unspecified bacterial toxins.

Year	Type of contaminated food	Contaminating microorganisms	Total outbreaks	Human cases	Hospitalized	Deaths
	Other foods					
	Vegetables, fruits, cereals, sprouts, herbs and spices, and products thereof	Calicivirus including norovirus (Norwalk-like virus)	289	13536	352	1
	Other foods	Hepatitis A	13	78	49	1
	Milk, cheeses, and dairy	Other viruses/unspecified[6]	99	1140	130	3
	-	*Cryptosporidium*	9	120	3	0
	Meat, and meat products	*Trichinella*	15	119	34	0
	-	Other parasites/unspecified[7]	28	63	7	0
	Fish, shellfish, mollusks, crustaceans, and products thereof	Other causative agents[8]	127	648	64	0
	Vegetables, fruits, cereals, sprouts, herbs and spices, and products thereof Fish, shellfish, mollusks, and crustaceans and products thereof	Unknown	1463	11941	788	1
2016	Milk, and milk products	*Campylobacter*	461	4606	140	0
	Meat, and meat products	*Listeria*	5	25	14	2
	Eggs, and egg products Other foods	*Salmonella*	1067	9061	1766	10
	Milk, and milk products Vegetables, fruits, cereals, sprouts, herbs and spices, and products thereof	Shiga toxin-producing *E. coli* (STEC)	42	735	125	3
	-	*Vibrio*	8	76	50	0
	Vegetables, fruits, cereals, sprouts, herbs, spices, and products thereof	*Yersinia*	8	41	3	0
	Eggs, and egg products Other foods	Other bacterial agents[9]	30	279	51	1
	Meat, and meat products	*C. botulinum*	18	49	39	0
	Meat, and meat products Mixed, food and Buffet meals	Other bacterial toxins[10]	830	8918	362	1
	Mixed food, and Buffet meals Other foods	Calicivirus including norovirus (Norwalk-like virus)	379	11993	404	1
	Meat, and meat products Other foods	Hepatitis A	16	155	63	0
	Milk, and milk products	Other viruses/unspecified[11]	75	937	97	0
	Meat, and meat products Vegetables, fruits, cereals, sprouts, herbs, spices, and products thereof	*Cryptosporidium*	6	62	0	0
	Meat, and meat products Other foods	*Trichinella*	5	14	9	0
	-	Other parasites/unspecified	7	17	0	0
	Fish, and Fisheries	Other causative agents	106	489	74	0
	Vegetables, fruits, cereals, sprouts, herbs and spices, and products thereof	Unknown	1723	12493	672	2

6 . 'Other viruses' include adenovirus, flavivirus, rotavirus, and other unspecified viruses.
7 . Other parasites include *Giardia* and other unspecified parasites.
8 . 'Other causative' agents include chemical agents, histamine, marine biotoxins, mushroom toxins, and scrombotoxin.
9 . Other bacterial agents include *Shigella* and other unspecified bacteria
10 . Bacterial toxins include toxins produced by *Bacillus, Clostridium* other than *Clostridium botulinum, Staphylococcus,* and other unspecified bacterial toxins.
11 . Other viruses include flavivirus and other unspecified viruses. Other causative agents include ciguatoxin and other unspecified toxins.

Year	Type of contaminated food	Contaminating microorganisms	Total outbreaks	Human cases	Hospitalized	Deaths
	Mixed food, and Buffet meals					
	-	*Brucella*	1	2	1	0
	Milk, and milk products[12] Meat, and meat products[13]	*Campylobacter*	395	1445	207	1
	Food of non-animal origin[14] Milk, and milk products	*Listeria*	10	39	22	2
	Eggs, and egg products Bakery products	*Salmonella*	1241	9600	2227	11
	Milk, and milk products	Shiga toxin-producing *E. coli* (STEC)	48	260	65	2
	-	*Vibrio*	3	59	7	0
	Food of non-animal origin Mixed food	Other bacterial agents/Unspecified[15]	46	816	67	0
	Meat, and meat products Other foods[16]	*C. botulinum*	9	26	26	2
2017	Buffet meals Mixed food	Other bacterial toxins[17]	809	8442	577	5
	Water Buffet meals	Norovirus, and other caliciviruses	211	6550	153	2
	Food of non-animal origin	Hepatitis A	90	591	452	2
	Milk, and milk products Food of non-animal origin	Other viruses/unspecified[18]	97	1379	107	0
	-	*Cryptosporidium*	5	15	0	0
	Meat, and meat products	*Trichinella*	11	199	125	0
	-	Other parasites/unspecified	13	28	1	0
	Fish, and Fisheries[19]	Histamine	117	572	51	0
	Fish, and Fisheries	Marine biotoxins[20]	54	170	14	0
	Other foods	Mushroom toxins	7	22	16	0
	-	Other/Unspecified	3	6	0	0
	Water Other foods	Unknown	1882	12794	423	6
	-	Unspecified	27	385	20	0
	Food of non-animal origin[21] Milk, and milk products[22]	*Aeromonas*	1	7	2	0
2018	Milk, and milk products Meat, and meat products[23]	*Campylobacter*	524	2335	135	0
	Food of non-animal origin Milk, and milk products	*Enterococcus*	1	4	4	0

12 . Milk and milk products include 'Cheese', 'Dairy products (other than cheeses)' and 'Milk'.
13 . Meat and meat products include 'Bovine meat', 'Pigmeat', 'Poultry meat', 'Sheep meat', 'Other or mixed red meat and their products, 'Meat and Meat products unspecified'.
14. Food of non-animal origin includes 'Confections, 'Fruits (and juices)', 'Herbs and spices, and 'Vegetables (and juices)'.
15 . Other bacterial agents include enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), *Shigella flexneri, Yersinia enterocolitica*.
16 . Other foods include 'Canned food products', 'Cereal products and legumes', 'Other foods (Unspecified)'.
17 . Bacterial toxins other than *Clostridium botulinum* include toxins produced by *Bacillus, Clostridium* other than *Clostridium botulinum* and *Staphylococcus,* and other unspecified bacterial toxins.
18 . Other viruses include adenovirus, flavivirus (TBE virus), rotavirus, and other unspecified viruses.
19 . Fish and fishery products include: 'Fish', 'Crustaceans, shellfish, mollusks, and their products.
20 . Marine biotoxins include ciguatoxin and other unspecified toxins.
21 . Food of non-animal origin includes fruits (and juices), herbs and spices, sweets and chocolate, and vegetables (and juices). Milk and milk
22 . Milk and milk products include cheese, dairy products (other than cheeses), and milk.
23 . Meat and meat products include bovine meat, pig meat, poultry meat, sheep meat, other or mixed red meat and products thereof, meat and meat products, unspecified.

Year	Type of contaminated food	Contaminating microorganisms	Total outbreaks	Human cases	Hospitalized	Deaths
	Food of non-animal origin Milk, and milk products	*E. coli* other than STEC	9	240	11	0
	Food of non-animal origin Milk, and milk products	*Leptospira*	1	8	6	0
	Buffet meals Food of non-animal origin Other foods[24]	*Listeria*	14	158	98	21
	Eggs, and egg products Bakery products	*Salmonella*	1581	11581	2298	8
	Milk, and milk products	Shiga toxin-producing *E. coli*	48	381	36	0
	Food of non-animal origin Milk, and milk products	*Shigella*	33	472	63	0
	Food of non-animal origin Milk, and milk products	*Vibrio parahaemolyticus*	10	31	0	0
	Food of non-animal origin Milk, and milk products	*Yersinia enterocolitica*	12	58	7	0
	Food of non-animal origin Milk, and milk products	Other unspecified bacteria[25]	3	29	4	0
	Mixed food, other foods, and unknown	*B. cereus*	98	1539	111	1
	Mixed food, other foods, and unknown	*C. botulinum*	15	48	35	2
	Mixed food, other foods, and unknown	*C. perfringens*	71	1783	18	2
	Mixed food, other foods, and unknown	*S. aureus*	114	1124	167	0
	Other foods Mixed food	Bacterial toxins, unspecified[26]	652	5232	203	1
	Food of non-animal origin Milk, and milk products	Adenovirus	1	2	0	0
	Food of non-animal origin Milk, and milk products	Flavivirus including tick-borne encephalitis virus	10	34	29	0
	Food of non-animal origin Water	Hepatitis A	56	380	281	0
	Food of non-animal origin Milk, and milk products	Hepatitis E	3	6	1	0
	Fish and Fisheries[27] Buffet meals, and Water	Norovirus, and other caliciviruses	389	8507	219	2
	Food of non-animal origin Milk, and milk products	Rotavirus	20	249	70	0
	Food of non-animal origin	Other viruses, unspecified[28]	50	748	6	0
	Food of non-animal origin	*Anisakis*	3	20	1	0
	Water	*Cryptosporidium*	9	43	1	0

24 . Other foods include canned food products, cereal products, legumes, drinks, including bottled water, and other foods, unspecified.
25 . Other bacterial agents include *Aeromonas hydrophila, Escherichia coli,* enteroinvasive *Escherichia coli* (EIEC)*,* enterotoxigenic *Escherichia coli* (ETEC)*, Enterococcus, Leptospira* spp.*, Shigella* spp.*, Shigella flexneri, Shigella sonnei, Yersinia enterocolitica,* and other unspecified bacteria.
26 . Bacterial toxins other than *Clostridium botulinum* include toxins produced by *Bacillus*, *Clostridium* other than *Clostridium botulinum, Staphylococcus,* and other unspecified bacterial toxins.
27 . Fish and fishery products include crustaceans, shellfish, mollusks, and products thereof, fish and fish products.
28 . Other viruses include adenovirus, flavivirus, hepatitis E, rotavirus, and other unspecified viruses.

Year	Type of contaminated food	Contaminating microorganisms	Total outbreaks	Human cases	Hospitalized	Deaths
2019	Food of non-animal origin	*Giardia*	18	45	2	0
	Meat, and meat products[29]	*Trichinella*	10	114	76	0
	Food of non-animal origin	*Taenia saginata*	1	2	0	0
	Fish, and Fisheries	Histamine/Scombrotoxin	80	488	115	0
	Fish, and Fisheries	Marine biotoxins	53	266	6	0
	Food of non-animal origin Other foods	Mushroom toxins/Mycotoxins	13	71	26	3
	Milk, and milk products Food of non-animal origin	Other causative agent/Unspecified[30]	21	296	32	0
	Unknown Water	Unknown/Unspecified	1223	12071	527	0
	-	*Arcobacter*	1	40	0	0
	Milk, and milk products	*Brucella*	1	2	1	0
	Meat, and meat products[31]	*Campylobacter*	319	1254	125	0
	Food of non-animal origin[32] Buffet meals	*E. coli* other than STEC	10	277	9	0
	Meat, and meat products	*Listeria monocytogenes*	21	349	236	31
	Eggs, and egg products Bakery products	*Salmonella*	926	9169	1915	7
	Food of non-animal origin Buffet meals	*Shigella*	22	106	19	0
	Water[33] Milk, and milk products[34]	STEC	42	273	50	1
	Food of non-animal origin Buffet meals	*Vibrio*	4	15	6	0
	-	*Yersinia*	15	149	14	0
	Food of non-animal origin Buffet meals	Other bacteria, unspecified[35]	3	33	0	0
	Mixed food Other foods[36] Unknown	*B. cereus*	155	1636	44	7
	Food of non-animal origin Other foods	*C. botulinum*	7	17	15	1
	Buffet meals Meat, and meat products	*C. perfringens*	75	2426	27	3
	Milk, and milk products Buffet meals	*S. aureus*	74	1400	141	0
	Unknown Mixed food	Bacterial toxins, unspecified	686	5076	134	3
	-	Adenovirus	1	8	0	0
	Milk, and milk products	Flavivirus including Tick-Borne Encephalitis virus	3	15	12	0

29 . Meat and meat products include bovine meat, pig meat, poultry meat, sheep meat, other or mixed red meat and products thereof, meat and meat products, unspecified.
30 . Other causative agents include atropine, lectin, monosodium glutamate, and chemical agents unspecified.
31 . Meat and meat products include bovine meat and products thereof, broiler meat (Gallus) and products thereof, other or mixed red meat and products thereof, other, mixed or unspecified poultry meat and products thereof, pig meat and products thereof, sheep meat and products thereof, turkey meat and products thereof.
32 . Foods of non-animal origin include 'Cereal products including rice and seeds/pulses (nuts, almonds)', 'Fruit, berries and juices and other products thereof', 'Fruit - the whole', 'Herbs and spices', 'Nuts and nut products, 'Vegetables', 'Vegetables - pre-cut, 'Vegetables and juices and other products thereof'.
33 . 'Water' includes Tap water, including well water.
34 . 'Milk and milk products' include cheese, dairy products (other than cheeses), and milk.
35 . Other bacteria' includes enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *Escherichia coli* (ETEC), *Escherichia coli, unspecified, Shigella, Vibrio parahaemolyticus, Yersinia,* and other unspecified bacteria.
36 . 'Other foods' include canned food products and other foods, unspecified.

Year	Type of contaminated food	Contaminating microorganisms	Total outbreaks	Human cases	Hospitalized	Deaths
	Food of non-animal origin Bakery products	Hepatitis A, and other Hepatitis viruses unspecified	22	135	99	0
	-	Hepatitis E	3	6	1	0
	Water Buffet meals	Norovirus	457	11125	279	0
	-	Rotavirus	8	85	51	0
	-	Sapovirus	1	89	0	0
	Milk, and milk products	Other viruses, unspecified[37]	59	764	14	0
	Food of non-animal origin Water	*Cryptosporidium*	11	468	4	0
	-	*Giardia*	14	233	2	0
	Meat, and meat products	*Trichinella*	5	44	12	0
	-	Other parasites, unspecified	1	2	0	0
	Fish, and fishery products[38]	Histamine / Scombrotoxin	96	428	52	0
	Fish, and fishery products	Marine biotoxins	48	214	14	0
	Other foods	Mushroom toxins	5	43	11	1
	Eggs, and egg products Other foods	Other causative agent/Unspecified[39]	6	88	3	0
2020	-	*Brucella*	1	2	2	0
	Milk, and milk products[40] Water (and other beverages)	*Campylobacter*	317	1319	112	0
	Meat, and meat products[41]	*E. coli* other than STEC	2	12	10	0
	Fish, and fishery products[42] Milk, and milk products	*Listeria monocytogenes*	16	120	83	17
	Eggs, and egg products[43] Meat, and meat products	*Salmonella*	694	3686	812	7
	Meat, and meat products	*Shigella*	5	58	14	0
	Water (and other beverages)[44] Milk, and milk products	Shigatoxin-producing *E. coli* (STEC)	34	208	30	1
	Meat, and meat products	*Vibrio parahaemolyticus*	4	56	0	0
	Composite foods, multi-ingredients, and other foods[45]	*Yersinia*	16	236	11	0
	-	Bacteria, unspecified	3	58	5	0

37 . 'Other viruses' includes flavivirus and other unspecified viruses.
38 . 'Fish and fishery products' include 'crustaceans, shellfish, mollusks, and products thereof, as well as 'fish and fish products.
39 . 'Other causative agents include atropine, mushroom toxins/mycotoxins, and unspecified toxins.
40 . Milk and milk products include 'Cheese', 'Cheeses made from cows' milk', 'Dairy products (other than cheeses)', 'Milk, cows' - pasteurized milk', 'Milk, cows' - raw milk', 'Milk, goats' - raw milk', 'Milk, sheep's - raw milk.
41 . Meat and meat products include 'Bovine meat and products thereof', 'Broiler meat (Gallus) and products thereof', 'Meat and meat products, 'Meat from bovine animals - meat products', 'Meat from bovine animals - meat products - ready-to-eat', 'Meat from a pig - fresh', 'Meat from a pig - meat products - fresh raw sausages', 'Meat from poultry, unspecified - meat products - non-ready-to-eat', 'Meat from wild boar - meat products – fresh raw sausages', 'Meat, mixed meat - meat products - ready-to-eat', 'Other or mixed red meat and products thereof', 'Other, mixed or unspecified poultry meat and products thereof', 'Pig meat and products thereof.
42 . Fish and fishery products include 'Crustaceans, shellfish, mollusks, and products thereof', 'Fish - smoked', 'Fish - smoked - hot-smoked, 'Fish and fish products, 'Live bivalve mollusks - oysters'.
43 . Eggs and egg products include 'Eggs', 'Eggs - raw material (liquid egg) for egg products', 'Eggs - table eggs - the mixed whole', 'Eggs and egg products.
44 . Water (and other beverages) includes 'Tap water, including well water', 'Water'.
45 . Composite foods, multi-ingredients foods, and other foods include 'Bakery products', 'Bakery products - cakes', 'Bakery products - cakes -containing raw cream', 'Bakery products - desserts - containing raw eggs', 'Bakery products - pastry - yeast leavened pastry', 'Buffet meals', 'Canned food products', 'Mixed food', 'Other foods', 'Other processed food products and prepared dishes', 'Other processed food products and prepared dishes-meat based dishes', 'Other processed food products and prepared dishes - pasta', 'Other processed food products and prepared dishes - pasta based dishes', 'Other processed food products and prepared dishes -sushi', 'Soups', 'Sweets and chocolate'.

Year	Type of contaminated food	Contaminating microorganisms	Total outbreaks	Human cases	Hospitalized	Deaths
	Foods of non-animal origin[46] Composite foods, multi-ingredients, and other foods	*B. cereus* toxins	71	835	10	1
	Foods of non-animal origin Composite foods, multi-ingredients, and other foods	*C. botulinum* toxins	9	34	34	0
	Composite foods, multi-ingredients, and other foods Foods of non-animal origin	*C. perfringens* toxins	32	682	10	2
	Milk, and milk products Meat, and meat products	*S. aureus* toxins	43	402	32	0
	Meat, and meat products Composite foods, multi-ingredients, and other foods	Bacterial toxins, unspecified	372	2564	96	3
	Raw sheep's milk and/or raw goat's milk	Flavivirus (including Tick-borne Encephalitis virus)	5	12	12	0
	Foods of non-animal origin	Hepatitis A	7	206	105	0
	-	Hepatitis E	3	6	2	0
	Fish, and fishery products Composite foods, multi-ingredients, and other foods	Norovirus, and other Calicivirus[47]	130	2633	90	1
	Milk, and milk products	Other viruses, unspecified	10	151	2	0
	-	*Anisakis*	2	6	0	0
	Foods of non-animal origin	*Cryptosporidium*	3	34	1	0
	-	*Enterocytozoon bieneusi*	1	77	0	0
	-	*Giardia*	2	4	0	0
	Meat, and meat products	*Trichinella*	6	119	13	0
	Fish, and fishery products	Histamine, and Scombrotoxin	43	183	17	1
	Fish, and fishery products	Marine biotoxins[48]	23	120	6	0
	Foods of non-animal origin	Other causative agents	3	55	0	0
	Fish, and fishery products Water (and other beverages)	Unknown / Unspecified	1229	6139	166	0

Discussions

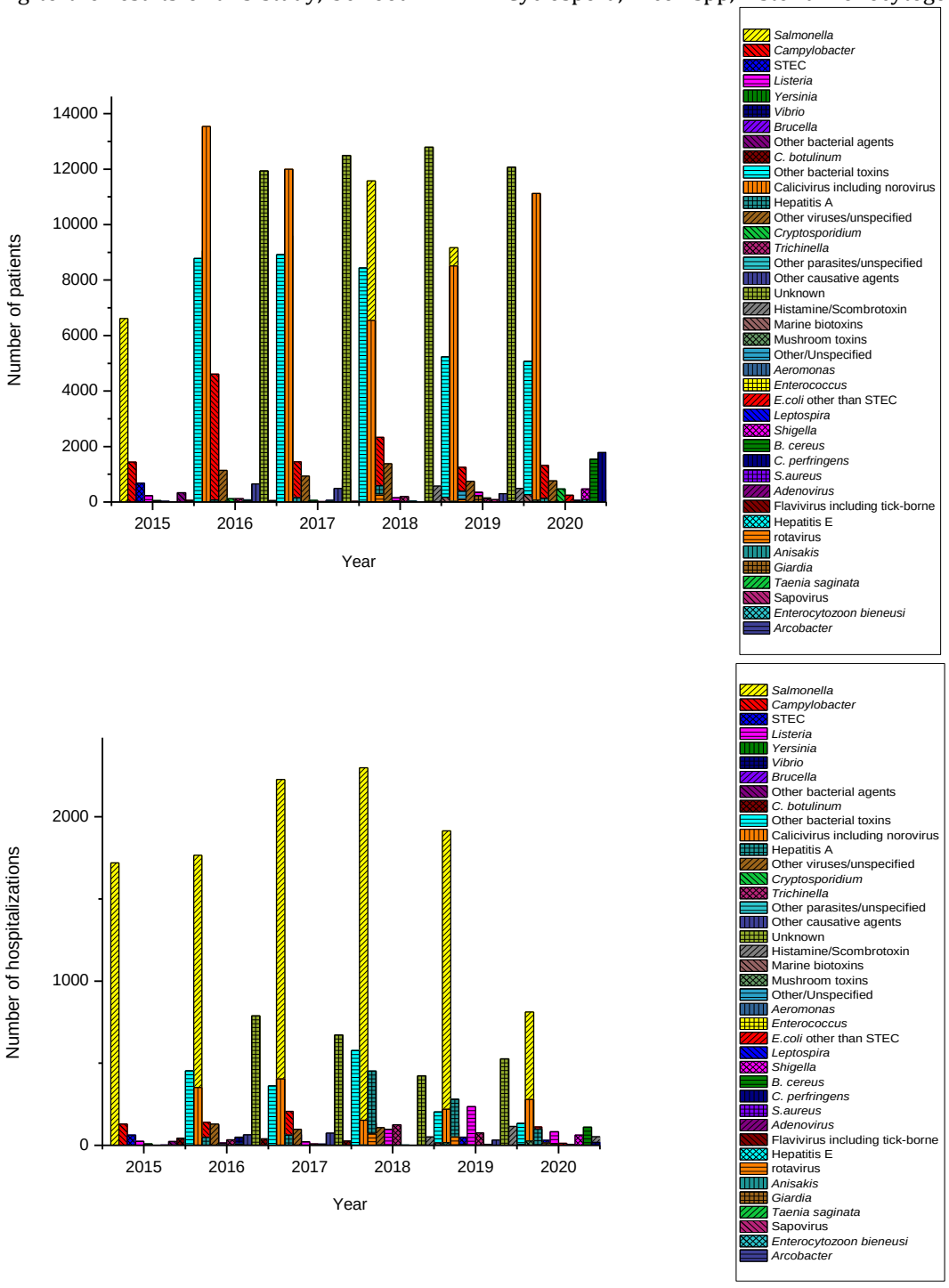
Factors such as the globalization of food supply, large-scale production, widespread distribution of food, emergence of new pathogens, eating out, and increasing proportion of consumers facilitate the risk of foodborne disease outbreaks, which are difficult to control (24, 25). A small percentage of foodborne diseases are reported, which may be due to the scattered pattern of foodborne diseases (26). In addition, more attention from authorities is paid to this issue due to the widespread interstate, restaurant-related outbreaks, or those that can cause serious illness, hospitalization, or even death (26). As a result, these systems represent only a small portion of the foodborne disease burden (26). This study does not include all outbreaks, but only those that are confirmed by surveillance systems (26). CDC reports that foodborne disease outbreaks (FBDOs) are increasing annually, which does not correspond to the actual increase in FBDOs and illustrates the positive effect of government oversight (26). Food contamination due to poor hygiene should be more considered in developing countries at every production stage, from farm to table (25). The cases reported in both databases include bacterial, parasitic and viral pathogens as well as

46 . Foods of non-animal origin include 'Cereal products including rice and seeds/pulses (nuts, almonds)', 'Fruit, berries and juices and other products thereof', 'Fruit - a whole', 'Herbs and spices', 'Nuts and nut products, 'Vegetables', 'Vegetables - pre-cut, 'Vegetables and juices and other products thereof'.

47. 'Norovirus and other caliciviruses include norovirus (Norwalk-like virus), sapovirus (Sapporo-like virus), and calicivirus unspecified.

48 . Marine biotoxins include ciguatoxin and other unspecified marine toxins.

chemicals and toxins (27). Based on this study, bacterial pathogens cause most outbreaks and infections among the mentioned outbreaks. According to the results of this study, 50 food- and water-borne pathogens led to morbidity and mortality in consumers in the US. The most important of which were Salmonella spp, Cyclospora, E. coli spp, Listeria monocytogenes.



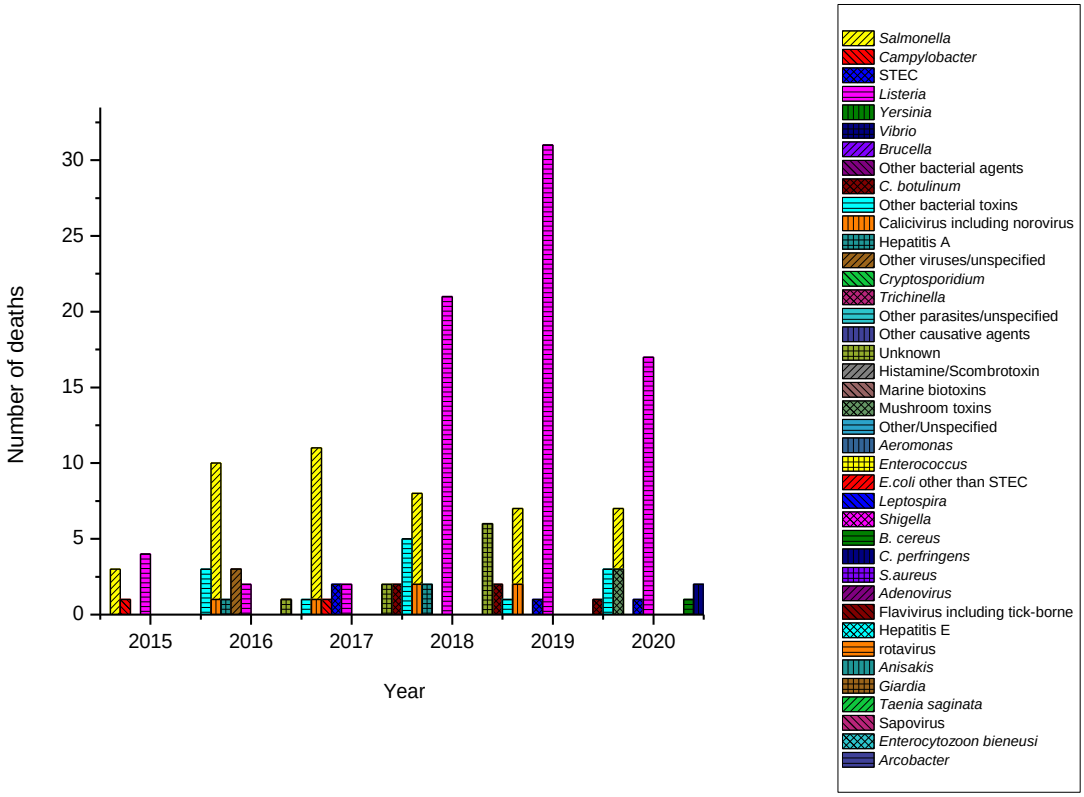


Figure 2. Reported cases of water- and foodborne outbreaks in Europe (ECDC)

In Europe, 36 food- and water-borne pathogens were reported by ECDC, and according to our review the most important of which were Salmonella, Norovirus, and other Calicivirus, Campylobacter, B. cereus, C. perfringens. Based on both monitoring systems, *Salmonella* resulted in the highest incidence of FBDOs and hospitalization of people and *Listeria monocytogenes* caused the highest mortality rate in the US and EU. As a part of the gut microbiota of animals, *Salmonella* spp. is one of the most common bacteria that contaminates food and causes hospitalization. Intestinal infections are commonly caused by *Salmonella.* Food poisoning caused by *Salmonella*, especially in children, is one of the public health problems nowadays (28). This microorganism has been growing rapidly due to the progress of the food industry, food preparation, and international transport and imports and exports (28). The use of raw and half-cooked foods such as chicken meat, eggs, and their products, milk and dairy products, meat and meat-based products and flour, as well as eating in restaurants, causes this disease (28). *Listeria* is another concerning foodborne pathogen causing deaths even in developed countries. There is a concern about the ability of *L. monocytogenes* to remain viable and grow at freezer temperatures equal and lower than 4°C. *L. monocytogenes* can also form biofilm on various surfaces, making it more resistant to environmental stress and leading to problems in disinfection and surface hygiene (29). The primary way of *L. monocytogenes* infection is through consuming contaminated food (29). Listeriosis can be asymptomatic or cause febrile gastroenteritis in healthy individuals. However, invasive infection cases can lead to septicemia, meningoencephalitis, and fetal loss (29). Although listeriosis has a low prevalence, it has the highest hospitalization rate (94%) among the main pathogens of food poisoning (29).

In the US, contaminated fruits were the primary cause of foodborne illnesses from 2015 to 2020, followed by ground beef as the second case. In Europe, the highest incidence of FBDOs was associated with eggs and egg-based products. Raw products have an increasing role in outbreaks. Crops such as fruits are the most common foods associated with raw produce outbreaks. The consumption of some fruits has increased, and improved transportation methods

for raw products may not accompany the increase in consumption. In addition, FDA recommendations are not always followed during the washing process. Consequently, improper food storage practices during washing and preparation can contribute to outbreaks associated with raw fruits. Fruits may be contaminated at many stages of production, from farm to table through contact with surfaces contaminated with feces of wild or domestic animals, soil, contaminated irrigation water or rainwater spray, equipment used during washing, chemicals, cooling, sorting, storing or packaging, and workers hands. In addition, using inappropriate time and temperature during storage may lead to the growth of bacteria from opening produce, such as cutting, slicing, shredding, or peeling. The lack of any further steps before consumption (e.g., cooking) and mentioned points emphasize the importance of promoting improved production and processing practices to reduce the contamination of raw products (30).

Contaminated food products of animal origin, especially eggs and egg-based products, are often implicated in outbreaks of human salmonellosis worldwide (31). Only 10^2 colony-forming units (CFU) of pathogenic *Salmonella* strains (*Salmonella Typhimurium* and *Enteritidis*) are required to cause disease in susceptible humans. Some *salmonella* species form a biofilm on the eggshell and spread the contamination. There is also evidence that Salmonella can survive on eggshells and grow in harsh conditions (31).

Undercooked or raw bovine products pose a risk for foodborne pathogens. Food items such as meat are considered to be among the most vulnerable perishable foods because of providing a favorable environment for microbe growth (32). Ground beef is a widely consumed food item in the United States, and diseases and outbreaks are commonly associated with the consumption of ground beef, especially undercooked ground beef (32). The small pieces of minced meat can act as a reservoir for bacteria, since it has a higher surface area and is cut into small pieces (32).

Viruses are also involved in FBDOs, and Chatziprodromidou (33) reported that norovirus and hepatitis A are viral pathogens commonly associated with fresh produce consumption. Fresh produce outbreaks are also often associated with *Cyclospora* and Cryptosporidium parasites (25, 34). From 2015 to 2020, in the US, contaminated fruits were the major cause of FBDs and the second main source of foodborne infections was ground Beef. While in Europe, the highest incidence of FBDs was attributed to eggs and egg products. Stricter hygiene measures are needed to reduce the cases of FBDOs with these interpretations.

Food safety risks can be controlled by the use of good agricultural practices, the HACCP program, good manufacturing practices, employee training to prevent food contamination, proper cleaning and disinfection of food contact surfaces, preventing cross-contamination, and maintaining good personal hygiene (25). Additionally, more attention should be paid to preventing foodborne diseases in homes (27).

Conclusion

According to the results, detecting foodborne outbreaks, determining the source of infection, and monitoring the food chain's hygiene are crucial to prevent and control FBDs. Therefore, establishing a comprehensive and specific system, such as the CDC and ECDC, for monitoring and assessing food safety is essential to improve public health, particularly in developing countries. Monitoring and prevention can effectively reduce the prevalence of FBDs in countries since most of them are considered controllable infections.

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Conflicts of Interest

The authors declared no conflict of interest.

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A Qualitative Analysis of Health Care Providers' Perceptions on Vitamin D Supplementation Program

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ABSTRACT

Previous studies have shown that the health burden associated with vitamin D deficiency is increasing globally. Vitamin D supplementation appears to be a feasible strategy for improving vitamin D status within populations. Little information is available on the perception and barriers to the widespread application of vitamin D supplements in Iran. Therefore, this study was conducted to explore the perception of health care providers regarding the implementation of vitamin D supplementation program in the Iranian cities of Mashhad, Qom and Zahedan.

This qualitative conventional content analysis study was conducted at 3 medical universities in Iran: Mashhad (MUMS), Qom (QUMS) and Zahedan (ZAUMS) University of Medical Sciences. These universities, are within regions with differing prevalence of vitamin D deficiency, and were selected based on the results of National Integrated Micronutrient Survey 2012 (NIMS-II study). Individual semi-structured in-depth interviews were performed with 103 participants (consisting of health professionals and health providers) to understand the perceptions of health professionals and health care providers'. The data were collected from December 2018 to July 2019. Guba and Lincoln's criteria were used to ensure the trustworthiness of the data. Data were analyzed using conventional content analysis based on the approach of Graneheim and Lundman's.

There were three categories of barriers to distribution and use of individual supplements, and the funding to implement the program; there were ten subcategories. Supplement distribution were affected by three subcategories of inadequate distribution of the supplement, irregular distribution of the supplement, and insufficient space to store the supplements. Individual barriers to the use of supplement comprised five subcategories: forgetting to take them, lack of knowledge about their benefits, accessing a health care center providing them, negative advertising for supplement use, and not taking them because of cost. Funding to implement the program contained the two subcategories of financial limitation in urban and rural area and financial limitation for all target groups.

The findings showed that health care providers reported a variety of barriers to supplement use. Applying a multiple strategy requires: training, conducting advertising campaigns, financial support, sufficient and regular distribution of the supplement and perhaps the use of alternative methods of supplement delivery, such as food fortification can be helpful.

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Introduction

There is substantial evidence for the important impact of vitamin D deficiency on public health; publications in this area have increased four-fold between1995 to 2015 (Quraishi et al, 2016). Vitamin D has been classically recognized as an important nutrient in the prevention and treatment of rickets and osteomalacia (Carpenter et al, 2017; Uday and Hoegler, 2017). However, new studies have demonstrated its possible roles in several other chronic conditions such as diabetes (Wu et al, 2017), cancer (Keum et al, 2019), autoimmune diseases (Altieri et al, 2017; Lin et al, 2016), and cardiovascular outcomes (Pliz et al, 2016). It has been estimated that 80% of the vitamin D requirements are derived from dermal synthesis due to UV-B exposure and 20% from dietary sources; several factors may effect vitamin D status: season/latitude, dietary habits, ethnicity, style of clothing, reduce optimal intake of this nutrient (Holick 2017; Macdonald et al, 2011). Several approaches have been suggested to improve vitamin D status, such as changing dietary habits toward vitamin D rich foods, increasing more outdoor activities and UV-B exposure, losing weight (to mobilize vitamin D and its metabolite from adipose tissues), supplementation, and fortification (Pilz et al, 2018). Although life style changes are potentially first steps in improvement of vitamin D status, they usually have a limited effect in the population; for example according to a recent meta-analysis, increasing fish consumption, a natural rich food in vitamin D, can increase serum 25(OH)D levels by 4.4 nmol/L (Lehmann et al, 2015). However greater degrees of exposure to UV-B may increase the risk of skin cancer. Hence, supplementation and fortification have emerged as feasible strategies for improving vitamin D status within populations.

Reports from *National Health and Nutrition Examination Survey* (*NHANES*) have indicated that supplement use in populations can significantly increase 25(OH)D across all age groups, race, gender and ethnics (Whiting and Calvo, 2005; Moore et al, 2005; Nesby-O'Dell et al, 2002). Nevertheless, the benefits of supplements were reported to be greater in individuals whose vitamin D intakes were above the median intake of population and individuals whose intake is less from foods had a lower beneficial gain from supplementation (Whiting and Calvo, 2005). One significant advantage of supplements is that there is no need to change dietary patterns. Supplements deliver a precise dosage of nutrients and minimize the risk of toxicity. On the other hand, supplementation may have several disadvantages; supplementation is more expensive than fortification in the context of public health and requires the adherence of individuals, which may be affected by its high cost and the knowledge and beliefs about their benefits (Vieth 2012). There are also several challenges to adopting vitamin D supplementation into public health policies, such as the variations in the requirement for adequate vitamin D intake due to multivitamins and prescribed or over-the-counter supplements. Furthermore, to determine the magnitude of vitamin D fortification, it is necessary to have information regarding the amount of vitamin D intake and barriers to using vitamin D supplements in populations. Therefore, this study was conducted to explore the perception of health care providers regarding to the vitamin D supplementary program implementation in Mashhad, Qom and Zahedan, Iran.

Study Design

This qualitative conventional content analysis study was conducted at the Universities of Medical Sciences at Mashhad (MUMS), Qom (QUMS), and Zahedan (ZAUMS) in Iran. The study population consisted of health professionals, health headquarters staff, public health practitioners, and health care managers. These universities are within regions with varying prevalence of vitamin D deficiency and were selected based on the results of the National Integrated Micronutrient Survey 2012 (NIMS-II study).

Study Population

The participants received both verbal and written information about the study. The age groups in our study include the elderly (over 60 years old), middle-aged (60-30 years old), youth (18-29 years old), pregnant mothers and students (6-17 years old) except for children with the age range of 2 to 5 years old. The study data were collected from December 2018 to July 2019. Purposive maximum variation such as Job Type in the health system and work responsibilities was used to select the participants. Individual semi-structured interviews were performed with 103 participants to understand health professionals

and health care providers' perceptions and to investigate the cultural and behavioural factors influencing the intake of vitamin D supplements in Mashhad, Qom, and Zahedan. The interviews were performed in the health centers, and the interviews were carried out in places that were convenient for the participants. The interview was conducted face-to-face or by telephone. The mean interview duration was 20 minutes (10-42 minutes).

Data Collection

For data gathering, a questions interview guideline was used to conduct in-depth semi-structured interviews. The interview questionnaires are available in Supplement 1. The interview began with general questions, such as "Have you encountered the use of vitamin D supplements as a health worker?" and moved to more specific, detailed questions as the interview advanced, such as "What kind of problems did you experience when attempting to supply vitamin D supplements? and "What are the barriers to taking and distributing vitamin D supplements?" Probe question, such as "Can you tell me more, please?" were asked to discover further data. Data collection was carried out until data saturation was achieved.

The Guba and Lincoln's criteria (Graneheim and Lundman, 2004), including credibility, confirmability, transferability, and dependability were used to assurance the trustworthiness of the data.

Data Analysis

Data were analyzed using the inductive, qualitative content analysis, which allows researchers to examine individual experiences (Graneheim and Lundman, 2004). After listening to the recorded interviews, the researcher transcribed and read them repeatedly to better understand their data. In the next step, meaning units (words, sentences, or paragraphs) that were related to each other through their content and context were identified (Graneheim and Lundman, 2004). The meaning units were condensed and given a descriptive code and were then organized into subcategories and categories. The categories were sets of different codes that shared the same content.

Results

A total of 103 participants were recruited, including Authorities of the Comprehensive Health centers, Healthcare Managers, Public Health Practitioners, Heads of Healthcare Centre, Heads of Network Development Group, Heads of Family Health Unit, Heads of Nutrition Unit, Deputy Heads of Healthcare, Executive Officers, Technical Officers, Heads of Network Development Unit, City Network Development Officer, Heads of Population/Family and schools Health, Heads of the Department of Nutrition Improvement, Heads of School, and Schools' Supplementary Implementation Officers.

Generally, the three categories of the supplement distribution, the individual barriers to use of supplement, and the funding to implement the program, and 10 subcategories emerged.

The Supplement Distribution

Supplement distribution comprised three subcategories of inadequate distribution of the supplement, irregular distribution of the supplement, and insufficient space to store the supplements. The likelihood of inadequate, or irregular distribution of the supplement based on age groups was a factor mentioned by the participants. "Distributing of supplements in different age groups has not been optimum for some time and has not been available for the whole year. It has been irregularly available in the Health Centers in a rural area). In this situation, people who are referred to Health Center were asked to get the supplement privately from the drugstore", (Contributor 39 in ZAUMS' Health Center). Another contributor emphasized that the supplement distribution was less than for the target population: "We gave the supplement to all centers but was not sufficient because the purchases were not sufficient to cover the target group", (Contributor 28 in the comprehensive Health Center). Prioritizing the distribution of supplements to the poorest areas and suburbs was also one of the factors contributing to the heterogeneous distribution of vitamin D supplement in the city: "The supplement is distributed to elderly and middle-aged subjects with the priority of suburban and rural area, but there is insufficient supplement available", (Contributor 47, Head of comprehensive Health Center).

In Zahedan, there was insufficient space to store the supplements, but stockroom capacity was reported as a problem to implement the program only in this city. The Head of the Family Health Unit in ZAUMS mentioned that "The supplement requests are set and delivered monthly because

of the lack of sufficient space in Health Centers and unsuitable conditions". Another contributor on hot weather and unsuitable storage conditions stated that "The health centers and schools have no suitable storage facilities for the supplements. Zahedan with hot and dry weather is warm all year round. Therefore, temperature <25º C as an important factor for storing vitamin D supplements, cannot be observed", (Head of Network Development Group).

A technical officer in health deputy stated that "There are currently insufficient vitamin D supplements available. A reason for this is that we faced an abrupt reduction in supplement distribution in the market; the drug distribution companies were not supplying many of the supplements, and so on. Because the prices have increased a lot, these increases have undoubtedly affected the program". Another contributor in a Health Center of the Qom said that "Despite sufficient funding, there were insufficient supplements available in the market. Then we had to start the vitamin D and iron supplementary program in February 2018. Based on the guideline, we should distribute nine vitamin D pearls (50.000 IU) to every student in high schools based on the guideline, only three supplements could be purchased and distributed to each student".

Solutions

To improve storage conditions, the necessary equipment for this and effective distribution were other important issues of contributors: "To supply cooling equipment during the hot seasons due to extremely high temperatures, for proper storage of all supplements are recommended in health posts, houses and health care centers of the city" (Contributor14, Head of Nutrition Unit, ZAUMS). "Supplement availability in the drug distribution companies is important because we had credit for a period of time, but there were no supplements available from the suppliers"(Contributor 52, Head of Nutrition Unit, ZAUMS). A public health practitioner in QUMS stated that "We should increase distributed supplements directly to people, especially to those who do not need other services and are merely referring to supplementation. This approach can help us to manage our time. For example, if we give them 6 supplements instead of 3, they will refer to the health care centers in a longer time than before".

Individual Barriers to Use of Supplement

Individual barriers to use of supplement comprisd five subcategories; forgetting to take them, lack of knowledge about their benefits, accessing a health care center, negative advertising for supplement use, and not taking because they are being provided free of charge. Most participants mentioned that going to the health care center is one of the important issues of using vitamin D supplements by people. Other barriers to the use of supplements were the lack of knowledge about the benefits and negative advertising for supplement use such as Family members, especially parents and teachers, not allowing the use of the supplements or being concerned about their potential negative health impacts.

A health care manager in Mashhad stated that "Resistance was greater in middle-aged subjects and they did not know what supplements were. Then, we taught them that the vitamin D supplement can be useful for osteoporosis and preventing heart disease, and they were then asking for the measurement serum vitamin D levels themselves and asking for vitamin D".

Another contributor mentioned that "Some elderly and middle-aged subjects cannot attend Health Centers, because they have mobility problems", (A public health practitioner, ZAUMS).

A Head of a High School said that "It has been rumored by the public that the vitamin D supplement may affect the fertility of students in the future".

Solutions

Training of the public was one of the main suggestions that contributors mentioned about taking vitamin D supplement.

"When some mothers are referred to Health Centers after delivery, we find that they have not consumed any vitamin D supplements at all. They mention that the doctor did not say anything about its consumption. Education and people's awareness should be raised. But generally, vitamin D supplement is much better consumed than iron because it is consumed once a month" (health care manager, MUMS). "Distributing brochures and educational posters on the vitamin D importance and its properties to people can be useful. Because some people sometimes forget in the follow-up what the supplement is. The brochure was already there, but in limited size", (public health practitioner in

QUMS). "Training about the importance of vitamin D intake and its deficiency through educational mass media should be increased because people do not even know how to get sunlight to make vitamin D in the body and to prevent vitamin D deficiency by taking Vitamin D pearls" (contributors 40, health care practitioner, ZAUMS). Practical training and counseling by health care providers to increase the willingness and awareness was the other participant suggestions, so that one of the participant stated this: "Education and counseling by health care professionals about vitamin D intake is important to people because if somebody is aware of the benefits, he/she can be persuaded to use vitamin D supplement and can be obtained from the drugstore", (Contributor 44 county of family health unit).

Funding to Implement the Program

Funding to implement the program contained the two subcategories of financial limitation in urban and rural area and financial limitation for all target groups. Funding was one of the most important issues raised by participants in 3 different provinces. Contributor 2, a health deputy in ZAUMS stated that "The urban per capita funding for health is much lower than the rural per capita, but the urban population is greater. So, there is a financial limitation in the city, and on the other hand given the current situation, there is also a financial limitation in a rural area. Moreover, much of the credit is also spent on providing the experts, and insurance deductions make it worse".

A contributor at MUMS' health deputy mentioned, "We have financial limitations to provide the required number of supplement for all target groups and prioritize our resources for vulnerable age groups. That is, we put the priority first on the pregnant women, then on students. Among the students, girls are the top priority".

Contributor 1, a Health Deputy in QUMS mentioned that "Supplements have been purchased for all age groups according to the guideline, but we could not completely provide all required supplements due to economic issues and increased prices". Another contributor is a health care center in Qom said, "Vitamin D supplements have been distributed as a guideline, but due to inadequacy, priority has been given to rural areas and city surrounds. So that, poor people hurt less, thus the supplement not distributed in some areas because there were no enough supplements".

Solutions

Timely funding allocation to purchase the vitamins was one of the most essential suggestions the participants mentioned: "Timely funding should be sent to the cities to purchase and distribute the supplements to students during educational year to prevent happening any problems" (Contributor 38, Head of school, ZAUMS).

Moreover, ordering the vitamins from drug distribution companies several months before starting the program was another subject that was mentioned: "Requests should be sent to pharmaceutical companies a few months in advance in order to prepare supplement in time" (Head of family health, contributor 44, ZAUMS).

"It is recommended that sufficient funding be provided for the preparation and distribution of supplements, together with supplying sufficient supplements in the market", (Head of Network Development Unit, QUMS).

Direct funding by the health ministry to make the program more effective was another issue raised by the contributors: "In the supplement distribution between units, the purchase and distribution depend on the city because the credit is allocated to every city .If the supplementary is effective, it is advisable that the supplementary budget be spent directly by the health ministry and if it is not effective, it should not be done" (Contributor 46, Public health manager, ZAUMS)

Others

Other problems encountered in implementing the vitamin D supplementary program were: insufficient supervision, lack of sufficient experts, hot weather, and transportation issues to get to health care center.

Solutions

Applying alternative methods for vitamin D supplements, such as sun exposure, proper diet, and food fortification was another suggestion that may be effective in preventing the vitamin deficiency and saving costs.

"The financial and economic cost of the supplements is important generally supplementation has early effectiveness and is one of the four recommended strategies of the World Health Organization. But food fortification, such as fortified bread with

vitamins D or other micronutrients including iron and folic acid has a very good effect, at a very low cost, with a high availability, and covers almost the whole of the country, therefore the costs are reduced nationally. For example, in 2001 and 2012, the Nutrition Improvement Office Ministry of Health reported that iron deficiency anemia was reduced by 50%, meaning we were successful about micronutrients. We suggest that the food should be fortified if possible. On the other hand, training people about vitamin D has a great effect and we are required to inform people about the supplementary benefits and vitamin deficiency side effects", (Head of the Department of Nutrition Improvement, QUMS). "In my view, the priority is a fortification, and people can select the type of goods themselves. I think having a financial contribution is better than not being paid too much", (Head of the Department of Nutrition Improvement, MUMS).

Discussion

Vitamin D deficiency has been identified as a health problem worldwide for all age groups, especially in Middle East countries (Ovesen et al, 2003). The high prevalence of vitamin D deficiency is an important concern of health officials in Iran (Pouraram H, et al, 2018). Since vitamin D deficiency would affect the health consequences and impairment of the function of many organs of the human body (Palacios and Gonzalez, 2014), the implementation of appropriate and targeted interventions can play a significant role to improve the public health in the country. In Iran, the vitamin D supplementary program has been applied by the Office of Nutrition Department Society in the whole of the country among different age groups since 2014. Therefore, it appears necessary to evaluate the status of the vitamin D supplementation program in Iran to identify the weaknesses and implementation problems of the program. Moreover, it seems that exploring the vitamin D supplementary program can also be used to identify the executive barriers and problems.

Qualitative analysis showed that there are several problems in implementing the program in Iran, such as distributing the supplement and the existence of barriers to using the supplement by people and funding. The barriers to use supplements were: 1- Forgetting to take them, especially among middle-aged or elderly, 2- Lack of knowledge about the benefits, 3- Access to the health care center, 4- Negative advertising for supplement use (Family members especially parents and teachers not allowing use of the supplements, being terrified of negative health effects), 5- Not taking supplements because of being free of charge, 6- Side effects, such as: nausea and headache, 7- Implementation of the project at schools with a delay of 3 months.

In MUMS and QUMS, supplying vitamin D supplementation was the most important problem, whereas in ZAUMS, distributing the supplement has been reported as the most critical issue as the barriers to the person's use of supplements. Among several problems, inadequate/irregular distribution of the vitamin D supplements and not providing them by drug distribution companies should be considered by health headquarters in QUMS and ZAUMS. The health professionals' solutions include a need to intervene a multiple strategy that contains training, campaign, financing, sufficient and regular distribution of the supplement and to apply the alternative methods such as food fortification.

In the current study, side effects such as nausea or headache were reported by 5.8% of health providers as a barrier to supplements. Most public health practitioners and health care managers have reported that people were willing to receive the vitamin D supplement because it lacks scent and bad taste.

In recent years, the supplementary price has increased by over 50%, but the budget dedicated to the supply of the supplement has not changed. Providing the vitamin D supplement as centralized (by health ministry/food-drug administration/provincial headquarters) and its sufficient and regular distribution was one of the solutions to the problems of providing supplements. Because in this case, the cost of purchasing decreased with the allocation of vitamins budget from the source to buy vitamins and the purchase of drugs at one time. The wandering of the authorities in the provision of supplements from different companies or the allocation of supplements budget to other essential requirements, such as the purchase of antibiotics, would be avoided.

Another strategy is taken to deal with the lack of funds was prioritizing the Health Center based on the richest and poorest areas. In the case of lack of supplement, distribution priority was in

poorest areas and in cities surrounding and in the richest areas; individuals were trained to purchase and consume vitamin D supplements. Moreover, some of the more vulnerable age groups, such as pregnant women and female students, were prioritized. Figure 1 has summarized some suggestions for improving the implementation of the Vitamin D supplementary program.

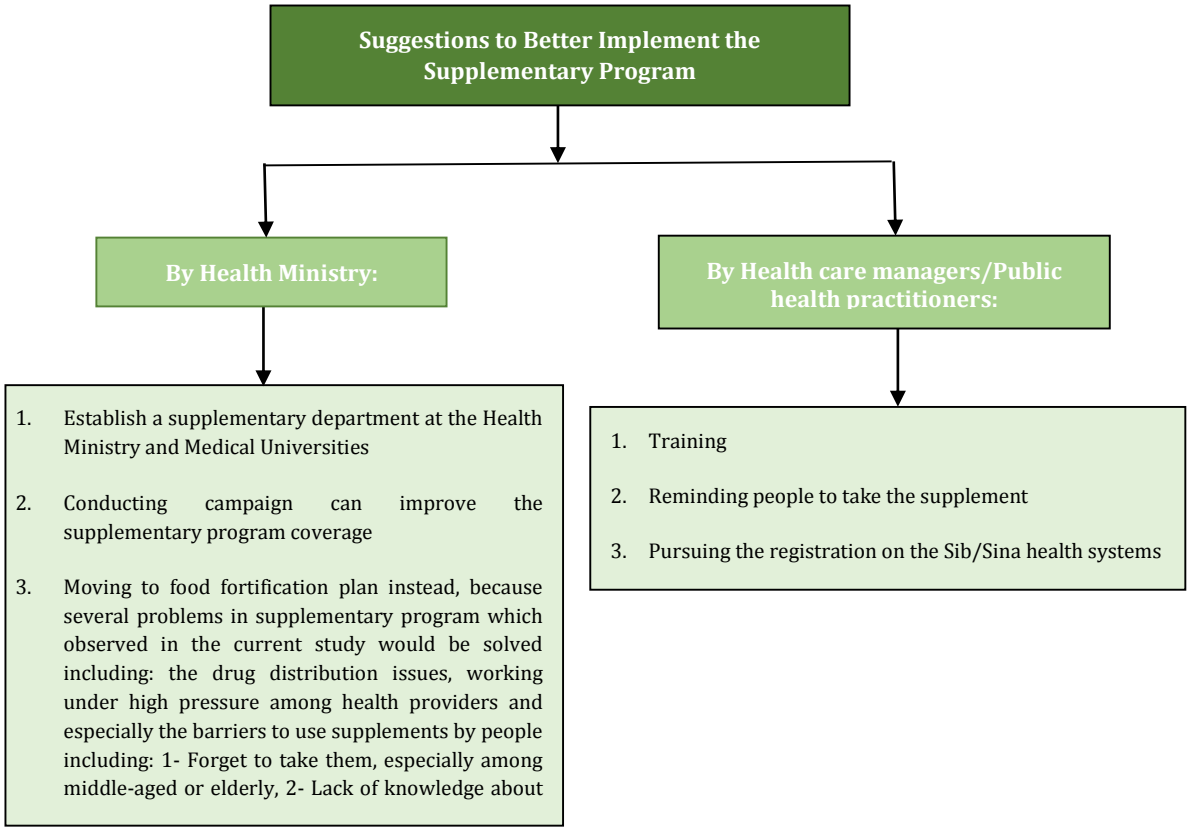

Figure 1. Suggestions for improving the implementation of the Vitamin D supplementary program

Conclusion

This study indicates that there are several problems in implementing the vitamin D supplement program, including issues related to distribution of the supplement, the barriers to take supplement by people, and funding. The findings can suggest applying national interventions through multiple strategies containing training, conducting campaigns, financing, sufficient and regular distribution of the supplement, and applying alternative methods such as food fortification.

Ethical Consideration

This study was approved by the Research Deputy of MUMS based on the MUMS's ethical guidelines and in accordance with the Declaration of Helsinki (ID: 970940; IR.MUMS.MEDICAL.REC.1397.387). Informed written consent was obtained from all participants. All participants' names have been removed from all of the manuscript.

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Conflicts of Interest

The authors confirm no competing interests.

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JOURNAL OF NUTRITION FASTING AND HEALTH

The Effect of Time Restricted Eating On the Steroid Hormones during Fasting and in Response to an Exercise Session in Active Boys

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ABSTRACT

Introduction: Nutrition programs can effectively influence physiological systems both at rest and in response to exercise. In the present study, the effect of time-restricted eating (TRE) on two steroid hormones, namely testosterone and cortisol and the ratio of testosterone to cortisol, at rest and in response to a session of exhaustive endurance exercise was investigated.

Methods: Participants of this study were 8 active boys (age: 22.63±3.50 years, body mass index: 23.46±5.61kg/m^2) who participated in the study voluntarily. Subjects performed TRE for two weeks. That is, they abstained from eating and drinking (except water) from 8 am to 4 pm, and continued their usual diet for the rest of the day. Before and after the two weeks of diet, they participated in the Yo-Yo exercise test. In both stages, blood samples were taken before the breakfast, and immediately after eating breakfast and the yo-yo test to measure the concentration of testosterone and cortisol. Paired t-tests were used to analyze the findings.

Results: Findings indicated that TRE increased fasting testosterone levels. Testosterone decreased after exercise following two weeks of TRE. Cortisol and the ratio of testosterone to cortisol at rest and in response to exercise after TRE were not significantly different compared to before TRE.

Conclusion: It can be concluded that TRE improved fasting testosterone as an anabolic index but reduced its response to exercise.

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Introduction

Testosterone and cortisol are two important steroid hormones and the end products of the hypothalamus-pituitary-gonadal and hypothalamus-pituitary-adrenal axes and their significance and physiological functions have been evaluated at rest and in response to stress/ exercise in various studies. A recent study's findings suggest that in the condition of high cortisol levels, testosterone may be neuroprotective. In contrast, low testosterone may be neuroprotective in the condition of low cortisol levels (1). High levels of testosterone and low levels of cortisol have been associated with social aggression (2). Also, the ratio of two steroids including testosterone to cortisol is known as possible biomarkers of physiological and psychological disorders (3) .

Testosterone is the strongest anabolic hormone in men which stimulates muscle growth, bone mass and androgenic potency in men. Inhibition of this hormone in men leads to a specific reduction in muscle strength and size (4). Physiological concentrations of testosterone cause a depot-specific reduction of catecholamine-stimulated lipolysis in subcutaneous fat cells, probably due to reduced expression of receptors and hormone-sensitive lipase (5). An association has been found between the low resting testosterone found in endurance-trained runners and cortisol (6). Diet and nutritional status including fasting can affect cortisol and testosterone level. A recent study indicated that resting and post-exercise cortisol increase during the first weeks of a low-carbohydrate diet. After this time, resting cortisol returns to baseline, while post-exercise

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cortisol remains elevated. High-protein diets decrease in resting total testosterone (7). Fasting could reduce stress hormones in patients with major depressive disorders (8). However, there are contradictions in some study findings which assessed the effect of fasting on cortisol and testosterone (9, 10).

Time-restricted eating (TRE) is a kind of eating pattern based on the circadian rhythm which limits duration of daily food intake (usually to ≤12 h/day), in which no obvious restriction is imposed on the quality, nor quantity, of food intake. TRE differs from other fasting protocols due to its aims on restricting eating time rather than caloric intake and can be more easily adopted by simply skipping a meal. The impact of TRE on fitness and athletic performance has been mainly related to use the Ramadan fasting protocol, which differs from TRF considering feeding schedule (11). In studies which compared TRE with usual diet, an increase in cortisol levels was observed in usual diet. Elimination of dinner has led to a significant reduction in evening cortisol at night, while non-significant increasing morning cortisol. In contrast, deleting breakfast significantly reduced morning cortisol (12).

Regarding the effect of exercise, high power resistance exercise may cause an anabolic hormonal response which partially explain the muscle hypertrophy observed in athletes who routinely employ high power resistance exercise (13). A study indicated that testosterone was elevated in the early recovery period following exhaustive endurance exercise but was reduced by 24 h after that (14). Acute response to endurance exercise stress focusing on cortisol and testosterone in professional athletes was evaluated in male professional athletes. It was found that high intensity endurance exercise induced catabolic response, but the level of response depended on a previous level of training (15).

Given the various adaptations of dietary restriction and response to short-term exercise, the aim of present study was evaluating the effect of TRE on cortisol and testosterone during fasting and in response to an exercise session.

Research Methods

The study method was a kind of quasi-experimental research with pre-test and post-test measurements. Participants of the study were 8 healthy and active young men in Shiraz whose age range was 17 to 30 years and participated in this study voluntarily and purposefully. Inclusion criteria were participating in moderate intensity exercise for at least one month, having cardiovascular health, not having a special diet in the last month, not smoking, not taking anti-anxiety and anti-depressant drugs, not taking hormonal drugs, and not suffering from obesity.

Exclusion criteria included non-adherence to the TRE plan, being affected with any metabolic or infectious diseases that affect the research variables.

Firstly, the participants were informed about the study procedures and the purpose of the study and signed the informed consent. Before participating in the research program, the subjects completed a questionnaire related to eating habits and physical activity to determine the basic information about the dietary and sports habits of the subjects. One day before the start of the research intervention program, as well as after that, the subjects' weight, height, body composition, waist and hip circumference were measured and participants were asked to avoid any changes in diet and physical activity compared to the last month, and also any dietary or energy supplements. During the TRE program, subjects were also asked to record their daily diet. The subjects were instructed to maintain their daily habits regarding physical activity, sleep and food quality and quantity during two weeks of study and their adherence to maintaining lifestyle was measured by comparing eating and physical activity before and during TRE generally and also asking the participants to report any stress or illness or any change in eating, physical activity and sleep habits within two weeks of the study. All of participants reported adhering to recommendation of maintaining lifestyle and none of them were excluded

YoYo test was used to measure cardiorespiratory fitness or VO2max of participants and also to assess its effect on testosterone and cortisol response. Validity and reliability of the test has been approved by previous studies (16). One week before taking the main test, the subjects performed the Yoyo test to be familiarized with the performance of the test and the learning effect of test can be reduced. The subjects participated in the Yo-Yo endurance exercise test twice (before and after the dietary restriction)

and between these two stages of the test, they observed TRE for two weeks. Blood samples were taken 4 times before and after TRE, during fasting and following eating breakfast and YoYo test.

Fasting blood samples were taken at 8 am, after 10 hours fasting and complete rest and sitting in a quiet situation for 30 min in the laboratory environment. Then, participants ate breakfast and 45 minutes after breakfast, subjects began warming up for 5 minutes and then performed the YoYo endurance test. Within two minutes after the YoYo test, blood samples were taken again, and the samples were sent to the laboratory in an ice chamber to assess the level of testosterone and cortisol. Subjects were then observed TRE for two weeks.

During TRE, participants were not allowed to eat any food from 8 am to 4 pm for two weeks and only drinking water was allowed. The three daily meals (50-60% carbohydrate, 25-35% fat, 10-20% protein, energy intake of 2137-2456 kcal) were designed to meet their usual energy requirements as before TRE. During the rest of the day and night, they could follow their normal diet and lifestyle and sleep pattern according to their normal routine (Jones et al., 2020).

Statistical Analysis Method

The collected data were analyzed by SPSS software version 26. Due to the normal distribution of findings approved by Shapiro-wilk test, the paired t-test was used to compare the variables in before and after TRE. Statistical significance was accepted at $p < 0.05$.

Table 1. Descriptive characteristics of participants

variables		Minimum	Maximum	Mean± Standard deviation	P value
Age(year)		17	29	22.625±3.502	-
Height (cm)		169	184	176.75±4.891	-
Weight(kg)	Before TRE	55.3	95.7	72.500±13.920	<0.001
	After TRE	54.6	94.8	71.600±13.369	
Waist circumference (cm)	Before TRE	72.8	113	88.662±14.780	<0.001
	After TRE	70	113	87.250±14.887	
Hip circumference (cm)	Before TRE	88	121.1	99.300±11.036	<0.001
	After TRE	86	117	97.887±10.425	
BMI(kg/m2)	Before TRE	17.2	33.5	23.462±5.611	<0.001
	After TRE	17.4	33.2	23.112±5.412	
VO2max (ml / kg / min)	Before TRE	20.5	39.7	31.07±7.51	0.525
	After TRE	19.3	44.5	32.37±9.32	

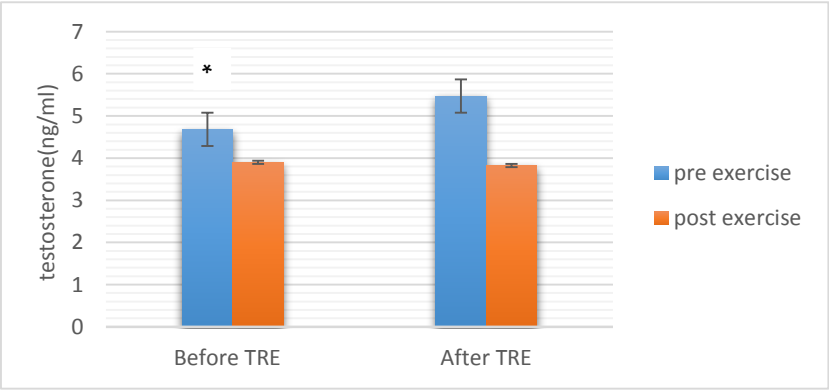

Figure 1. Comparison of testosterone before exercise (fasting) and after exercise between before and after TRE, * Significant differences with pre-exercise (pre vs post TRE), #Significant difference in testosterone post exercise (pre vs post TRE)

Results

The descriptive characteristics of the subjects are shown in Table 1 and the descriptive characteristics of the variables are shown in Table 2.

Among the variables presented in table 1, BMI, weight, and waist decreased significantly after

TRE (p<0.001), while Vo2max did not change significantly (p> 0.05).

The description and comparison of the study variables are presented in Table 2. Main aims of the study were comparison of variables before exercise and the changes (post prandial and exercise-fasting) of variables before and after TRE.

The results of comparison of fasting testosterone in before and after TRE are presented in Table 2. According to table 2, there was a significant difference between fasting testosterone levels before and after TRE (p = 0.016 and t = -.169). This means that fasting testosterone levels increased significantly after TRE compared to before TRE (Figure 1).

In order to compare the changes in testosterone after exercise, firstly the changes in testosterone in every session, including post exercise and eating breakfast –fasting state, were calculated and then the changes in each session before and after TRE were compared using paired t-test (Table 2).

As indicated in table 2, there was a significant difference in testosterone between before and after TRE (p = 0.006 and t = -3.869). This means that testosterone changed significantly following TRE compared to before TRE.

Regarding cortisol, as shown in Table 2, there was no significant difference in pre exercise cortisol between before and after TRE (p = 0.546 and t = -0.635).

In order to compare the changes in cortisol after exercise, first the changes in cortisol in every session, before and after eating breakfast were calculated and then the changes in each session (before and after TRE) were compared using paired t-test.

As indicated in table 2 there was no significant difference in post exercise changes of cortisol between pre and post TRE (p = 0.464 and t = -0.774).

Table 2. Description and comparison of variables before and following TRE

variable	Measurement time	Mean ±Standard deviation	Mean difference	t	p
testosterone ng /ml	Fasting before TRE Fasting following TRE	4.683±1.488 5.470±1.838	0.786±0.701	3.169	0.016
	Changes before TRE Changes following TRE	3.900±1.068 3.852±0.947	0.833±0.609	3.869	0.006
	Post exercise before TRE Post exercise following TRE	3.900±1.067 3.852±0.947	0.047±0.576	0.233	0.823
cortisol ng /ml	Fasting before TRE Fasting following TRE	13.837±4.592 14.600±4.737	0.762±1.397	0.635	0.546
	Changes before TRE Changes following TRE	11.712±5.997 10.587±3.197	1.887±6.895	0.774	0.464
	Post exercise before TRE Post exercise following TRE	11.712±5.997 10.587±3.197	1.125±5.173	0.550	0.599
Ratio of testosterone to cortisol	Fasting before TRE Fasting following TRE	0.448±0.404 0.441±0.271	0.033±1.515	0.063	0.951
	Changes before TRE Changes following TRE	0.399±0.155 0.377±0.084	0.570±3.552	0.454	0663
	Post exercise before TRE Post exercise following TRE	3.315±2.694 2.778±0.691	0.536±2.171	0.699	0.507

As shown in Table 2, the fasting ratio of testosterone to cortisol in before and after TRE was compared with the paired t-test. The results showed that there was no significant difference in testosterone to cortisol ratio between before and after TRE (p = 0.951 and t = 0.063).

Also as presented in table 2, the changes in testosterone to cortisol ratio between the two conditions before and after TRE were not significantly different (p = 0.663 and t = -0.454).

Discussion

The aim of the present study was to evaluate the effect of TRE on testosterone, cortisol, and testosterone to cortisol ratio during fasting and in response to an exercise session.

Fasting testosterone levels increased significantly after TRE compared to before TRE.

Also, the decreasing changes in testosterone following eating breakfast and exercise and after TRE were greater than before TRE.

Although no study was found on the subject of the present study, studies with almost similar subjects have been performed and contradictory results have been obtained. In a study, Moro et al. (2020) evaluated the effect of time TRE as limiting calories for 8 hours from 10 am to 6 pm and compared it to receiving the same calories in three meals from 7 am to 9 pm. and found that testosterone levels decreased following TRE(17). Stratton et al (2020) also found that four weeks of time-restricted feeding combined with resistance training reduced testosterone level (18). Regarding the reducing effect of fasting or TRE on testosterone, it seems that TRE could have an inhibitory effect on the Leydig cells responsible for producing testosterone. In addition, a diet with a time limit similar to calorie restriction may stimulate the AMP-activated protein kinase / acetyl-coa-carboxylase (AMPK / ACC) signaling pathway. AMPK is a central metabolic regulator that is activated during a low cellular energy state, when activated, stimulates ATP production through fatty acid oxidation and glycolysis, while simultaneously inhibiting anabolic processes. Studies in rodents have shown that short-term fasting (19-39 hours) increases AMPK and ACC in fat cells, but not in muscle. However, this hypothesis has not been confirmed in humans. In addition, calorie restriction does not appear to decrease IGF-1 concentration, although it may increase IGFBP-1(17). Malnutrition (e.g., protein restriction or protein-energy deficiency) has been suggested to impair Leydig cell function and affect testosterone biosynthesis(19). However, in the present study, testosterone increased following TRE, which indicates that TRE was not accompanied with caloric restriction or malnutrition. We wanted the participants to maintain their previous quality and quantity of diet program which may be effective on the present findings.

Considering the reduction of testosterone following eating breakfast and exercise session, it seems that eating breakfast has been effective on reducing testosterone even following exercise and this effect was more prominent following TRE. There are discrepancies about the effect of content of food on testosterone. Carbohydrate intake may affect male sex hormones. A low-carbohydrate diet (less than 5% of total energy content) decreased total plasma testosterone levels, while increased circulating levels of adrenaline, noradrenaline, and growth hormone(20). A study by Anderson et al(1987) found that a high-carbohydrate diet increased circulating testosterone and globulin-binding levels of the steroid SHBG(21), while a high-protein diet has reversed this effect. Mikolski and Zimbabwe and Nazar (2010) showed that in both low (35% protein, 64% fat, 1% carbohydrate) and high (4% protein, 1% fat, 95% carbohydrate) carbohydrate intake status, serum testosterone levels elevated in physically active people(22). However, carbohydrates appear to be positively correlated with circulating testosterone and SHBG levels in men. Increased intake of refined carbohydrates is associated with low serum SHBG levels in men and women. While according to another study no significant relationship was found between carbohydrate intake and total and free testosterone levels in healthy women (23). According to the mentioned mechanisms, it seems that TRE has probably increased testosterone by lowering blood sugar.

Regarding the reduction of testosterone following exercise and breakfast, although some studies have indicated the increasing effect of exercise on testosterone, not all sports induce the same effect. Some factors including gender, biology, and the type of exercise are effective on exercise response. Research has shown that resistance training helps short and long term increasing of testosterone levels. Resistance training such as weightlifting is the best type of exercise to boost testosterone especially in men(24) Testosterone needs many stimuli for release. The increase in testosterone due to exercise is largely influenced by the intensity, duration and type of exercise. However, debilitating activity can have a negative effect on testosterone release. Two main systems, namely the autonomic nervous system and the two axes of HPA and HPG, in response to stressful stimuli in sports, regulate the secretion of testosterone (25). Both of these systems can be influenced by blood sugar. However, in the present study, the reason for the decrease in testosterone after exercise compared to fasting could be related to food intake before exercise , as high blood sugar causes a decrease in testosterone(20). About the more decreasing effect of TRE, it is possible that this period of TRE has led to adaptations to

maintain blood sugar even under exercise conditions and may have caused maintaining blood sugar for longer duration, which is associated with a decrease in testosterone, which requires further studies in the future.

Findings of the present study indicated that there were not any significant differences in fasting and post exercise cortisol and testosterone to cortisol ratio between before and following TRE. A study by Queiroz et al (2020) found that there was an association between eating hours and fluctuations in the secretion of hormones such as cortisol(26). Solianik et al. (2020) in a study examined the effects of 48-hour fasting on several factors in elderly and obese women who were in the age range of 63 to 80 years with a body mass index of more than 25 kg / m2. Drinking water was free during this time. They found that cortisol levels increased after fasting (27).

Relevant to the present study, a review by Tinsley & La Bounty (2015) discussed studies that examined the effects of 30 or 40 hours of fasting for 3 weeks on cortisol secretion and found that cortisol did not change significantly (9). The short diet with limited time in the present study could be one of the factors affecting the lack of change in cortisol or the ratio of testosterone to cortisol in the present study. Cortisol is the final hormonal product of the hypothalamic-pituitary-adrenal axis (HPA) and plays a major role in the adaptation and regulation of homeostasis in response to internal and external challenges. As a result, cortisol mediates many metabolic processes, including increased mobility of energy substrates and increase energy delivery to the brain and muscles (28). Cortisol levels are also affected by nutrition. During the day, food intake leads to a sharp rise in salivary cortisol levels, which begins 30 minutes after the end of the meal and peaks approximately 1 hour after the start of the meal. Restricted feeding leads to increased cortisol at sunset and peaks before meals (29). The related mechanisms are not well understood, but the hypotheses that justify this phenomenon suggest that the there is an interaction between the insulin response and glucocorticoid secretion (30).

Witbracht et al. (2015) found that women who did not eat breakfast had significantly higher cortisol after meals, especially at noon(31). According to some other studies, not eating dinner led to a significant decrease in cortisol at night and a slight increase in morning cortisol, indicating that TRE increases the amplitude of the cortisol rhythm. Conversely, not eating breakfast resulted in a low daily cortisol pattern with a significant reduction in morning cortisol(32). Stratton et al. (2020) compared the effect of fasting with a typical diet pattern, both of which were 25% reduction in calories. Higher concentrations of cortisol were observed following a normal diet compared to a fasting diet (18). Similarly, another study examining the effect of an 8-week fasting diet intervention on firefighters showed that salivary cortisol levels increased in response to a fire test, decreased following diet, which may have implications for reducing the stress response (33).

In addition, the fasting can change the normal circadian rhythm increase cortisol (34, 35). However, previous research by Tinsley et al. showed no change in cortisol awakening response or change in mean cortisol concentration after eight weeks of resistance training with TRF in women (36). Discrepancies are obvious in previous study findings. The lack of change in cortisol and the ratio of testosterone to cortisol in the present study could be due to due to adaptive responses to TRE, short period of TRE, or limited number of subjects. However, maintaining the quantity and quality of eating can also limit changes in cortisol. Also, the low intensity of the exercise program can also be effective on non-significant change of cortisol following acute exercise and TRE.

The strength of the study was its novelty as well as evaluating two important hormones of testosterone and cortisol in health and exercise aspects. However, this study included some limitations. One of the limitations of present study was the wide range of BMI of participants and its possible effect on testosterone and testosterone to cortisol ratio. Another limitation of the study was the small number of participants; because of the necessity of observing time restricted eating accurately, small numbers volunteered to participate in the study which may affect the generalization of the findings.

Conclusion

In general, two-week TRE increased testosterone, which can be explained by existing background mechanisms. After eating breakfast

and exercising, testosterone decreased following TRE. Cortisol and testosterone to cortisol ratio did not change at rest and after exercise before and after TRE. In general, it can be recommended that a time restricted eating can be used to increase basal testosterone.

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Seasonal Investigation of Aflatoxin M1 Level in Afyon Tulum Cheese

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ABSTRACT

Introduction: The milk produced from dairy animals fed with feed intake aflatoxin may include Aflatoxin M1 (AFM1). Dairy products, produced with milk containing AFM1 represent an important problem in terms of public health.

Methods: This study investigated AFM1 in Afyon Tulum cheese (ATC) taken in Afyonkarahisar province. For analysis, 80 samples of Afyon Tulum cheese were collected and stored at -20°C. All samples were then analyzed using a commercial Aflatoxin M1 ELISA test kit.

Results: AFM1 was found between 0.007-0,017 ug/kg in spring-summer season and between 0.006-0,041 ug/kg in autumn-winter season. AFM1 levels were found under the Turkish Food Codex (TFC) limit (0,05 µg/kg) in all samples.

Conclusion: It is recommended that milk containing AFM1 should not be used in production and the feeds used in animal feeding and storage conditions should be checked.

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Introduction

Milk is the first nutrient consumed by humans and other mammals after birth. Rich in content and complex structure, milk is one of the essential nutrients for human nutrition. Milk is converted into durable products such as cheese or yoghurt to extend storage time. Cheese is the most popular of these products (1).

White, cheddar and Tulum cheese are among the most produced cheeses in Turkey. In addition to these cheeses, there are also many local kinds of cheese. Tulum cheese is the most known and made among the local cheeses. According to the Turkish Food Codex (TFC), Tulum cheese is a product produced by fermenting the curd obtained by coagulating with rennet, crumbling and salting it. It is a cheese that is then pressed into a suitable packaging material or leather overalls and consumed after ripening (2). Tulum cheese is produced in many Turkey regions, except the Thrace region. Tulum cheese is named differently according to areas and production methods. For example; Divle, Erzincan, Erzurum, Çimi and İzmir tulum cheeses (3, 3, 4). Another traditionally made cheese with its unique production technology is Afyon Tulum cheese (5).

The presence of Aflatoxin M1 (AFM1) in milk and milk products poses a problem worldwide, especially in developing countries (6). Aflatoxins are toxic fungal metabolites generally produced by *Aspergillus parasiticus*, *A. flavus* and *A. nominus* and occur in foods and animal feeds. When ruminants consume feed contaminated with Aflatoxin B1 (AFB1) during the lactation period, this toxin is metabolized in the digestive system and causes the formation of AFM1 in milk (7). Aflatoxins have severe adverse effects on human and animal health. It causes liver damage, immune system suppression, tumour formation, and teratogenic, mutagenic and carcinogenic effects (8).

Material & Method

Afyon Tulum Cheese Samples

This study collected Afyon Tulum cheese samples between December 2019 and November 2020 in Afyonkarahisar province. Eighty Afyon Tulum cheese samples (40 samples autumn-winter season and 40 samples spring-summer season) were collected, belonging to different sales

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points. The samples were stored at -20°C until analysis.

ELISA Kit

A commercial ELISA test kit (Bio-Shield M1 ES, Larissa, Greece) was used in the study. Aflatoxin M1 detection limit is 0.005 µg/kg.

Preparation and Extraction Process

Dichloromethane (DCM) was used for extraction. For this purpose, 8 ml of DCM was added to a 2 g cheese sample and mixed. It was then incubated at room temperature (21±2°C) for 30 minutes. After incubation, the tubes were centrifuged at 3000xg for 10 minutes at 21±2°C. The extractant was transferred to a different tube with 4 ml and evaporated at 60°C under N_2. Then 0.5 ml of methanol (100%), 0.5 ml of distilled water and 2 ml of hexane were added to each sample. The tubes were mixed and centrifuged at 3000xg for 10 minutes. After centrifugation, top of the hexane layer and the bottom of the methanolic-aqueous phase were removed with the help of a pasteur pipette. The extract was diluted 1/10 with AFM1-free milk from the kit. 100 µL of each sample was used for ELISA measurement.

Preparation of ELISA Plates

All reagents of the kits to be used were brought to room temperature before use. 100 µL of standards and extracted samples were added to the wells. The wells were covered with a transparent film, shaken manually for 30 seconds, and incubated for 45 minutes at room temperature. The wells were washed four times with 1X wash buffer. After washing, 100µl of fixation solution was added to all wells. The wells were covered with a transparent film, shaken by hand for 30 seconds and incubated for 15 minutes at room temperature. The wells were washed four times with 1X wash buffer. 100µl of TMB Substrate was added to all wells. Finally, the wells were covered with a transparent film, shaken manually for 30 seconds, and incubated for 15 minutes at room temperature for colour development. After incubation, 100µl of Stop solution was added, and absorbance was read.

ELISA Reading and Evaluation

Absorbance reading was done at 450 nm in the prepared plate ELISA Reader (ThermoFisher, Multiskan-go, Vantaa, Finland), and the results were evaluated with the program belonging to the kit (Prognosis Data Reader).

Results & Discussion

AFM1 standard results (0, 0.005; 0.01; 0.025; 0.05, 0.1 and 0.25 µg/kg) are shown in Figure 1. In the study, a total of 80 Afyon Tulum cheese samples (40 autumn-winter seasons, 40 spring-summer seasons) belonging to different sales points were collected. As a result, AFM1 was detected in 87.5% of the samples of the Autumn-Winter period; 97.5% in the spring-summer period samples. However, AFM1 was not detected above the TFC limit of 0.05 µg/kg in the Tulum cheese samples (Table 1). In addition, while the average of the samples belonging to the autumn-winter period was 0.02 µg/kg, the average of the samples belonging to the spring-summer period was determined as 0.01 µg/kg. According to the results, the AFM1 level in the autumn-winter period was higher than in the spring-summer period.

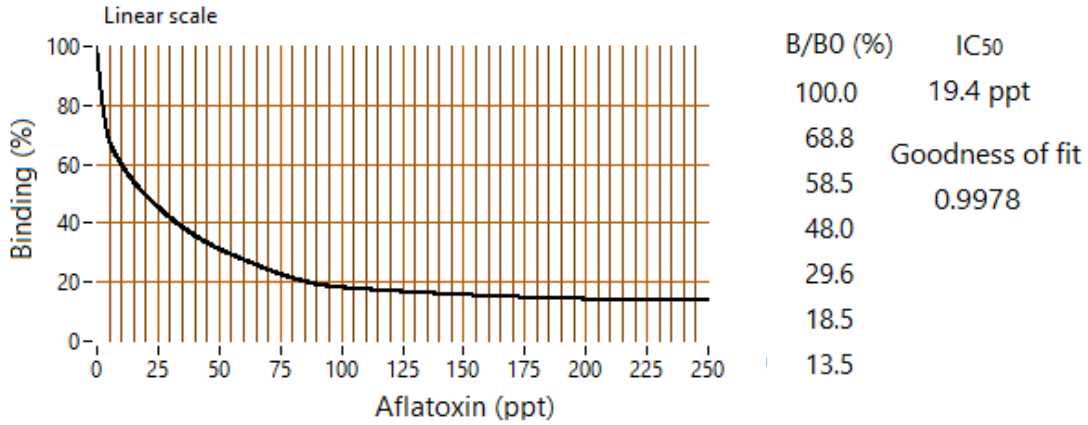

Figure 1. Standards of Aflatoksin M1

In similar studies, Sarımehmetoğlu et al. (2004) detected AFM1 in 81% of Tulum cheese and reported that 24% of the samples were above the Turkish Food Codex AFM1 limit (9). Hampikyan et al. (2010) AFM1 was detected in 55% of Tulum cheese samples (between 0.057-1.36 ng/kg levels) (10). They also reported that 10% of the Tulum cheese samples were above the TFC limit. In the study of Gücükoğlu et al. (2010) did not detect AFM1 in Tulum cheese (11). Ertas et al. (2011) AFM1 was detected in 16 of 20 Tulum cheese samples (in the range of 13.0-378.0 ng/kg). In two samples, AFM1 detections were above the TFC limit (12). İşleyici et al. (2011) AFM1 determined in amounts varying between 5.15 ng/kg and 26.44 ng/kg (13). Ayyıldız (2012) AFM1 detected 113.07 ng/kg in one (14.3%) Tulum cheese sample (14). Bakırdere et al. (2014) found AFM1 detected in 18.75% (3) of 16 Tulum cheeses (0.05-0.10 µg/kg) (15). They did not find the AFM1 level of the samples above the limit set by the TFC (0.05 µg/kg) (2).

Table 1 Aflatoxin M1 Levels in Afyon Tulum Cheese

	Spring-Summer Season (n: 40)		**Autumn-Winter Season (n:40)**		**Total (N:80)**	
	n	%	n	%	n	%
< 0.005	1	2.50	5	12.50	6	7.25
≥0.005 – <0.01	12	30.00	3	7.50	15	18.75
≥0.01 – <0.02	27	67.50	28	70.00	55	68.75
≥0.02 – <0.03	0	0.00	3	7.50	3	3.75
≥0.03 – <0.05	0	0.00	1	2.50	1	1.25
≥0.05	0	0.00	0	0.00	0.00	0.00
Min*	0.007		0.006			
Max	0.017		0.041			
Average*	0.01		0.02		0.014	

* Samples above the detection limit (0.005 µg/kg) were calculated.

In all studies, AFM1 in different rates and levels were detected in Tulum cheeses. Although AFM1 was detected in Tulum cheeses in this study, the AFM1 level was found below the TFC maximum limit. Similarly Gücükoğlu et al. (2010), İşleyici et al. (2011), and Bakırdere et al. (2014) found the AFM1 level in Tulum cheese to be below the maximum limit of the TFC (11, 13, 15). On the other hand, Sarımehmetoğlu et al. (2004), Ertas et al. (2011), Hampikyan et al. (2010), and Ayyıldız (2012) reported that they determined the level of AFM1 in Tulum cheeses above the TFC limit (9, 10, 12).

Differences between studies can be attributed to the Aflatoxin content of milk and animal feed used in production. Inappropriate stages may cause aflatoxin contamination in feed, harvesting, and storing feeds. In addition, seasonal air temperature changes also affect the formation and amount of Aflatoxin in feed. In the study, the AFM1 level in the autumn-winter period's Tulum cheese samples was higher than in the spring-summer period. This situation may be related to the consumption of animals in closed feeding and stored feed materials.

Conclusion

Aflatoxin-free milk should be used in the production of Tulum cheese. Routine checks for Aflatoxin should be made for milk and cheese. It is recommended to prevent mould formation in the processing, transportation and storage stages of the feed used in animal feeding, starting from the growing and harvesting stage. The necessary hygienic conditions must be observed in the production area for the hygienic quality of milk and dairy products. The Farm to Fork principle should control the products at all stages. Manufacturers and consumers should be informed about Aflatoxin.

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Conflicts of Interest

The authors declares no potential conflict of interest.

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Short-Term Effect of Aerobic Exercise and High-Fat Diet and Consumption of Curcumin Extract on Leptin Gene Expression in Liver Tissue in Male Rats

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Leptin gene expression

ABSTRACT

Introduction: Leptin is a protein hormone with a spiral structure similar to cytokines, which is mainly synthesized and released by subcutaneous fat cells in a steady pulsating manner with a peak secretion near midnight. The aim of this study was to compare the short-term effects of aerobic exercise and high-fat diet and curcumin extract on leptin gene expression in the liver tissue of male rats.

Methods: Fifty male rat with two-month-old were prepared and divided into five groups: control (G1), high-fat diet (G2), curcumin and high-fat diet (G3), exercise and high-fat diet (G4), curcumin+exercise+high-fat diet (G5). Data analysis was compared using one-way analysis of variance. Bonferroni's post hoc test was used to accurately determine the differences between groups. The mean of intra-group differences was also compared using the t-test of the sample.

Results: The results showed a significant difference in the average leptin gene expression of subjects among 5 groups. The results of Bonferroni's post hoc test also showed an increase in the high-fat diet group compared to the control group(P=0.0001), and a decrease in the high-fat diet + curcumin group(P=0.0001), the high-fat diet + exercise group(P=0.0001), and the high-fat diet + exercise + curcumin group compared to the control group(P=0.0001).

Conclusion: Leptin gene is expressed under the influence of short-term exercises and the consumption of curcumin, which is an antioxidant. So that the use of each agent alone causes a decrease or increase in the leptin gene expression in the liver, and the simultaneous use of both factors causes a decrease in the leptin gene expression of these cells in the muscle tissue.

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Introduction

Leptin was discovered in 1994 by isolating the obesity gene. This substance is a protein hormone with a spiral structure similar to cytokines (Larijani and Ghodsi, 2004), which is mainly synthesized and released by subcutaneous fat cells in a steady pulsating manner with a peak secretion near midnight. According to the family to which it belongs, it can be an internal regulator on the thymus gland and the factors secreted during the reaction such as interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α). Since the increase in plasma concentration of leptin is proportional to the fat content of fat cells and the degree of obesity is influenced by dietary interventions or daily exercise activity, leptin can report the long-term state of body fat tissue accumulation to the brain. Obesity in mice and in some people is caused by leptin gene mutation, which results in defective protein synthesis. Obesity in mice is a consequence of mis-transcriptional splicing of leptin mRNA, which produces a short leptin receptor (Furukawa et al., 2017). As mentioned, the level of leptin hormone can be affected by the nutritional status, neuroendocrine and immune function of the body. In addition, hormones such as sex hormones, catecholamines and thyroid hormones play a role in leptin regulation. These hormones are effective on leptin production by regulating the gene responsible for obesity. In addition, cortisol and growth hormones are the most important hormones that help to increase the level of leptin secretion (Baek et al., 2018). There is a relationship between leptin changes with negative energy balance, sympathetic activity and some metabolites. Among the potential regulators of leptin secretion are

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pressure caused by exercise, changes in fuel displacement, concentration of systemic hormones and the effect of the amount of energy consumed. The reduction of fat mass is one of the reasons for which leptin levels change (Milan et al., 2015).

Among the plant and natural factors effective on leptin, polyphenols and flavonoids are very effective (Perl et al., 2015). Among these polyphenols, curcumin, or the yellow extract from the turmeric plant, is a major biological polyphenol that is widely used as a spice, food additive, and as a herbal medicine in Asia. ¬Gird (Martins et al, 2016). Research has shown that curcumin has anti-inflammatory, cell protection, apoptosis and antioxidant effects. By activating AMPK, it increases the expression of PGC-1α and further inhibits Adipo-R2 (Relling et al., 2006). In addition, curcumin has other cardiovascular protective effects that can improve heart health in patients and people at risk of obesity from high-fat diets (Kandula et al., 2016). Although the beneficial effects of curcumin have been well demonstrated; however, based on the searches, there are still not enough studies to prove the effect of curcumin on leptin in the liver of obese rats and inhibiting the protein breakdown pathway. In his study, Jin (2018) stated that many natural products in nature are usually consumed by healthy or sick people to prevent or treat chronic diseases. Among them, several dietary polyphenols, including the compound curcumin, have attracted the most attention of biomedical researchers and drug developers. Unlike many so-called "good drug candidates", curcumin and several dietary polyphenols do not have a known therapeutic target or a defined receptor. In addition, the available bioavailability of these polyphenols is usually very low due to their poor intestinal absorption. These recently discussed properties have created enormous problems for drug manufacturers. This review does not discuss how curcumin, other dietary polyphenols or their derivatives are made into pharmaceuticals. Instead, it discusses how curcumin and dietary polyphenol research has enriched our knowledge of insulin signaling, including providing researchers' perspectives on how these studies have added to the understanding of the well-known hepatic insulin function. On the other hand, the extent of permanence of leptin changes after performing sports activity is a subject that has received less attention and the findings of existing studies are contradictory (Jin et al., 2016). The effect of exercise training in preventing the occurrence of non-alcoholic fatty liver disease has shown that 16 weeks of optional running exercise with an intensity of 50 to 75% of the maximum oxygen consumption on a treadmill can be used in the expression of non-alcoholic fatty liver leptin gene in rats. In addition, the effect of regular daily exercise on increasing the oxidation of fatty acids, in order to prevent fatty liver disease, has been studied on laboratory rats, and along with these regular exercise exercises, feeding with fatty foods was also done in the control and experimental groups. Investigated and studied. Researchers' findings of aerobic activities can have a significant effect on non-alcoholic fatty liver disease. In their experimental studies, many other researchers and researchers have investigated the effect of regular daily exercise activities on the oxidation of fatty acids in preventing non-alcoholic fatty liver disease in laboratory rats. Undoubtedly, the relationship between energy consumption and physical and sports activities is one of the strongest reasons for the benefits of exercise for the treatment of fatty liver disease (Sonoli et al., 2016). Sirico et al. (2020) in a study titled "Effects of Physical Exercise on Leptin and Markers of Obesity in Children: A Systematic Review and Meta-Analysis" stated that new findings on adipose tissue physiology and obesity-related inflammatory status suggest that modifying levels Adiponectin may be relevant for long-term prevention of obesity-related chronic disease. They conducted a systematic review with meta-analysis of electronically identified randomized controlled trials. A database search was conducted to investigate the effect of physical exercise without concurrent dietary intervention on leptin or other inflammatory markers in children up to 18 years of age with a body mass index greater than the 95th percentile for age and sex. Seven trials were included in the meta-analysis, with a total of 250 participants. The findings showed that compared to the control group without any lifestyle modification, physical exercise led to a decrease in leptin. They concluded that whether the effects of physical exercise improve inflammatory status in obese children is still unclear (Sirico et al., 2016). According to the mentioned materials regarding the effect of exercise, diet and curcumin extract

supplementation, the present study investigated the short-term effect of aerobic exercise, high-fat diet and curcumin extract on leptin gene expression in liver tissue in male rats.

Material & Methods

The current research is fundamental and experimental. This research was conducted in a laboratory and controlled manner (Code of Ethics No. IR.IAU.SHAHROOD.REC.1400.059 from Islamic Azad University). Considering that it was not possible to access human subjects due to space, ethical and time constraints, therefore, animal subjects (male rats) were used. At first, the necessary permits were obtained and then according to the instructions of the Iranian Society for the Protection of Laboratory Animals, they were kept in separate cages. The statistical population of female rats and sampling method is random and its volume includes fifty male rat with two-month-old. The sample size was determined using G POWER software based on the statistical method of analysis of variance and alpha error level of 0.05 and power of 0.85 equal to 50 mice, which were randomly divided into five groups: control group, high-fat diet, curcumin and high-fat diet. Exercise and high-fat diet, curcumin + exercise + high-fat diet were divided.

In the present study, rats kept in controlled conditions for two weeks in order to familiarize and adapt to the living environment, nutritional and training conditions; they were divided into five groups. In order to avoid stress and changing physiological conditions, the samples were kept for two weeks under new conditions (temperature (22±2°C), ambient humidity (50±5%) and light-dark cycle of 12:12 hours). During this period, all subjects freely consumed standard food and water. During these two weeks, the samples were subjected to the familiarization program on how to operate on the animal electric treadmill (ST008, manufactured by Tabriz University). This smart animal treadmill had five separate channels, all related factors such as the amount of slope (positive and negative), speed and time were controlled by the smart program. During this period, the amount of electric shock was constant at 0.1 mV. During the familiarization period, the incline of the treadmill was 0%, the speed was 10-15 meters per minute, and the training duration was 5-10 minutes per day. Chips and wood chips were used to absorb the urine and feces of animals and also for their comfort. Once every two days, the wood chips were replaced and the cages were washed and cleaned once a week. In the present study and during this period (compatibility with the environment), five rats were kept in each cage. These mice are sensitive to respiratory diseases, so dust or ammonia from animal urine should not accumulate in the breeding and maintenance hall. To prevent such a situation, it is necessary to change the air flow in the hall 10-15 times per hour. In this research, an ordinary device was used to ventilate the air flow of the animal house. This device was on all day and night. At the end of this period, rats were randomly replaced in five groups after weight matching.

Table 1. A 6-week program of aerobic training with an intensity equivalent to 70-75% of maximum oxygen consumption

Aerobic exercise protocol	Weeks of training					
	1	2	3	4	5	6
Training duration (minutes per day)	10	20	30	40	45	50
Reel speed (m/min)	25	26	27	28	29	30
Slope of the turntable (percentage)	15	15	15	15	15	15

Table 2. Research protocol

group	First and second week	Day 14	3rd to 8th week (six weeks)	Day +2
G1	Keeping in controlled conditions with the aim of getting to know and adapting to the living environment, nutritional and training conditions	Weight measurement	----------	Measurement of research variables
G2			High-fat diet gavage	
G3			Gavage of high-fat diet and curcumin	
G4			High-fat diet gavage and aerobic exercise	
G5			Gavage of high-fat diet and curcumin and performing aerobic exercise	

High-fat diet

All the groups receiving high-fat food used a high-fat food emulsion containing the compounds of the following table in the amount of 1.5 mg per kilogram of body weight daily for six weeks, which, in addition to the regular rodent food, in the composition The diet of mice was intended (Mirghani et al., 2019).

Aerobic Exercise

The exercise group participated in the aerobic exercise program on the animal smart electronic treadmill for five days a week (Sunday, Monday, Tuesday, Thursday and Friday) and for six weeks.

RNA extraction, cDNA synthesis and gene expression

Real Time-PCR method was used to check the gene or mRNA expression of the desired proteins. To prepare the PCR master mix to prepare cDNA, first all the following items are poured into a microtube using a sampler in the same proportion as mentioned in the kit protocol, and then we add 0.5 microliters of the desired RNA to the master mix. The ingredients were mixed in an ice pool. cDNA was used according to the recommended protocol to perform PCR by Viragen's Mix Red. The primers were received in lyophilized vials and then diluted with Sina Gene's TE buffer according to the ratio indicated on the vial. After that, 180 microliters of TE buffer, 10 microliters of forward primer (F) and 10 microliters of reverse primer (R) were poured into the designated tubes. The sequence, length and type of primer designed for leptin gene are as follows:

Table 3. Specifications of the primers used in the Real Time PCR process

Gene name	Primer sequence	Gene access code	Product length (open pair)
leptin	F 5′-CCTGTGGCTTTGGTCCTATC-3′ R 5′-ATACCGACTGCGTGTGTGAA-3′	NM_013076.3	138

Data analysis was compared using one-way analysis of variance. Bonferroni supplementary test was used to accurately determine the differences between groups. The mean of intra-group differences was also compared using the one-way statistical test.

Results

The results of the Shapiro-Wilk test show that the distribution of data in all variables is normal. Therefore, one-way analysis of variance test was used to analyze the data. The findings related to the descriptive characteristics of the subjects including pre-test and post-test weight and leptin gene expression of mice in the groups are shown in Table 4.

According to the results of the present study, there is a significant difference between the short-term effect of aerobic exercise and high-fat diet and curcumin extract on leptin gene expression in liver tissue in male rats.

Table 4. Findings related to the descriptive characteristics of the subjects and Average difference between the groups and the control group related to leptin gene expression

group	Pre-test weight		Post-test weight		Leptin gene expression		t	P Value
	Mean	S.D	Mean	S.D	Mean	S.D		
G1	2.06	3.02	2.66	3.65	-	-	-	-
G2	2.06	3.50	2.93	4.32	2.38	0.050	87.12	0.001
G3	2.06	2.91	2.83	2.87	1.79	0.049	30.11	0.001
G4	2.06	3.02	2.47	6.25	0.57	0.044	50.24	0.001
G5	2.06	3.29	2.40	3.7	0.48	0.048	33.72	0.001

Table 5. Results of analysis of variance between groups of leptin gene expression in five groups

group	P	F
Control **high-fat diet** **Curcumin and high-fat diet** **Aerobic exercise and high-fat diet** **Aerobic exercise and curcumin and high-fat diet**	***<0.0001**	**8.11**

As can be seen in Table 4, the difference in means between the control group and other groups shows that leptin gene expression has increased in the high-fat diet group compared to the control

group. But in the three groups of high-fat diet + curcumin, high-fat diet group + exercise and high-fat diet + exercise + curcumin group, it decreased, which is significant at the error level of 0.

As can be seen in Table 5, the results of analysis of variance showed that the average leptin gene expression of the subjects showed a significant difference among the 5 groups (Table 6).

Table 6. Bonferroni post hoc test results

Variable	group	High-fat diet group	High-fat diet and exercise group	High-fat diet group and curcumin	High-fat diet group, exercise and curcumin
Leptin	Control	M=-1.614*, P=0.0001	M=0.099*, P=0.0001	M=-0.32*, P=0.0001	M=0.095*, P=0.0001
	high-fat diet	----------	M=1.713*, P=0.0001	M=1.294*, P=0.0001	M=1.709*, P=0.0001
	Aerobic exercise and high-fat diet	----------	----------	M=0.419*, P=0.0001	M=-0.004*, P=0.0001
	Curcumin and high-fat diet	----------	----------	----------	M=0.415*, P=0.0001

The results of the Bonferroni post hoc test also showed a significant difference between the groups, but no significant difference was observed between the high-fat diet and exercise group and the high-fat diet, exercise and curcumin group (Table 6).

Discussion

The findings of the research indicated a difference in the means between the control group and other groups, so that the leptin gene expression increased in the high-fat diet group compared to the control group. But it decreased in the three groups of high-fat diet + curcumin, high-fat diet + exercise group, and high-fat diet + exercise + curcumin group. Many previous researches have reported a significant increase in leptin following the consumption of high-fat meals, which have been expressed in different amounts depending on the type of diet and dosage. This significant increase in leptin levels can indicate the presence of inflammatory conditions following the consumption of a high-fat diet. The exact mechanism of leptin response to high-fat diet is not yet known. A possible mechanism is the recruitment of neutrophils following the consumption of high-fat food, which causes the production of leptin (Babalwa et al., 2019). It has been suggested that cytokines are the bridge between obesity metabolism disorder and inflammation. It has also been suggested that one third of leptin production originates from fat tissue. Therefore, it is likely that the level of leptin change in subjects with similar amount of fat tissue is close to each other (Singh et al., 2016). On the other hand, short-term aerobic exercise caused a significant decrease in leptin levels. Gu et al observed that short-term exercise did not significantly alter leptin levels. Another study showed that 60 minutes of exercise with an intensity of 75% of the maximum oxygen consumption significantly increased leptin levels. They stated that leptin is probably released during exercise from active muscles and immune organs. On the other hand, some researches have shown that leptin levels increase quickly and immediately after intense and acute exercise and return to their resting levels 24 hours later (Frayn et al., 1996). . Several possible explanations for the different results of studies on the effect of exercise on cytokines have been stated. First, the type, intensity and duration of physical activity can have a different effect on the profile of cytokines. For example, a greater increase in cytokines has been seen after extroverted exercises compared to introverted exercises. Also, the range of cytokines increase depends on the duration of training. In addition; It should be kept in mind that the activity of cytokines after training is not limited to one of them, for example, leptin is after training. In the present study, short-term aerobic training significantly reduced leptin levels compared to the control group. However, in the groups with high-fat diet and no exercise, the levels of leptin increased significantly compared to the control group. Significant changes in leptin levels following exercise and a normal diet can be related to the exercise program and duration of exercise. It is also possible that the sampling of the subjects caused leptin levels to return to resting levels. The findings obtained from the Copenhagen Marathon studies (1996, 1997 and 1998) suggest that there is a negative relationship between increasing running time and increasing leptin levels. Therefore, runners showed lower levels of leptin (Frayn, 2002),

which results were in line with the present research. In addition, the significant decrease seen in leptin levels after intense exercise in subjects on a high-fat diet indicates the beneficial effect of exercise on the reduction of inflammatory factors following a high-fat diet. Physical activity protects the body against diseases related to low-grade chronic general inflammation. This long-term effect of physical activity may be caused by the response of short-term physical activity, which is partly modulated by muscle-derived leptin. It has been shown that physiological concentrations of leptin stimulate the release of anti-inflammatory cytokines that accept the leptin antagonist in circulation and inhibit the production of pro-inflammatory cytokines. In addition; Leptin stimulates lipolysis. The anti-inflammatory effects of physical activity may protect against TNF-a-induced insulin resistance (Park et al., 2005). Dabidi Roshan and Barzegarzadeh (2011) observed that 8 weeks of high-fat diet increased leptin levels in rats. However, 8 weeks of aerobic exercise decreased leptin in subjects, which is in line with the results of the present study (Dabidi Roshan and Barzegarzadeh, 2011).

Conclusion

Finally, according to the results, the leptin gene is expressed under the influence of short-term exercises and the consumption of curcumin, which is an antioxidant. So that the use of each agent alone causes a decrease or increase in the leptin gene expression in the liver, and the simultaneous use of both factors causes a decrease in the leptin gene expression of these cells in the muscle tissue.

Conflict of Interest

The authors declare that there is no conflict of interest in the present study and the present study was carried out at the expense of the authors.

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The Effects of Nanochitosan Coating Integrated to *Zataria Multiflora* Boiss and *Polylophium Involucratum* Essential Oils on the Shelf-Life Extension of Silver Carp Fillets

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ABSTRACT

Introduction: Active antimicrobial food packaging prevents the growth of foodborne pathogens and spoilage microorganisms by incorporating antimicrobial agents into the film materials.

Methods: The effects of Nanochithosan (NC) coating containing various concentrations of *Polylophium involucratum* essential oil (PIEO) and *Zataria multiflora* Boiss. Essential oil (ZMEO) were investigated on microbial, chemical, and sensory characteristics of silver carp fillets within 12 days during refrigerated storage.

Results: The aerobic plate count (APC) exceeded 7 log CFU/g after day four and day six for the control and samples coated with pure NC, respectively. The samples coated with NC containing ZMEO 0.6% and PIEO 0.6% showed the lowest microbial count. In a control sample with NC containing ZMEO 0.6% and PIEO 0.6%, the total volatile base of nitrogen (TVB-N) reached 33.15 mg/100 g after eight days, but this value remained lower than 25 mg/100 g for the coated samples with NC containing ZMEO 0.6% and PIEO 0.6%. Generally, integrating the ZMEO and PIEO did not significantly and negatively affected the sensory characteristic of coated samples compared with those of control.

Conclusion: According to the results, NC coatings containing ZMEO and PIEO were capable of being used as novel active packaging for fish meat products without compromising their organoleptic characteristics.

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Introduction

The global aquaculture production for silver carp in 2016 was about 5301 tons and ranked second among freshwater fish species due to the low-cost production, availability, high feed efficiency ratio and easy cultivation (1-3). Fish meat is a valuable source of proteins and healthy lipids, which is classified as a highly spoilable food with a relatively short shelf-life under refrigerated conditions. The reduction of fish meat shelf-life could be attributed to its intrinsic factors like neutral pH, low connective tissue, high water holding capacity, free amino acids content and high concentration of polyunsaturated fatty acids (PUFAs) (4). The two major methods of preserving fish meat are freezing and canning, but these methods cannot be used for fresh fish meat.

Active packaging such as antimicrobial films and coatings based on chitosan and its derivatives has been used to prolong the shelf-life and improve food safety (3, 5-8). Chitosan, prepared by chitin deacetylation, is a nontoxic, biocompatible, and biodegradable polyaminosaccharide. The European Union considers chitosan safe at a daily consumption of 3 g (6, 9). Several published studies have reported that chitosan nanoparticles (sizes from 10 to 1000 nm) have higher antioxidant and antibacterial activity properties than chitosan due to their larger surface area and high affinity for bacteria (5, 8).

Avishan Shirazi, with the scientific name *Zataria multiflora* Boiss., belongs to the Lamiaceae family, which mainly grows in Southeast Asia and its leaves are utilized as a flavoring agent in foods and in treating diseases in folk medicine.

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Researchers have reported that Zataria multiflora essential oil (ZMEO) and its major components, carvacrol and thymol, are good antioxidants and antimicrobials.
Polylophium involucratum is a plant belonging to Umbelliferae, which is often wildly grown in northwestern Iran and extensively integrated into foods as a condiment in herbalism.
This study aimed to evaluate the effects of 2 g/100 ml NC solution containing different concentrations of ZMEO and PIEO on shelf-life, prolonging silver carp fillets within a 12-day refrigerated (+4°C) storage period.

Material and Methods

Essential Oil Extraction and Analysis

The dried leaves of *Zataria multiflora* Boiss. and seeds of *Polylophium involucratum* were ground and transferred to a Clevenger and steam distillated for three hours. The gathered EO was stored in a glass tube under the dark condition at 4°C until further use. The constituents of the obtained EO were analyzed by gas chromatography-mass spectrometry (GC-MS) as described previously by Khanjari et al. (2013) (10).

Sample Preparation

The fresh silver carp with an average weight of 2100 g were obtained from a fish farm in Tehran and delivered to the food hygiene laboratory in approximately 45 minutes. Then, the fish fillets were prepared in portions of about 60 g under a safety cabinet (JTLVC2X, Jaltajhiz, Iran).

Sample Treatment and Packaging

The coating solution was made by adding 2 g of chitosan nanoparticles (Nanonovin polymer Mazandaran, Iran) to 100 ml distilled water and stirring for 3 h on a magnetic stirrer at room temperature. Then, glycerol (0.75 ml per gram of nano-chitosan) was incorporated as a plasticizer, and the solution was stirred for another 10 minutes. The coating solution was then mixed with varying concentrations of ZMEO (0, 0.3, and 0.6%) and PIEO (0, 0.3, and 0.6%) mixed with Tween 80 (0.25% v/v). The final coating solution was homogenized at 12,000 rpm for 2 minutes. Afterward, fillet pieces were put in bowls containing 500 ml of coating solution with various EO concentrations for 1 min. In the next stage, the fillets were removed aseptically from the solution and placed on sterile metal mesh under a safety cabinet for 2 min to remove excess coating. Subsequently, the samples were taken into bags and stored at 4°C for 12 days (6, 7, 11).

Microbiological Analysis

Samples of each treatment (10g) were homogenized using stomacher bags containing 90 ml of diluent for 1 min each interval day. Then, suitable serial decimal dilutions of samples were made and cultured on plate count agar for both Aerobic plate count (APC), psychrotrophic bacteria counts (PSB), *Pseudomonas* agar for *Pseudomonas* spp. (PSE), on De Man Rogosa, and Sharpe agar for lactic acid bacteria (LAB) enumeration at seven intervals (0, 2, 4, 6, 8, 10 and 12 days), respectively (12).

Chemical Evolution

The samples were assessed for total volatile basic nitrogen (TVB-N) according to Wang et al. (2018).

Sensory Evaluation

The sensory properties were examined using a 6-member trained panel under the same light, location, and dish conditions. The chicken samples were cooked at the high power of a microwave oven (700 W). The color, taste and odor indexes were assessed using the acceptability scale, with 0 corresponding to the least liked sample and five corresponding to the most liked sample (7).

Statistical Analysis

First, the microbiological count of each treatment was calculated based on the logarithms of the number of colony-forming units (log CFU/g). Then, the logarithmic microbiological and chemical data were analyzed using a one-way ANOVA followed by Tukey's test (12).

Result and discussion

Microbiological analysis of samples

Figure 1 illustrates the changes in aerobic plate count within storage at 4oC. The APC of the silver carp fillet was 4.49 log CFU/g at the beginning day of the study. However, the APC of the control samples reached the maximum number of bacteria for acceptable quality in fresh fish recommended by the ICMSF (higher than 7 log CFU/g) on the fourth day (7.01 log CFU/g) (13). APC of NC-coated samples was significantly reduced (P0.05) by coating, possibly due to the antimicrobial properties of NC, ZMEO, and PIEO. The growth of PSB is an important factor in reducing fish fillet shelf-life under refrigeration.

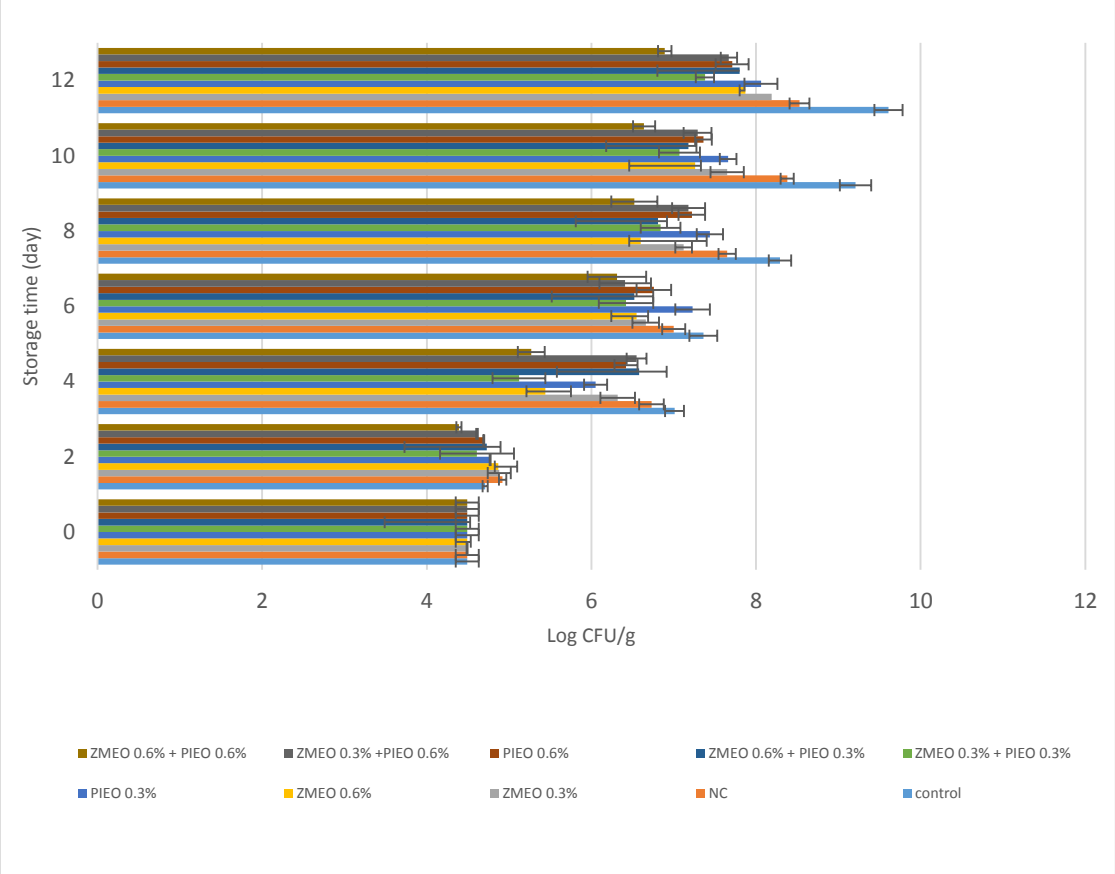

Figure 1. Changes in aerobic plate count (APC) of silver carp during refrigerated storage (*Zataria multiflora* Boiss. (ZMEO), and *Polylophium involucratum* essential oil (PIEO) and nanochitosan (NC))

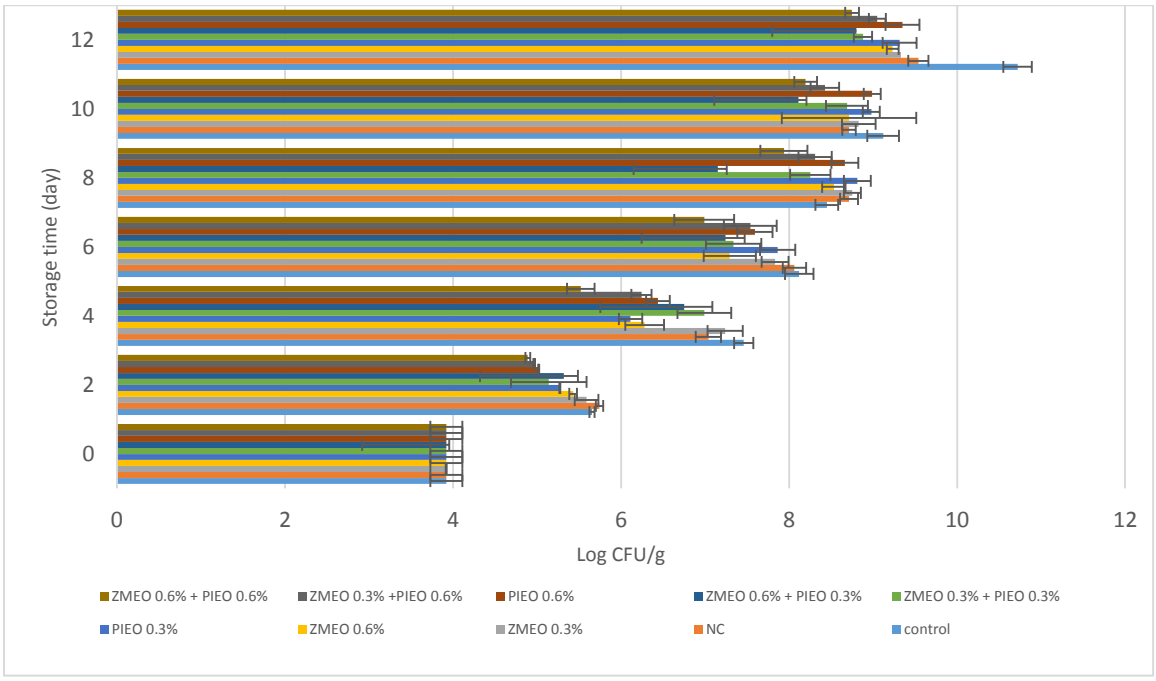

Figure 2. Changes in psychrotrophic bacteria (PSB) of silver carp during refrigerated storage (*Zataria multiflora* Boiss. (ZMEO), and *Polylophium involucratum* essential oil (PIEO) and nanochitosan (NC))

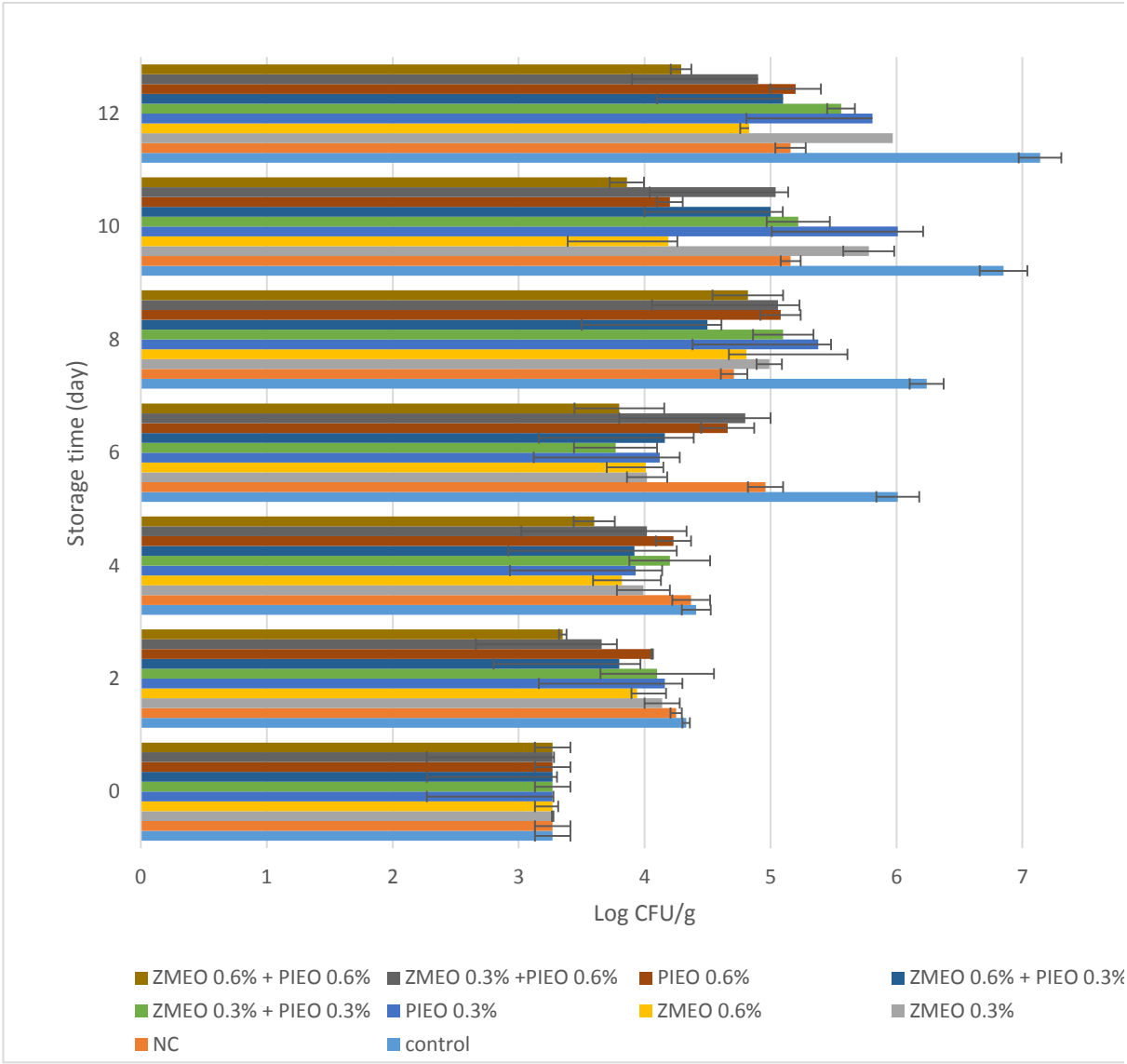

Figure 3. Changes in lactic acid bacteria (LAB) of silver carp during refrigerated storage (*Zataria multiflora* Boiss. (ZMEO), and *Polylophium involucratum* essential oil (PIEO) and nanochitosan (NC))

In this study, the number of PSB in fish fillets was 3.92 log CFU/g, which varied between 2 to 6 logs CFU/g in previous studies depending on the water temperature and conditions (14). The coating with pure NC and NC containing different concentrations of ZMEO and PIEO significantly decreased the PSB ($P < 0.05$) (Figure 2).

As shown in Figure 3, coating samples with NC containing ZMEO and PIEO alone or in combination resulted in a lower LAB count than control samples ($P<0.05$).

The PSE count of silver carp fillets was 2.73log CFU/g and a significant ($P<0.05$) decline in PSE was observed in samples coated with NC incorporated with both EOs compared to the control at the end of the study (Figure 4).

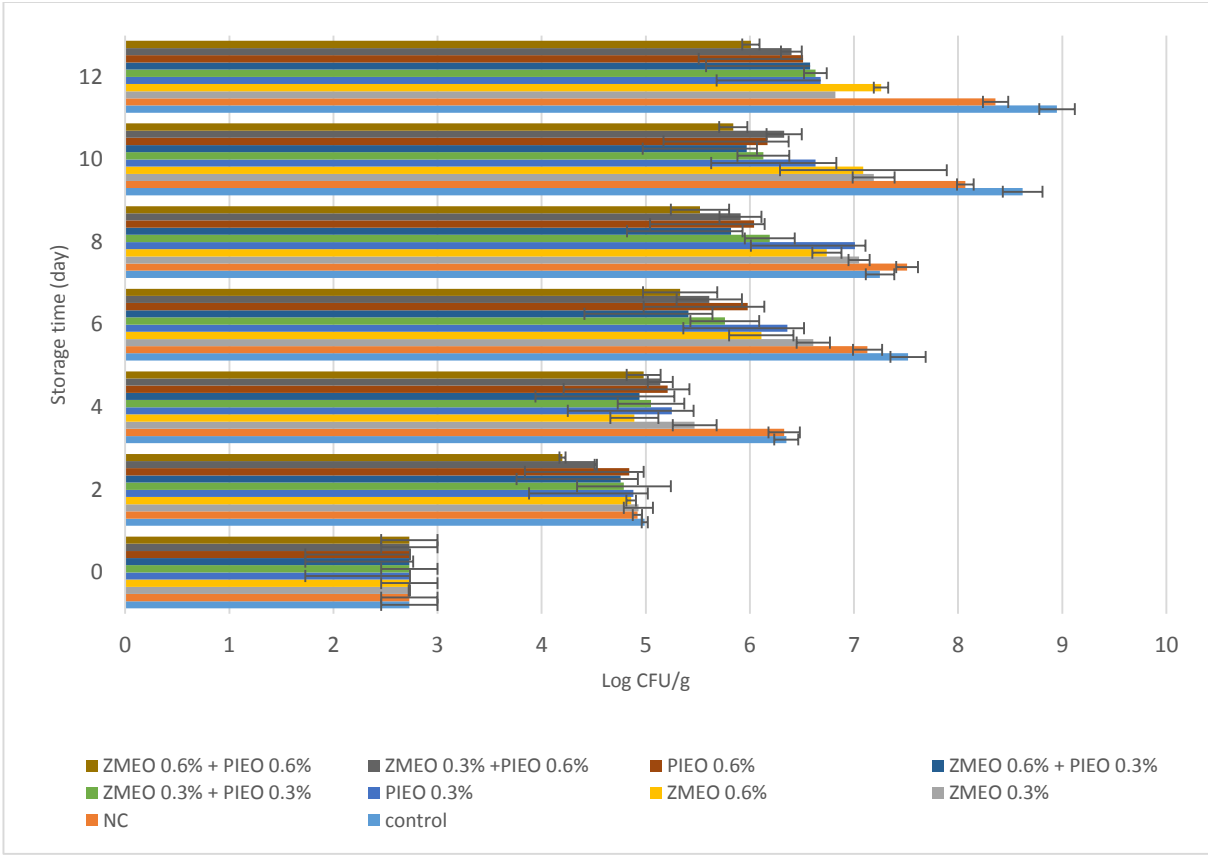

Figure 4. Changes in *pseudomonas* spp. (PSE) of silver carp during refrigerated storage (*Zataria multiflora* Boiss. (ZMEO), and *Polylophium involucratum* essential oil (PIEO) and nanochitosan (NC))

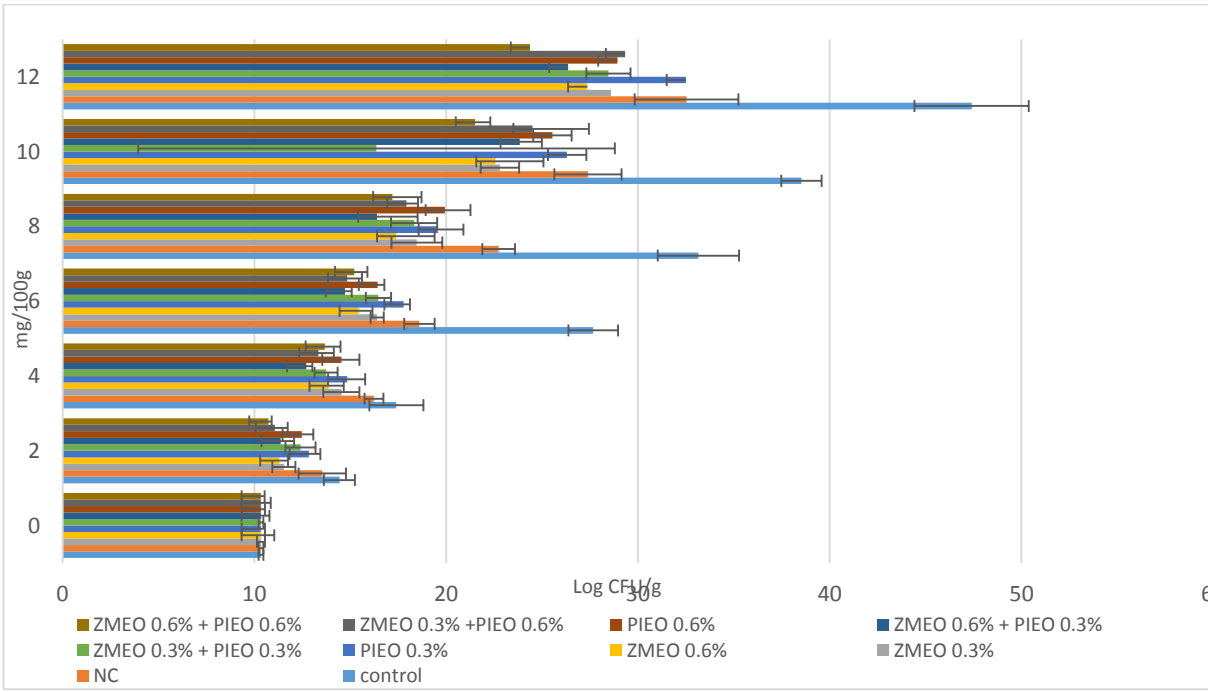

Figure 5. Changes in TVN of silver carp during refrigerated storage (*Zataria multiflora* Boiss. (ZMEO), and *Polylophium involucratum* essential oil (PIEO) and nanochitosan (NC))

Chemical Analysis Evaluation

According to Figure 5 a, both control and NC-coated samples increased TVB-N levels during the study. Nevertheless, the values were significantly lower in the NC-coated samples vs. control samples ($P < 0.05$). In this study, the amount of TVB-N in control samples was 10.34 mg/100g on the first day and reached and afterwards reached to 47.40 mg/100g after 12 days.

Sensory Evaluation

The studied sensory properties (color, odor and taste) of cooked fish fillets for all groups are presented in Figure 6 (a, b and c). The color, odor, and taste scores of the samples were vice versa to microbial and chemical values during storage time. The flavor of low concentrations of ZMEO and PIEO was very attractive to the sensory panel members, and Nanochitosan did not change sample sensory properties.

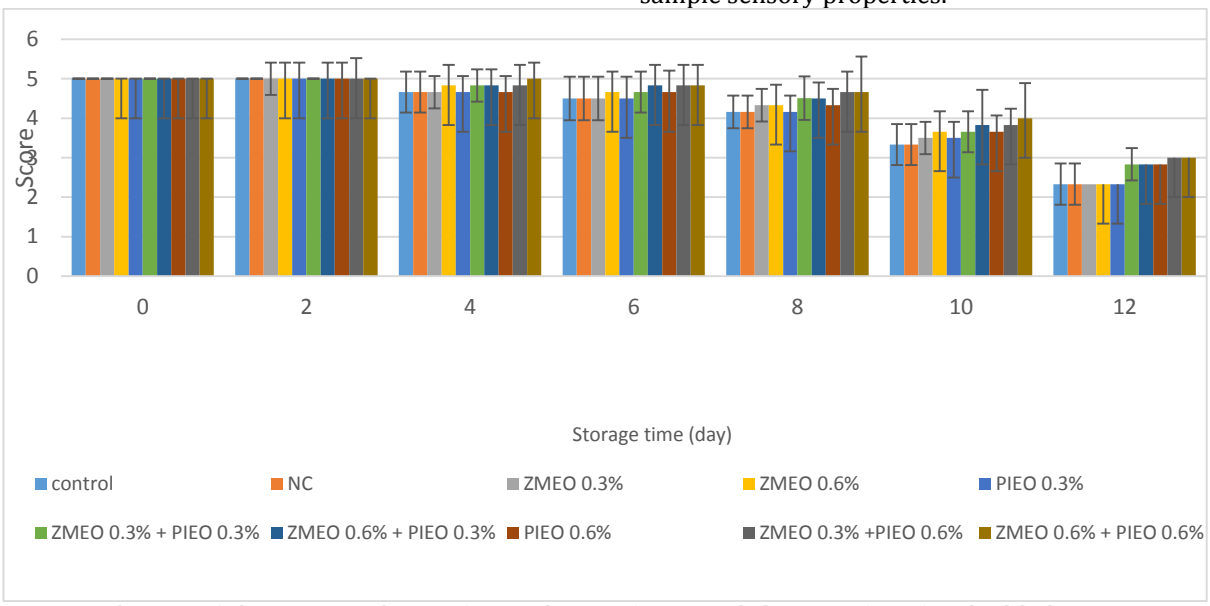

Figure 6. Color score of silver carp meat during refrigerated storage (*Zataria multiflora* Boiss. (ZMEO), and *Polylophium involucratum* essential oil (PIEO) and nanochitosan (NC))

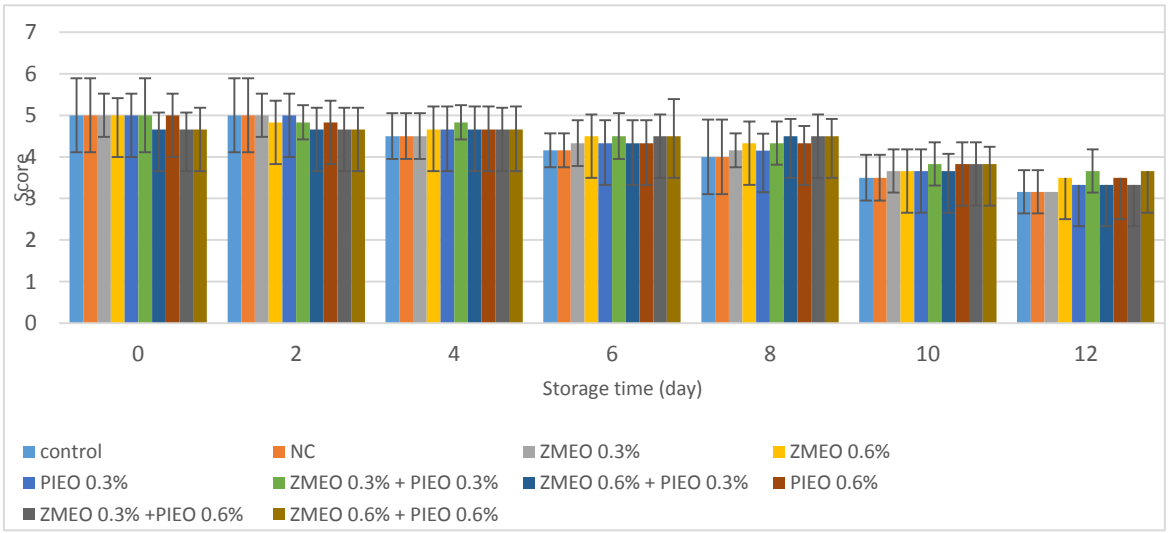

Figure 7. Odor score of silver carp meat during refrigerated storage (*Zataria multiflora* Boiss. (ZMEO), and *Polylophium involucratum* essential oil (PIEO) and nanochitosan (NC))

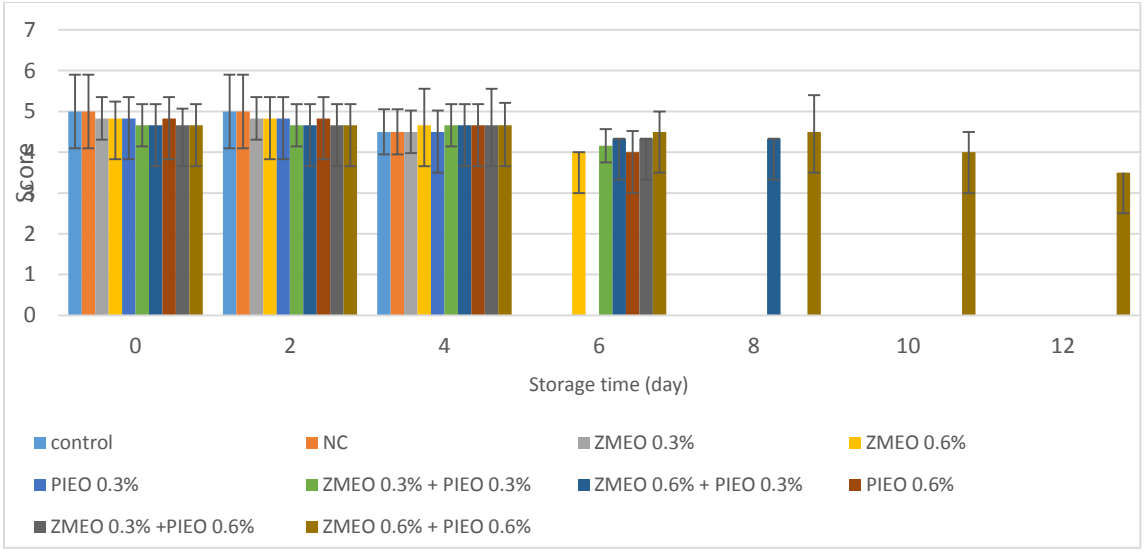

Figure 8. Taste score of silver carp meat during refrigerated storage (*Zataria multiflora* Boiss. (ZMEO), and *Polylophium involucratum* essential oil (PIEO) and nanochitosan (NC))

Table 1. Essential oil composition of *Polylophium involucratum* identified by GC-MS.

Row	Compounds	Kovats Retention Index	Percentage
1	Alpha-pinene	997	4.66
2	Sabinene	1037	.57
3	Beta-pinene	1044	.60
4	Myrcene	1052	1.83
5	Limonene	1106	33.08
6	Gamma-tripene	1139	.11
7	Linalool	1175	.23
8	Cis-limonene oxide	1219	.6
9	Trans-limonene oxide	1225	.83
10	Methylbenzoate	1393	49.15
11	Perilla Alcohol	1428	1.12
12	Limonene Glycol	1461	.21
13	Alpha-Copaene	1552	.12
14	Beta-Cubebene	1564	.21
15	Germacrene D	1682	.26
16	Spathulenol	1795	.16

Table 2. Essential oil composition of *Zataria Multiflora* Boiss. identified by GC-MS.

Row	Compounds	Retention Time (minutes)	Percentage
1	α– Pinene	6.291	2.08
2	β- Pinene	7.765	.26
3	Octanone	8.181	.74
4	β–Myrcene	8.356	.84
5	α–Terpinene	9.342	1.02
6	Cymene	9.727	5.52
7	1,8-Cineole	9.927	.24
8	γ–Terpinene	11.156	2.56
9	Linalool Oxide	11.74	.61
10	α–TERINOLENE	12.432	.72
11	Linalool	13.393	23.91
12	Borneole	15.884	.19
13	4-Terpineol	16.439	.93
14	α–Terpineol	17.312	.44
15	Carvacrol methyl ether	19.776	1.97
16	Thymol	22.447	4.13
17	Carvacrol	23.325	48.19
18	Carvacrol Acetate	25.723	1.27
19	Trans-Caryophyllene	27.372	.89
20	Caryophyllen oxide	32.913	.55

Discussion

The major compounds of ZMEO and PIEO used in this study were Carvacrol (48.19%) and Methyl benzoate (49.15%), respectively, with good antimicrobial activity (15). Hosseini et al. (2015) reported the same outcomes regarding the use of EO, and observed that the APC of rainbow trout fillets exceeded 6 log CFU/g in control samples and samples coated with oregano EO after 8 and 12 days, respectively (16). Fadiloglu and Coban (2018) found that the APC values of control samples were 6.77 log CFU/g at day 9, and the APC value of coated samples with chitosan alone and chitosan+sumac was lower than the 7 log CFU/g at days 9 and 12, which consistent with this study (17). In addition, these results are in line with those of Shahbazi and Shavisi (2019), who concluded that coating of silver carp fillets with sodium alginate incorporated with *Mentha spicata* EO significantly diminished the APC of treated samples in comparison with control ones (18). However, Andevari and Rezaei (2011) reported inconsistent findings so that coating the rainbow trout fillets with gelatin integrated with cinnamon EO did not significantly differed in the APC of coated samples from those of the control samples (19). The possible reasons for the observed differences could be attributed to the different compositions of ZMEO and PIEO and the general microflora of fresh fish. The dominant microflora of temperate water fish is gram-negative rod-shaped psychrotrophic bacteria (PSB) (20). The outcome of this research was consistent with Ojagh et al. (2010), who stated that the coating of chitosan enriched with *Cinnamomum zeylanicum* EO delayed the PSB growth in coated rainbow trout (7). Similar results were declared by Ramezani et al. (2016), who explained that the NC treatment significantly diminished the PSB bacteria population in silver carp fillets (8).

The most important spoilage bacteria in refrigerated proteinaceous products are lactic acid bacteria. This type of bacteria can metabolize amino acids and produce ammonia and biogenic amines, such as histamine, putrescine, and tyramine (21). Researchers have declared that coating prepared with various EOs can retard the LAB growth in refrigerated fish (22-24). LAB is among the most resistant Gram-positive bacteria against the antimicrobial activity of EOs (25). A possible explanation for this resistance may be their ability to adjust to osmotic stress conditions by producing ATP and responding more effectively to EO-induced K+ efflux (24, 26).

Pseudomonas (PSE) are among the commonest microorganisms associated with fish fillets' spoilage within cold storage. Species of this genus can produce protease enzymes and use nitrogen-containing compounds as energy sources, causing fish fillets to have an off-odor and off-flavor. Based on Jouki et al.'s (2014) study, NC coating incorporated with ZMEO and PIEO yielded similar results. Rainbow trout samples wrapped with quince seed mucilage edible films infused with 2% thyme extract were found to have a significant reduction in PSE populations (24). The results of this study are also consistent with those of Shahbazi and Shavisi (2019), who reported a significant reduction in silver carp PSE of 4.14-5.44 log CFU/g when treated samples were compared with untreated samples. By adding Mentha spicata EO (0.5 and 1%) to edible sodium alginate coatings after 14 days of storage, the control samples were significantly reduced in PSE (18).

TVB-N level of meat is related to the microbial growth and endogenous protease activity of meat. Thus, the shelf-life and freshness of meat could be evaluated by measuring the TVB N (27). Similar findings were declared by Raeisi et al. (2020), Andevari and Rezaei (2011), and Wang et al. (2018) regarding the utilization of NC coating integrated with ZMEO and PIEO. As a result of the antibacterial effect of Nanochitosan combined with ZMEO and PIEO, the TVB-N values are lower in the coated samples (2). Some compounds such as dimethyl disulfide, dimethyl sulfide and propylene sulfide are formed during the metabolism of Pseudomonads by the growth of the *Pseudomonas* spp. population. LAB metabolizes the amino acids to ammonia and biogenic amines after exhausting carbohydrates in fish meat. The taste and odor were unfavorable due to these compounds. Furthermore, producing chemical substances such as aldehydes and ketones from lipid oxidation could be the reason for the off-odor of fish fillets (21, 28).

Previously, color parameters were used to describe the freshness and acceptability of fish muscle. In general, the use of different concentrations of ZMEO and PIEO within the research period had no significant negative

effects on NC-coated samples' sensory characteristics compared to those of the control samples.

Conclusion

According to the results, NC coatings containing ZMEO and PIEO could be used as novel active packaging for fish meat products without adverse organoleptic effects. Silver carp fish fillets can be controlled by using alternative and non-toxic matrices that reduce spoilage bacteria populations.

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JOURNAL OF NUTRITION FASTING AND HEALTH

The Simultaneous Effect of Endurance Training and Octopamine Supplementation on Octopamine and its Receptors in the Visceral Fat Tissue of Rats Treated with Deep Fried Oil (DFO)

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ABSTRACT

Introduction: The role of exercise and some supplements in lipolysis has been reported, but given limited information on the simultaneous effect of endurance training (ET) and octopamine (Oct), the present study aimed to investigate the interactive effect of these two interventions on adipose tissue lipolysis with emphasis on octopamine receptors in rats treated with deep fried oil (DFO).

Methods: Thirty male Wistar rats (20-18 weeks old and 280-320 g) were divided into five groups, including (1) healthy control (C), (2) DFO, (3) Oct+DFO, (4) ET+DFO and (5) DFO+Oct+ET. Aerobic training was performed for four weeks, five sessions per week with an intensity of 16-26 m / min and equivalent to 50-65% VO_{2max}; also, 81 µmol/kg octopamine supplementation was intraperitonally injected to rats 5 days a week. Two-way analysis of variance and Bonferroni's *post hoc* test were used to analyze the data.

Results: ET increases *Octβ-R* expression (P=0.02) and Oct protein concentration (P=0.001) in the visceral adipose tissue of rats exposed to DFO. Oct supplementation increases *Octα-R* (P=0.01) expression in the visceral adipose tissue of rats exposed to DFO. Also, ET and Oct do not have a synergistic effect on *Octβ-R* (P=0.91), *Octα-R* (P=0.65) and Oct protein concentration (P=0.16) in the visceral adipose tissue of rats exposed to DFO.

Conclusion: It seems that although training and octopamine supplementation alone play a role in increasing the protein concentration of octopamine and its receptors, these two interventions do not have a synergistic effect on lipolysis by emphasizing the octopamine receptor pathway.

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Introduction

Today, due to the individuals' busy everyday activities and industrialization of societies, a large number of people have turned to fast foods and fried foods. Because a lot of oil is used to cook such foods and these oils are not cost-effective, they may be continuously exposed to heat and have harmful effects on people (1). In other words, high and long-term heat in frying oils leads to an increase in free radicals in the cooked foods and at the same time with the destruction of the structure of vitamin E due to deep heating in these oils, inflammation and vascular dysfunction increase (2). In addition, this style of diet due to the increase of ample unsaturated fatty acids leads to increased fat cell hypertrophy, increased fat mass, obesity and finally related diseases (3).

Researchers believe that increased fat mass is associated with inhibition of lipolysis pathways and its mechanisms. Octopamine (Oct) is a mammalian norepinephrine analog that binds to adrenoceptor receptors (ARs) such as beta-adrenoceptors (4), octopamine receptors (*Oct.R*), beta-adrenergic receptors *Octβ1R*, *Octβ2R* and *Octβ2R*, and α-adrenergic octopamine receptor (Octα.R), and exerts its lipolysis effects (5); therefore, the addition of this dietary supplement, which has long been used by researchers, is recommended to take advantage of its beneficial effects.

In addition, the researchers believe that the octopamine pathway and its receptors act like adrenergic pathways; in other words, the Octβ1R receptor is similar to beta-adrenergic, and similar to it, it phosphorylates and activates the

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downstream pathways such as cAMP/AMPK/HSL (5).

In this vein, in previous studies, the use of honey-derived Oct resulted in an increase in Octβ1R receptors (6); also, in our previous study, Oct consumption increased tyramin-R receptor, increased hormone-sensitive lipase (HSL) and decreased G protein-coupled receptors (GPCR) in adipose tissue (7). Other studies have also shown an improvement in fat profile in rats fed with deep-fried food (DFO) (8).

Also, octopamine supplementation led to an increase in HSL activity and lipolysis pathways in visceral adipose tissue (7). In addition, in another study, researchers showed that the consumption of octopamine led to an increase in β1- or β2-ARs proteins in fat cells derived from mammals (4).

On the other hand, regarding reduced physical activity due to lifestyle changes and its effect on weight gain and obesity, sports scientists believe that the most appropriate and non-invasive way to lose weight is regular exercise (9). In other words, endurance training leads to a reduction in fat mass by activating androgenic pathways, activating HSL and activating the Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) pathway (10). Past studies have also shown that endurance training improves *Octβ1R* expression in heart and skeletal muscle tissue of Dorsophila (5).

In this regard, the researchers showed that Octβ1R expression decreases after aging, however endurance exercise in Dorsophila increases the expression of Octβ1R and OAMB in the heart and skeletal muscle tissue, and this causes changes in body fat tissue (5). Also, in a previously-conducted study, endurance training increased the expression of Octβ1R, Octβ2R and Octβ2R, and Octα.R receptors in the visceral fat tissue of rats exposed to DFO (7).

In addition, researchers' attention has recently been drawn to the interactive effects of training and Oct supplementation on animal models; for example, the study of Kianmehr et al. showed that aerobic training (ET), Oct and interaction of ET and Oct increased the expression of PGC-1α and uncoupled protein-1 (UCP-1) (11), and augmented inhibition of apoptotic factors (12) in the heart tissue of rats exposed to DFO. In a study, the results showed that the interaction of the two (ET and Oct) increased heat shock protein-70 (HSP70) and decreased caspase-3 in the brown adipose tissue of rats exposed to DFO (13).

Although the individual and favorable role of aerobic training and octopamine supplementation on lipolysis pathways has been reported, the lipotic mechanism dependent on different receptors such as Oct-α and Oct-β in the visceral adipose tissue of rats exposed to DFO is still not well understood.

In addition, the simultaneous effect of training and octopamine supplementation on lipolysis pathways is a very interesting topic for researchers; therefore, considering the increase in weight and obesity, the increase in deaths caused by it, and the globalized spread of this problem, conducting basic research that can increase the understanding of researchers to provide more practical solutions and prevent diseases caused by obesity seems necessary.

Therefore, the aim of this study was to investigate the interactive effects of ET and Oct on the octopamine receptor pathway in the visceral adipose tissue of rats exposed to DFO.

Materials and Methods

Preparation and Maintenance of Animals

In this basic and experimental study, 30 male Wistar rats with an age range of 20-18 weeks and a weight range of 280-320 g were prepared from the Histogen Research Center.

The selection of the sample size in this study was based on analogous animal model studies. In this vein, given the ethical codes of research, the researchers sought to use the lowest number of animals in the sample and obtain the optimal results (14,15).

Samples were kept in the laboratory for one week for compatibility. It should also be noted that during the course of research, the ethical principles of working with laboratory animals were observed in accordance with the Helsinki Agreement. In this study, rats were kept in the standard conditions of 22-24° C, relative humidity of 55%, 12-12 hours of light-darkness cycle, in special washable cages, with *ad libitum* access to water and food. Subsequently, after adaptation, they were randomly divided into five groups of six animals, including: (1) healthy control (C), (2) DFO, (3) Oct + DFO, (4) ET + DFO and (5) DFO + Oc + ET.

Preparation of Deep Fried Oil (DFO)

To prepare the deep fried oil, 8 liters of sunflower oil was heated at 190 to 200 ° C for 8 hours for 4 days, and according to the literature review, every 30 minutes some food including chicken

nuggets, potatoes, chicken and protein products (sausages) were dipped in oil. On the fourth day, the deep fried oil was fed orally to rats (0.1 cc per 100 grams of body weight by gavage) for 4 weeks as a poisoning intervention (16).

Consumption of Octopamine

In this study, octopamine supplement prepared by Sigma Aldrich Company was first dissolved in 9% normal saline and then, each time, 81 µmol / kg of it was injected peritoneally into rats for four weeks, five days a week (16).

Endurance Training Protocol

To perform endurance training, first, three introductory sessions were performed with a treadmill. For this purpose, rats were placed on the treadmill for 20 minutes at a speed of 9 m / min for 5 days. The main endurance training protocol was performed for 4 weeks. In this training protocol, the intensity of training was considered 50% VO_{2max} in the first week and 65% VO_{2max} in the last week. In order to comply with the principle of training overload, the intensity of training started at 16 m / min on the first day and reached 26 m/min on the last day. Also, in addition to the main training, a duration of 5 minutes at a speed of 7 meters per minute were considered for warming up and cooling down (11).

Dissection and Sampling

48 hours after the last training session and supplementation, rats were anesthetized with a combination of ketamine (55 mg / kg) and xylazine (18 mg / kg) following 12 hours of fasting. After experts' confirming complete anesthesia and ensuring analgesia and unconsciousness, rats' abdominal cavity was cut with a razor blade size 20 and visceral adipose tissue was carefully extracted. For physiological measurements, 500 mg of tissue was placed in a tissue storage microtube and immediately frozen at -70° C. Also, for pathological examinations, the required amount of visceral adipose tissue was placed in 15% formalin solution to examine tissue incisions and hematoxylin-eosin and trichrome methods in the future.

Assessment of Octopamine by Western Blotting

Octopamine levels in this study were measured by Western blotting. For this purpose, 40 mg of the extracted octopamine protein were boiled for 5 to 10 minutes and then placed on polyacrylamide gel and run for 100 to 2 hours at a voltage of 100. Then the electrophoresis gel was prepared for transfer and the nitrocellulose paper, device pads and sponges were sandwiched inside the transfer buffer and electrophoresed at 350 amps for one hour. The nitrocellulose paper was then washed using TBS buffer for 5 to 10 minutes. The nitrocellulose paper was then left at room temperature for one hour and immersed and shaken in a TBS (or blocking) buffer. This operation was repeated several times and then nitrocellulose paper was diluted with secondary antibody (dilution of 1/3000 antibody) at room temperature for 1 to 2 hours and then incubated in TBS buffer. This operation was repeated twice to ensure stabilization. The nitrocellulose paper then appeared on the photographic film in a dark room by means of the exposure solution and the stabilizer using ECL, and after the bands appeared, the nitrocellulose paper was washed with distilled water. At the end, the paper with B actine antibody was placed on the paper and incubated with secondary antibody. After B actine control appeared as an internal control in the radiology film, the images banded by Image J program were densiometered to obtain quantitative values.

Assessment of Oct-α and Oct-β Gene Expression Levels

In order to evaluate the expression levels of *Oct-α* and *Oct-β* in qReal Time PCR, initially 20 mg of adipose tissue was used to extract RNA.

RNA extraction was performed using the manufacturer's protocol (Trizole, manufactured by Kiagen Company, Made in the United States with catalog number 79306). Then, to determine the quality of RNA extraction, the light absorption property at 260 nm wavelength was used. After ensuring the proper concentration of RNA, cDNA synthesis was performed according to the RevertAid First Strand cDNA Synthesis protocol manufactured by Thermo Scientific Company in the USA with catalog number K1622. The synthesized cDNA was then used to perform the reverse transcription reaction. First, the designed primers were added to the cDNA according to the instructions on the PUBMED site. It should be noted that before performing the test, the researcher made sure that the primer worked using the software available on the PUBMED site. The GAPDH internal control gene was then inserted into the qReal Time PCR

together with the target gene, and the transcription reaction was allowed to continue transcribing in different cycles to reach the threshold cycle (CT). Then, after completing the operation of the device, the formula $2^{-\Delta\Delta CT}$ was used to quantify the data. The sequence of the primers used in the study is presented in Table 1.

Table 1. Sequence of gene primers measured in the research

Genes	Primer Sequences	Size (bp)
GAPDH	Forward: *5'- AAGTTCAACGGCACAGTCAAGG -3'* Reverse: *5'- CATACTCAGCACCAGCATCACC -3'*	112
Oct-α	Forward: *5'- GGTACTTTGGTAAGGTGTGGTG -3'* Reverse: *5'- AGTGGCGGGAAGGAGATGA -3'*	126
Oct-β	Forward: *5' GGTGGAGCAGGATGGGAGGA -3'* Reverse: *5' GTAGCCAGCAGAGGGTGAAG -3'*	118

Histological Examination by Hematoxylin-Eosin Method

Hematoxylin-eosin method was used to evaluate changes in visceral adipose tissue in terms of cell volume and size, cell density and size of fat vacuoles. For this purpose, some adipose tissue was fixed in 15% formalin solution; the tissue was then immersed in an alcohol solution (80, 96, and 99%, respectively) to dehydrate. After clarification and impregnation with paraffin and alcohol, the samples were molded. Blocks of tissue were prepared for molding. Then molten paraffin was poured on the blocks, and then a BASCET was placed on the samples and some molten paraffin was poured on them again. The samples were then placed in the ambient temperature to solidify. The tissue was then prepared for incision and the tissue was cut with a microtome with a diameter of 1 micrometer and after placement on a laboratory slide, it was fixed with alcohol. The samples were then stained with hematoxylin and eosin dyes for three minutes and kept at room temperature. Finally, the tissues were examined by light microscope after dehydration.

Statistical Procedures

The Shapiro-Wilk test was used to evaluate the normality of the distribution of findings. To evaluate the effect of DFO on the levels of research variables, the DFO and HC groups were compared by independent samples t-test.

Given that the present design had two factors of training and octopamine supplementation, a two-factor design of two-way analysis of variance was used.

Two-way analysis of variance was used to evaluate the main effect of training, the main effect of octopamine supplementation as well as the interactive effect of training and octopamine supplementation. Also, Bonferroni's *post hoc* test was used to evaluate the effect of each intervention. The findings of the present study were analyzed in SPSS software version 22 and the significance level was set at 0.05.

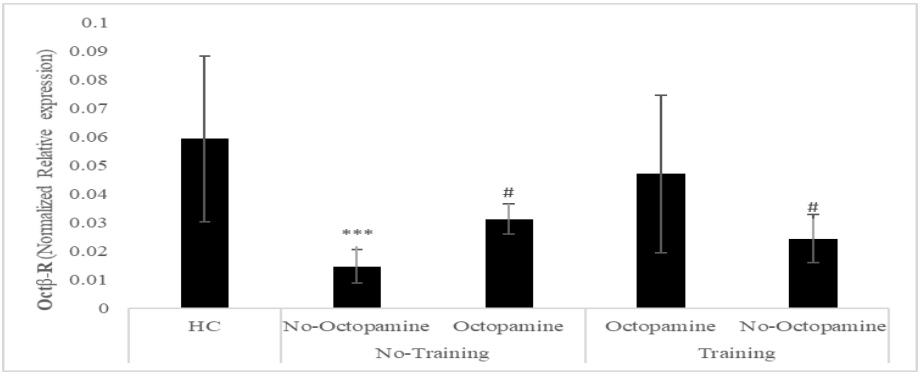

Figure 1. Results of two-way analysis of variance to evaluate the interactive effect of ET and Oct on *Octβ-R* receptor levels in the visceral adipose tissue of rats exposed to DFO

**(P = 0.01) Significant decrease in the DFO group compared to the HC group

#(P = 0.05) Significant increase in the Training and Oct variables compared to the DFO group

The results show that although *Octβ-R* levels are higher in the ET + Oct group, but these two variables alone increase *Octβ-R* receptor expression and their effect is not synergistic

Results

The results of independent samples t-test showed that *Octβ-R* (P = 0.002), *Octα-R* (P = 0.01) and Oct (P = 0.001) in the HC group were significantly higher than the DFO group.

The results of two-way analysis of variance showed that ET (P = 0.02, F = 6.46 and effect size 0.28) and Oct (P = 0.01, F = 8.18 and effect size 0.33) had a significant effect on changes in *Octβ-R* gene expression levels in the visceral adipose tissue of rats exposed to DFO; but the interaction of ET and Oct on changes in *Octβ-R* receptor levels was not significant (P = 0.91, F = 0.011 and effect size 0.001). Also, the results of Bonferroni's *post hoc* test showed that *Octβ-R* receptor levels in the ET (P = 0.022) and Oct (P = 0.011) groups were significantly higher than the DFO group (Figure 1).

ET has no significant effect on Octα-R changes in the visceral adipose tissue of DFO-poisoned rats (P = 0.075, F = 3.64 and effect size 0.18), but Oct has a significant effect on changes in *Octα-R* levels in the visceral adipose tissue of DFO-poisoned rats (P = 0.01, F = 8.64 and effect size 0.35). Also, the interaction of ET and Oct on changes in *Octα-R* gene expression levels is not significant (P = 0.65, F = 0.21 and effect size 0.013); in other words, Octα-R levels in the Oct groups are significantly higher than the DFO group (P = 0.01) (Figure 2).

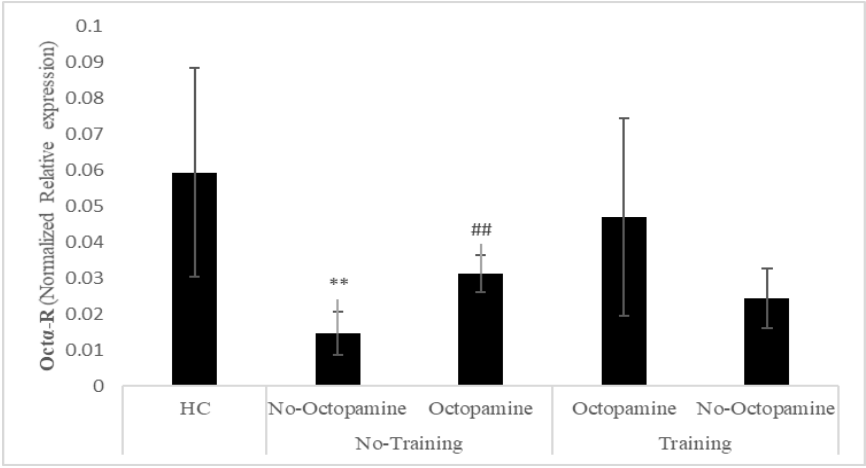

Figure 2. Results of two-way analysis of variance to evaluate the interactive effect of ET and Oct on *Octα-R* receptor levels in the visceral adipose tissue of rats exposed to DFO

**(P = 0.01) Significant decrease in the DFO group compared to HC group

##(P = 0.01) Significant increase in the Oct variable compared to the DFO group

The results show that although Octα-R levels are higher in the ET + Oct group, only the Oct supplement variable is effective in increasing the expression of Octα-R receptor and the training variable modulates the effect of Oct supplement.

ET has a significant effect on changes in Oct expression levels in the visceral adipose tissue of rats exposed to DFO (P = 0.001, F = 61.38 and effect size 0.83), but Oct supplementation has no significant effect on changes in gene expression levels (P = 0.055, F = 4.51 and effect size 0.27). Also, the interaction of ET and Oct on changes in Oct expression in the visceral adipose tissue of rats exposed to DFO is not significant (P = 0.16, F = 2.18 and effect size 0.15). Oct levels in the ET groups are significantly higher than the DFO group (P = 0.001) (Figure 3).

Examination of images obtained from different groups of visceral adipose tissue show that the number of fat cells in the healthy control group (CH) is significantly lower than the other groups, while the diameter of each fat vacuole in this group is larger than the other groups. Also, the number of these cells in the training + octopamine group is low and there has no significant difference with the normal group. In groups such as the training + octopamine, the number fat cells increase compared to the healthy control (CH) group. In the DFO group, the number of these cells increase significantly compared to the other groups, while the diameter of each fat vacuole is smaller than the other groups. The results of histological images showed that the higher the amount of oil intake in the groups, the number of fat cells initially increases and then the cells begin to become obese and hypertrophic, which indicates a gradual increase in adipose tissue.

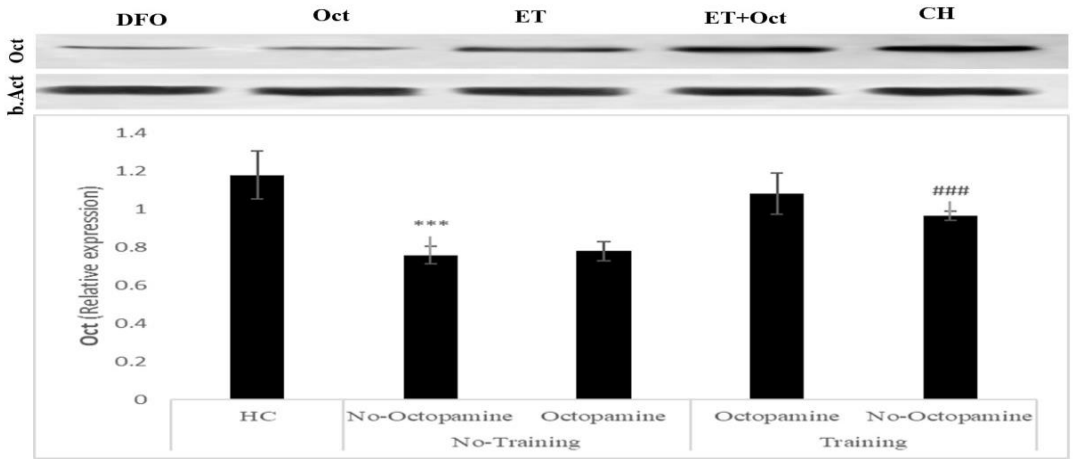

Figure 3. Results of two-way analysis of variance to evaluate the interactive effect of ET and Oct on Oct protein levels in the visceral adipose tissue of rats exposed to DFO
***(P = 0.001) Significant decrease in the DFO group compared to the HC group
###(P = 0.001) Significant increase in the Training variable compared to the DFO group The results show that training and octopamine have a synergistic effect on Oct; However, octopamine supplementation somewhat modulates the effect of training in increasing Oct in the visceral adipose tissue of DFO-exposed rats.

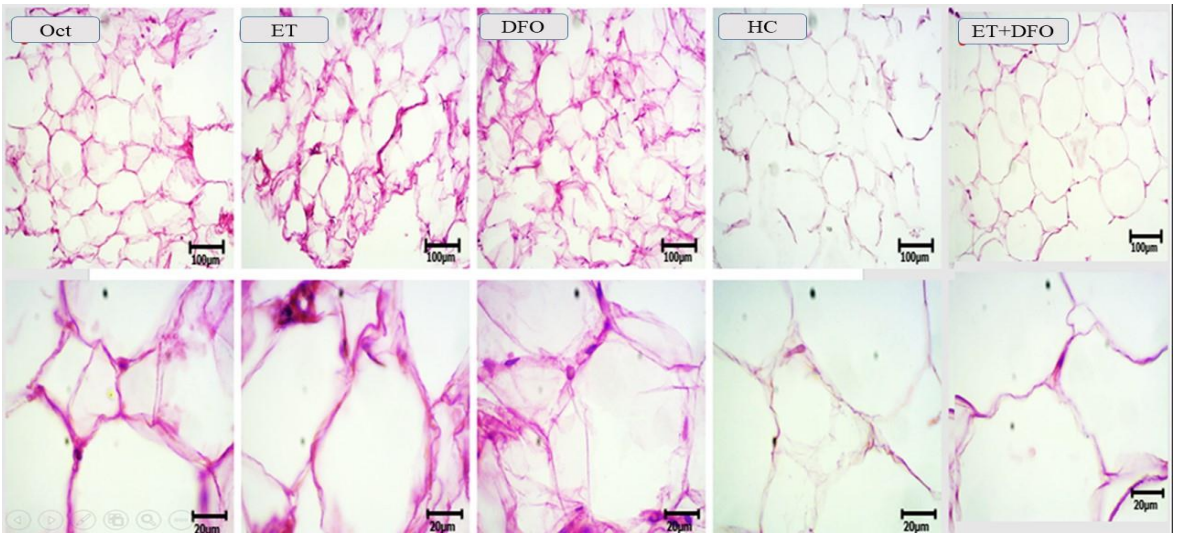

Figure 4. Results of microscopic examination of visceral adipose tissue cells in the research groups

Discussion

According to the previous studies, it seems that improper diet, especially oils that have been heated at high temperatures and for a long time, increases the amount of TG and cholesterol in plasma and liver, and this leads to the activation of the lipogenesis pathway in the visceral fat tissue, because in this case, the amount of energy received from high-fat food increases compared to the calories consumed with physical activities and leads to an increase in body fat percentage (17). In addition, DFO seems to lead to the activation of lipogenic pathways by inhibiting GPCR and tyramine receptors as well as inhibiting beta-adrenergic receptors (7). Furthermore, studies show that octopamine signaling is one of the vital pathways of metabolism, thus the present study aimed to investigate the synergistic effect of endurance training and octopamine supplementation on

protein concentration and two types of octopamine receptor subunits in the visceral adipose tissue of DFO-poisoned rats. The results showed that ET increased *Octβ-R* gene expression levels and Oct protein concentration in the visceral adipose tissue of rats exposed to DFO. Studies show that octopamine, especially *Octβ-R* subunits, is a beta-adrenergic receptor analog such as norepithephrine, and as the beta-adrenergic pathway is activated following training, Octβ-R signaling is activated as well (5). Nonetheless, it appears that the effect of training on lipolysis activation is through emphasis on the octopamine pathway of PKA/cAMP and Ca2+/IP3/CaMK, which ultimately, by activating gene transcription pathways, leads to increased expression of octopamine receptors, especially with *Octβ1R* and *Octβ3R* subunits, and is effective in lipolysis (18). In this regard, Sujkowski's study (2020) showed that endurance training increased Octβ1R in heart tissue, improved physical function in Dorsophila (5). In another study, the researcher increased octopamine levels following endurance training, which improved neuronal function and the octopaminergic pathway in Dorsophila (18).

In addition, in a study it was shown that endurance training leads to the inhibition of myocardial apoptosis in rats exposed to DFO (12). Still, in another study, researchers showed that aerobic training leads to an increase in HDL/LDL ratio as well as in acetyl co-enzyme A, and through this pathway increases lipogenic proteins such as malonyl co-A (8).

Also, in a researchers' previously-conducted study, endurance training increased tyramine receptor levels and expression of hormone-sensitive lipase in the visceral adipose tissue of rats exposed to DFO (7). Therefore, in line with previous studies, our study also showed that different types of training increase lipolysis by activating beta-adrenergic subunits and inhibiting alpha-adrenoceptors.

The results showed that Oct increased *Octβ-R* and *Octα-R* gene expression levels in the visceral adipose tissue of rats exposed to DFO. According to studies, octopamine is known as a biogenic amino acid that has a high affinity for *Octβ3R*; it can also activate the protein by activating beta G subunits and lead to phosphorylation of cAMP by activating adenylate cyclase, thereby activating the transcription pathway of metabolic genes including *Octβ-R* and *Octα-R* receptors as well as lipolysis in adipose tissue (7,19). In this regard, researchers have stated that the use of octopamine as a beta-adrenergic analogue led to the modulation of nervous system function and improved dopamine type 3 receptor levels (20). Furthermore, the consumption of octopamine leads to the inhibition of myocardial apoptosis (12) and an increase in HDL/LDL ratio as well as in acetyl coenzyme A (8) in rats exposed to DFO. In confirmation of these results, another study stated that honey-derived octopamine supplementation increased the expression of Octβ-R and Octα-R receptors; Octopamine supplementation has also been shown to be beneficial in lipolysis and glucose metabolism pathways, and improved genes associated with these thermogenesis pathways (6). In a study, researchers also showed that taking octopamine increased β-3 adrenergic expression and improved glucose metabolism (21); in another study, octopamine increased epinephrine and norepinephrine (22); therefore, octopamine seems to play a role in activating gene transcription from beta-adrenergic pathway, and further transcribes both of its receptors through this pathway.

The results also showed that implementing ET and Oct individually increases the expression of *Octβ-R*, but only Oct supplement is effective in increasing the expression of the *Octα-R* receptor and training modulates the effect of Oct supplement. Octopamine supplementation also partially modulates the effect of training on increasing Oct in the visceral adipose tissue of DFO-exposed rats. It seems that the effect of octopamine supplementation along with training is dose-dependent, so that in a study, researchers showed that low dose (150 mg) of octopamine before training had no significant effect on improving performance in active men (23). In addition, training and octopamine had an interactive effect in reducing G protein-coupled receptor (GPCR) in visceral adipose tissue (7).

Considering the review of literature, few studies have been performed on the synergistic effect of training and octopamine supplementation on its receptor pathways that cause lipolysis; however, it appears that endurance training by increasing catecholamines (epinephrine and norepinephrine) and gene expression transcription pathways increase the expression of octopamine receptors (5); also, octopamine supplementation plays a role genes transcription

through the *octβ3R* and cAMP pathways, and hence both variables are involved in improving fat metabolism by similar mechanisms. Therefore, as can be seen in the above Figures, the training and octopamine supplementation group had a better performance in *Octβ-R* and *Octα-R* gene expression levels than either alone, however non-significance of synergistic effect of these two variables could be attributed to thr intensity of endurance training in lipolysis activation (24,25) and the difference in the dose of octopamine in the lipolysis pathway along with training (23) which can sporadically moderate each other.

It seems that aerobic training plays a role in improving lipolysis by increasing catecholamines as well as increasing angiogenesis and mitochondrial biogenesis, while Oct supplementation plays a role in fat catalysis by increasing HSL activity in the visceral adipose tissue; Therefore, although the interactive effect of the two interventions on the lipolysis pathway was not observed, it appears that both interventions can work together and affect each other mutually.

Given the role of the proteins responsible for transcription in the downstream octopamine-dependent lipolysis pathways, the lack of measurement of these proteins is one of the limitations of the present study. Therefore, it is suggested that NRF1/ 2 and PGC1-a be measured in adipose tissue in future studies.

Conclusion

Even though training and octopamine supplementation alone seem to play a role in increasing the protein concentration of octopamine and its receptors, these two interventions do not have a synergistic effect on lipolysis by focusing on the octopamine receptor pathway.

Acknowledgments

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Conflicts of Interest

The authors have not declared any conflict of interest.

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JOURNAL OF NUTRITION FASTING AND HEALTH

Dietary Supplementation of Clove (*Syzygium Aromaticum*) Essential Oil Improves Growth Performance, Oxidative indices, and Lipid Profile of Japanese Quails Exposed to Lead

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ABSTRACT

Introduction: The current survey investigated the alleviating effect of dietary clove (*Syzygium aromaticum*) essential oil (CEO) in comparison with vitamin C (VC), against the adverse effects of Pb on growth performance, serum oxidative indices, and lipid profile in Japanese quails (*Coturnix japonica*), following oral administration.

Methods: 480 one-day-old quails were randomly segregated into 8 groups, which fed with the following diets, via 35 days: basal diet (negative control), basal diet + VC (500 mg/kg), basal diet + CEO (450 mg/kg), basal diet + CEO (100 mg/kg), basal diet + VC (500 mg/kg) + Pb (100 mg/l), basal diet + CEO (450 mg/kg) + Pb (100 mg/l), basal diet + CEO (100 mg/kg) + Pb (100 mg/l), and basal diet + Pb (100 mg/l) (positive control). The data were analyzed using a one-way analysis of variance and Duncan's post hoc test.

Results: Quails exposed to Pb and treated with CEO had reduced oxidative stress as evidenced by lower concentrations of TBARS and CP, higher activities of SOD, GPx, and CAT, and more improved lipid profile, compared to positive control. Moreover, the alleviating effects of CEO were dose-dependent.

Conclusion: The CEO (450 mg/kg) was potentially as effective as or even more potent than VC (500 mg/kg) in alleviating the adverse effects of Pb.

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Introduction

Heavy metal pollution, such as lead (Pb) contamination, has become an actual significant health problem for humans and animals. Pb is one of the most well-recognized prevalent environmental toxicants, with cumulative and non-biodegradable nature (1-3), which raised the risk of their possible transmission from animals' meat to humans' bodies through the food chain (1). FAO and WHO have recognized no safe threshold of Pb in humans (4). Pb affects a broad range of body systems and leads to chronic and acute complications, including carcinogenicity, immunotoxicity, neurotoxicity, growth retardation, hearing loss, short-term memory disorder, intelligence decrease, and even death (2, 3). The main mechanisms of its toxic action have been announced to provoke oxidative stress and boost the production of reactive oxygen species (ROS), thereby leading to cell structure impairment through DNA and protein oxidation and also lipid peroxidation (1, 2, 5). The antioxidant defense mechanism of the body could also be disrupted by Pb (2).

According to the aforementioned, applying approaches to lowering these toxic metals seems to be a health priority. With respect, the application of natural substances like plant essential oils (PEOs), due to having both antioxidant and chelating properties, has been discussed to be a good candidate for protection against Pb toxicity (1, 6). In this respect, clove (*Syzygium aromaticum*), a spice plant and an FDA-approved food additive, was highlighted (7). Clove essential oil (CEO) is a primary source of polyphenols, especially eugenol which represents 89% of this oil, so it is a potent radical

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scavenger (8). There is a strong relationship between the polyphenol percentage and the antioxidant properties of essential oils (9, 10).
Ultimately, the Japanese quail (*Coturnix japonica*) was nominated to be used in the current study due to being a well-known laboratory animal model (3) and also being a valuable source of meat and egg (11, 12). Therefore, the current study was designed to evaluate the alleviating effect of CEO in comparison with VC against the toxic effects of Pb on the growth performance, serum oxidative indices, and lipid profile of Japanese quails.

Materials and Methods

Sample Collection and Preparation

The current survey was performed in the Poultry House of the Department of Animal Nutrition, Shahrekord University, Chaharmahal and Bakhtiari, Iran (Ethical code: IR.SKU.REC.1392.122.531).With a magnitude of 3.5 m in width × 4 m in length × 17 m in height. All experimental procedures were carried out according to the animal welfare guidelines of the Veterinary Control and Research Institute of Shahrekord University, Iran. 480 one-day-old chicks were purchased from a local farm in Chaharmahal and Bakhtiari province, Iran. And fed with a basic diet for up to seven days. At 7 days after weighing, the chicks with equal initial body weight were used in a randomized design trial with eight groups, each consisting of 15 pieces, with three replications. Briefly, quails were housed in an environmentally controlled pen under a 24 lighting cycle and following a standard temperature, which gradually decreased from 36 to 25 °C, at the rate of 2 °C per week. They were fed basal diets (Table 1) and daily refreshed water for 35 days. Quails were vaccinated against Newcastle by B1 serotype on day 7th. The experimental treatments were as listed: 1. Basal diet (negative control); 2. Basal diet + VC (500 mg/kg); 3. Basal diet + CEO (450 mg/kg); 4. Basal diet + CEO (100 mg/kg); 5. Basal diet + VC (500 mg/kg) + Pb (100 mg/l); 6. Basal diet + CEO (450 mg/kg) + Pb (100 mg/l); 7. Basal diet + CEO (100 mg/kg) + Pb (100 mg/l); 8. Basal diet + Pb (100 mg/l) as lead acetate in water (positive control).
On the 35th day, 30 blood samples from each group were randomly collected and stored in sterilized test tubes after that their serum was isolated. To separate the serums, the samples were allowed to clot for 30 min at room temperature and then centrifuged at 5000 rpm for 10 min. All serum samples were stored at -70°C before analysis.

Growth Performance Measurement

-Bodyweight (BW)
At the end of each week, after being starved for 2 hours, the chicks in each group were weighed individually, and the average weight was documented.
-Feed intake (FI)
The amount of grain given to each treatment group was recorded daily for five weeks, and at the end of each week, the leftover grain was collected and weighed and the amount of feed consumed was deliberated.
-Feed conversion ratio (FCR) was calculated based on the following equation:
FCR = total feed consumed weekly / (initial weight) - (weight of losses + final weekly weight)
-Identification of volatile oil compounds (GC / MS)
The pure CEO was obtained from Barij Essence Company (Kashan, Iran). The gas chromatography-mass spectrometry (GC-MS) analyses were carried out using an Agilent Technologies GC (Model HP-7890, Palo Alto, CA, USA), with a capillary column (Model HP-5MS, length: 30 m, film thickness: 0.25 µm, internal diameter: 0.25 mm), coupled with a mass spectrometer (Model HP 5975, Agilent Technologies, Palo Alto, CA, USA), which have an electron impact ionization potential (70 eV). The oven temperature was initially held at 60°C for 5 min and gradually was elevated at the rate of 4°C per min until it reached 240°C. Eventually, it was raised at the rate of 15°C per min until reached 290°C, and then maintained at this degree for 3 min. Helium was used as the inert gas, which flowed past at a speed of 0.8 ml per min, and its purity was 99.999%. Samples of 1.0 µl were injected using a Hamilton syringe. The injection temperature was 300 ° C. The separation ratio was set to 100: 1. The mass range was 50-50 m / z. Quantitative data were obtained by using the area of the peaks. The EO ingredients were quantified by assimilating their retention indexes (Table 2) relative to a set of n-alkanes (C5 to C25), available in literature or our laboratory, and confirmed by matching their mass spectra analysis patterns. The essential oil dosage was determined based on previous studies (13, 14) .
-Measurement of biochemical parameters

The thiobarbituric acid reactive substances (TBARS) value was calculated based on the following formula and represented as mg malondialdehyde (MDA)/kg of serum samples (15):

TBARS = (A × 288)/156

The absorbance (A) of the acquired upper layer was read at 532 nm versus a blank (1 ml of DDW + 2 ml of TBA/TCA).

The serum carbonyl protein (CP) was measured by the Levine et al. (1990) (16) method.

The activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were calculated according to the methods described by Sun, Oberley, and Li (1988) (17), Goth (1991) (18), and Paglia and Valentine (1967) (19), respectively.

Triglyceride (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) concentrations were evaluated by commercial kits (Pars Azmoon, Iran). Serum low-density lipoprotein cholesterol (LDL-C) concentration was acquired based on the Friedewald formula [LDL-C = (TC) – (HDL-C) – TG/2.2] (20).

-Statistical analyses

All data were evaluated by one-way ANOVA, accompanied by Duncan's test in SPSS software (version 22). The values of all the Pb intoxicated groups were compared to the control group and the significance level was considered as $P < 0.05$. The values were described as mean ± SEM.

Table 1. Composition of basal diets during the experiment

Ingredient (g/kg)	0-35 days
Maize	509.6
Soybean meal	438.4
Soybean oil	20.6
Calcium carbonate	12.6
Dicalcium phosphate	8.3
DL- Methionine	1.6
Mineral premix[a]	2.5
Vitamin premix[b]	2.5
Salt	1.6
vitamin D_3[c]	1
vitamin E[d]	1.5
Calculated analysis	
Metabolizable energy (KCal/kg)	2850
Crude protein%	25.3
Calcium%	0.8
Phosphorus%	0.3
Sodium%	0.15
Lysine%	1.34
Methionine%	0.5
Arginine%	1.57
Methionine + cysteine%	0.84
Threonine%	1.02
Valin%	1.12
Leucine%	1.8
Isoleucine%	1.22
Histidine%	0.22
Phenylalanine%	1.13
Mn (mg)	21.17
Fe (mg)	146.6
Cu (mg)	17.44
Zn (mg)	41.35
Se (mg)	0.11

Composition of basal diets' ingredients were calculated based on NRC table.

[a] Provided per kilogram of diet: 1200 mg Mn (as manganese oxide), 1000 mg Zn (as zinc oxide), 1800 mg Fe (as ferrous sulphate), 400 mg Cu (as copper sulphate),8 mg Se (as sodium selenite), 38 mg Iodine (as calcium iodate), 180 g Ca (as calcium carbonate).

[b] Provided per kilogram of diet: 200000 IU vitamin A, 80000 IU vitamin D_3, 1600 IU vitamin E, 700 mg vitamin B_{12}, 35 mg vitamin K_3, 1200mg vitamin C, 30 mg vitamin B_1, 130 mg vitamin B_2, 1300 mg nicotinic acid, 225 mg panthotenic acid, 8200 mg choline chloride, 3.3 mg biotin.

[c] Provided per kilogram of vitamin D_3: 100000 IU

[d] Provided per kilogram of vitamin E: 100000 IU

Table 2. Constituents of *CEO* and their Relative Percentages of Retention time, Kovats Index and Total Chromatogram Area.

Ingredients	Retention time (min)	Kovats Index	Area (%)
Eugenol	18.175	1365.335	77.63
Iso-eugenol	18.86	1387.06	0.65
β-Caryophyllene	19.937	1421.934	9.54
α-Humulene	20.984	1456.262	1.33
delta-Cadinene	23.07	1524.656	0.2
Eugenol acetate	24.852	1587.019	7.07
Caryophyllene oxide	24.852	1587.019	0.28

Results

GC/MS Identification of CEO

The major abundant components of the CEO were shown in Table 2.

Growth Performance (BW, FI, FCR)

Based on Table 3, at 28 and 35 days of age, BW was significantly (P < 0.05) improved in CEO (450 mg / kg), CEO (100 mg / kg), and VC (500 mg / kg) groups, while it was significantly (P < 0.05) reduced in Pb (100 mg / l) group, compared to the control group. Nevertheless, it remained unchanged in all Pb-exposed groups supplemented with dietary inclusions (CEOs and VC), compared to the control group. Expect, in the CEO (100 mg/kg) group, on day 35th, which was significantly (P < 0.05) decreased, compared to the control group. Additionally, there was no significant (P < 0.05) difference in FI and FCR during the whole experiment. Overall, the CEO (450 mg/kg) was as effective as VC in alleviating the adverse effect of Pb on growth performance parameters.

Table 3. The effect of the dietary inclusion of clove essential oil and vitamin C and lead acetate on body weight (BW; gr) and feed intake (FI; gr) and feed conversion ratio (FCR) of quails (0-35 days)

Groups	Control	Vit C 500 mg/kg	Clove oil 450 mg/kg	Clove oil 100 mg/kg	Vit C 500 mg/kg + Pb 100 mg/l	Clove oil 450 mg/kg + Pb 100 mg/l	Clove oil 100 mg/kg + Pb 100 mg/l	Pb 100 mg/l
	mean ±sem	sem ± mean	sem ± mean	sem ± mean	sem ± mean	sem ± mean	sem ± mean	sem ± mean
BW (gr)								
7 days	32.85±0.51	34.15±0.82	33.33±0.38	33.83±1.23	33.88±0.34	32.82±0.62	34.08±0.58	32.80±0.86
14 days	68.13±0.93	71.70±3.52	67.33±4.26	69.74±3.31	69.71±0.97	62.80±1.33	64.20±1.75	62.88±1.17
21 days	112.03±2.90	128.55±1.68	125.18±4.15	125.39±3.46	119.83±8.15	123.57±4.07	119.39±2.17	110.40±1.37
28 days	162.52±1.61^{a}	174.30±5.25^{b}	172.80±4.69^{b}	170.47±1.87^{b}	165.00±2.50^{a}	164.83±3.70^{a}	164.20±0.90^{a}	150.57±1.53^{c}
35 days	192.92±3.09^{b}	204.01±6.42^{a}	202.91±2.86^{a}	202.41±4.00^{a}	192.78±4.72^{b}	196.69±2.58^{b}	189.99±0.58^{c}	179.05±1.12^{d}
FI (gr)								
7-14	84.96±5.49	85.11±0.71	72.59±8.88	84.55±4.98	77.97±1.07	71.47±4.24	71.67±3.73	82.55±1.82
14-21	114.68±5.56	136.06±3.68	135.39±4.53	134.83±0.23	137.50±11.60	149.90±0.90	142.72±4.72	140.40±1.37
21-28	178.12±1.70	158.67±11.58	163.81±13.93	156.07±7.77	162.69±11.99	156.55±4.70	163.60±3.33	164.31±4.92
28-35	153.85±2.77	161.80±5.17	148.43±5.79	157.17±3.16	162.56±10.40	157.36±2.79	145.48±2.49	167.33±0.82
7-35	531.60±2.89	541.64±11.48	520.22±17.15	532.62±5.17	540.71±22.26	535.28±4.88	523.47±10.59	554.59±5.78
FCR								
7-14	2.41±0.16	2.33±0.28	2.16±0.24	2.36±0.07	2.18±0.04	2.38±0.09	2.41±0.26	2.74±0.05
14-21	2.62±0.06	2.40±0.12	2.37±0.23	2.43±0.05	2.83±0.25	2.48±0.12	2.60±0.08	2.97±0.13
21-28	3.54±0.14	3.52±0.19	3.47±0.20	3.46±0.04	3.73±0.43	4.01±0.62	3.68±0.27	4.09±1.14
28-35	5.12±0.36	5.46±0.19	5.20±0.84	4.99±0.47	5.87±0.13	5.12±0.71	5.65±0.19	5.88±0.08
7-35	3.32±0.06^{b}	3.20±0.8^{c}	3.07±0.10^{e}	3.17±0.10^{d}	3.40±0.04^{b}	3.27±0.03^{c}	3.36±0.7^{b}	3.80±0.09^{a}

Pooled s.e.m. - pooled standard error of the mean.

^{a,b,c,d,e} Means within rows with different superscripts differ significantly P < 0.05.

BW: Body Weight, FI: Feed Intake, FCR: Feed Conversion Ratio.

Biochemical Analyses of Serum on Day 35th

According to Table 4, a significant (P < 0.05) enhancement in TBARS and CP levels has been observed in Pb (100 mg / l) and CEO (100 mg / kg) + Pb groups. Moreover, TBARS value was significantly (P < 0.05) increased in CEO (450 mg / kg) + Pb group, while remained unchanged in VC + Pb group, compared to control. While, CP values remained unchanged in CEO (450 mg / kg) + Pb and VC + Pb groups, compared to control. Consequently, it has been determined that CEO (450 mg/kg) was as effective as VC in restoring these values to that of control in the presence of Pb.

Moreover, a significant (P < 0.05) reduction in CAT, SOD, and GPx activities has been detected in Pb (100 mg / kg) group, compared to control. Furthermore, the activities of SOD and GPx, despite CAT, which remained unchanged, were significantly (P < 0.05) decreased in the CEO (100 mg/kg) + Pb group, compared to the control. Eventually, CEO (450 mg/kg) was as effective as

VC in alleviating the adverse effect of Pb on the activities of antioxidant enzymes.

In addition, the contents of the serum lipid profile (TG, TC, HDL-C, and LDL-C) remained unchanged in all groups compared to the control group. Except in CEO (450 mg/kg) and CEO (450 mg/kg) + Pb groups, which showed a reduction for TG, TC, and LDL-C and an increment for HDL-C. So, the CEO (450 mg/kg) was a more positively improved serum lipid profile than VC.

Table4. Serum analysis on day 35th; (TBARS; µM/mg protein), (CP; nmol/mg protein), (TG, TC, HDL-C and LDL-C; mmol/L), (CAT; U/mg protein), (SOD; % inhibition/mg protein) and (GPx; U/mg protein)

Groups	control	Vit C 500 mg/kg	Clove oil 450 mg/kg	Clove oil 100 mg/kg	Vit C 500 mg/kg + Pb 100 mg/kg	Clove oil 450 mg/kg + Pb 100 mg/kg	Clove oil 100 mg/kg + Pb 100 mg/kg	Pb 100 mg/kg
	mean ±sem	sem ± mean	sem ± mean	sem ± mean	sem ± mean	sem ± mean	sem ± mean	sem ± mean
Antioxidant Status								
TBARS	1.99±0.23^{a}	1.93±0.30^{a}	1.87±0.29^{a}	2.07±0.28^{a}	2.23±0.39^{ac}	3.07±0.22^{c}	4.18±0.45^{bc}	4.99±0.32^{b}
CP	0.57±0.08^{a}	0.51±0.07^{a}	0.49±0.09^{a}	0.53±0.05^{a}	0.65±0.19^{a}	0.63±0.12^{a}	1.48±0.22^{b}	1.56±0.18^{b}
CAT	25.41±2.13^{a} b	27.83±1.98 a	26.03±1.02 a	24.12±1.13^{a} b	22.83±1.95 b	25.11±1.20^{a} b	22.36±0.85 b	16.93±0.99 c
SOD	19.35±0.95^{a}	21.80±2.05 a	20.72±1.53 a	18.35±1.01^{a}	18.97±1.76 a	18.09±1.52^{a}	13.90±0.71 b	12.35±0.58 b
GPx	29.94±2.57^{a}	28.40±2.13 a	28.15±2.09 a	27.81±2.29^{a}	23.18±2.17 a	23.95±2.98^{a}	15.81±0.63 b	13.39±0.70 b
Lipid Profile								
TG	2.67±0.11^{a}	2.54±0.16^{a}	2.12±0.09^{b}	2.59±0.13^{a}	2.57±0.09^{a}	2.15±0.08^{b}	2.62±0.17^{a}	2.70±0.20^{a}
TC	4.37±0.35^{a}	4.32±0.19^{a}	3.21±0.12^{b}	4.19±0.28^{a}	4.40±0.17^{a}	3.15±0.08^{b}	4.33±0.19^{a}	4.50±0.31^{a}
HDL-C	1.72±0.20^{a}	1.75±0.09^{a}	2.25±0.18^{b}	1.85±0.19^{a}	1.63±0.22^{a}	2.21±0.15^{b}	1.75±0.22^{a}	1.69±0.14^{a}
LDL-C	1.43±0.11^{a}	1.41±0.13^{a}	0.51±0.03^{b}	1.15±0.11^{a}	1.59±0.11^{a}	0.59±0.07^{b}	1.38±0.12^{a}	1.57±0.18^{a}

Pooled s.e.m. - pooled standard error of the mean.

^{a,b,c} Means within rows with different superscripts differ significantly $P < 0.05$.

TBARS: Thiobarbituric Acid Reactive Substances, CP: Carbonyl Protein, CAT: Catalase, SOD: Superoxide Dismutase, GPx: Glutathione Peroxidase, TG: Triglyceride, TC: Total Cholesterol, HDL-C: High-Density Lipoprotein Cholesterol, LDL-C: Low-Density Lipoprotein Cholesterol.

Discussion

Pb is a ubiquitous and long-standing environmental toxicant (3) that can induce adverse effects like growth retardation stress while CEO has alleviating properties like growth promotive and oxidation mitigative effects (9). So, the CEO was assumed as a compensatory agent that can protect quails from Pb toxic effects. To confirm this assumption, this study was carried out to evaluate the alleviating effect of CEO vs. the adverse effects of Pb on mentioned parameters. Furthermore, vitamin C (VC) is an accepted and well-known antioxidant, that has been used widely in the poultry nutrition industry to increase its antioxidant capacity (21, 22) and improve its growth performance (23). It is a terminal reductant ROS scavenger that can inhibit lipid peroxidation (24) and exhibit a hypolipidemic effect (25). Thus, it is eligible to study the ability of the CEO in comparison with VC, as a scale, in alleviating the oxidative stress induced by Pb.

CEO Ingredients

The main components of CEO in the current study were found to be eugenol (77.63%), β-caryophyllene (9.54%), and eugenol acetate (7.07%), which were in good accordance with recent findings of Yu et al. (2017) (26). While another study reported eugenol and eugenol acetate as the major constituents (27). These differences could be associated with such parameters as weather conditions, soil composition, genetics, age, stage of maturity, type of plant sections, and distillation protocols (28).

Effects of Pb and CEO on Growth Performance Parameters

In the present study, despite BW, the values of FI and FCR have remained unchanged, as affected by Pb, which is in line with other results (27, 29). Dislike other reports that showed an inhibitory effect of Pb on FI and FCR (1, 30). In the current research, BW was only decreased at the end of the trial, which showed the cumulative effect of

Pb. The growth-retarding effect of Pb in poultries is evidenced by poor performance, weight loss, loss of visceral and subcutaneous fat, atrophy of breast muscle, and even starvation of birds, which is mainly and primarily associated with inducted anorexia by this metal (1). Although the exact mechanism by which Pb induces anorexia is not well recognized (31), it is hypothesized that the possible main mechanisms of Pb for inducing anorexia are probably as follows: (i) muscular paralysis of the digestive system and cellular deteriorations which induced by oxidative stress; (ii) suppression of nutrients absorption by downregulation of main nutrient transporter genes in the small intestine; (iii) induction of metabolic disorders by inhibition of glycol-metabolism and haem synthesis key enzymes (30, 32). In line with our results, other scientists have demonstrated that anorexia is a primary characteristic of Pb toxicity in the Japanese quail, which results in poor performance (9, 33).

Nevertheless, in the present study, the depressive effect of Pb on BW has recovered by the improving impact of the CEO (450 mg/kg) supplement. And the alleviating effect of CEO (450 mg/kg) on BW was the same as VC. It has been demonstrated that CEOs can serve as growth promotors in the poultry industry due to: (i) their antimicrobial properties, which repress pathogen colonization in the gastrointestinal tract of quails and therefore lessen their fatality during the growth period; (ii) their ability to improve the palatability of foodstuffs, which stimulate appetite and FI; (iii) their ability to enhance nutrients digestibility by increasing digestive enzymes and bile salt secretion. Furthermore, CEO is a valuable source of manganese, trace minerals, and a minor source of omega 3 fatty acids and vitamins K and C, which are crucial for improving growth performance (8, 9). Under our findings, many investigations have also described affirmative effects of CEO on BW (9, 10), while other researchers reported no effect (8, 25).

Effects of Pb and CEO on Biochemical Parameters of Serum

According to our findings, the CEO had reduced oxidative stress induced by Pb, as evidenced by lower concentrations of TBARS and CP, higher activities of SOD, GPx, and CAT, and a more improved lipid profile of serum, compared to the positive control group. Moreover, the antioxidant properties of CEO were dose-dependent.

Pb induces oxidative stress by disturbing the oxidative and antioxidative balance of the cells and ultimately leads to cell apoptosis (3, 5, 24), via (i) promoting the surplus production of ROS, which are highly reactive and attack substantial biomolecules in cells including DNA, proteins, and lipids (9). MDA (TBARS) is a terminal product and the indicator of lipid peroxidation. The final product and the protein's oxidation indicator is carbonyl protein (CP) (8). (ii) depleting the antioxidant enzymes (SOD, GPx, and CAT) capacity, which might be attributed to the high affinity of Pb to sulfur and selenium in these enzymes and producing an immobile form of the enzyme or might be due to the capability of Pb to suppress the uptake of selenium and ferric, as enzymes' co-factors, from the gastrointestinal tract (3). The findings of the present study were following previous studies, which concluded that Pb suppressed the activities of antioxidant enzymes, and elevated the TBARS levels in Japanese quail (3, 9). Whereas, a recent study announced that the activities of serum CAT and GPx were increased in poultries treated with Pb (34). In addition, another researcher detected no significant changes in CAT, SOD, and GPx activities, as a result of Pb-intoxication in Japanese quail (5).

In light of our results, Pb did not affect the lipid profile of quails. The contrary to the reports which elucidated that Pb increases the risk of dyslipidemia, as implicated by high levels of TC, TG, and LDL-C and low levels of HDL-C, which results in atherosclerosis and other cardiovascular diseases (1, 2, 9). In contrast to our result, an article claimed no significant alterations in TC, along with a reduction in the level of TG as Japanese quails exposed to Pb (35). On the other hand, our results have depicted that CEO (450 mg/kg) has positively improved the lipid profile of serum by increasing the level of HDL-C and decreasing the levels of TC, TG, and LDL-C. It has been confirmed that CEO is a potent free radical scavenger and metal chelator due to its hydrogen donating property from its hydroxyl and carbonyl groups in its aromatic ring (28). It has pharmacological activities such as antioxidant, anticancer, anti-inflammatory, anti-atherogenic, hypolipidemic, and hepatoprotective activities, which are attributed to its high amount of phenolic compounds (i.e.,

eugenol and caryophyllene) (28). It acts as a hypocholesteric agent by inhibiting a key regulatory enzyme in cholesterol synthesis (25) and also acts as a hepatoprotective agent by manipulating cell membrane permeability and preventing the entrance of hepatotoxic substances to hepatocytes (9). Accordingly, the anti-obesity effects of CEO by reducing the serum TG and TC levels were well established (28). Compared to the lipid-lowering drug lovastatin, eugenol lowered the concentration of TC, TG, and LDL by 55.88%, 79.48%, and 64.30%, respectively, thereby exerting anti-hyperlipidemic effects (9). Dislike our findings, other authors have declared that the serum lipid profile of broilers was not changed by any dietary inclusions of CEO (36) or other EOs supplements (8, 37).

Limitation

This study had some limitations, including the environmental concerns regarding the disposal water which contained lead. Furthermore, due to the low volume of blood in quail, the birds had to be slaughtered in order to obtain a sufficient volume of blood for serum tests. So, in order to comply with the ethics of sampling, the blood sampling was limited to the last day of the breeding period, which was the slaughter time and fewer birds were killed.

Conclusion

Overall, it seems that, due to the unavoidable exposure of poultries to heavy metals through various sources (including diet, water, soil, air, etc.), the use of natural antioxidants, especially PEOs, in poultry nutrition, was found to help reduce their risks. With respect, we have observed that CEO (450 mg/kg) was well managed to compensate for the adverse effects of Pb on growth performance, serum antioxidant status, and lipid profile, as compared to VC (500 mg/kg). It was as effective as or even more potent than VC (500 mg/kg) in alleviating the adverse effects of Pb.

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Conflict of Interests

The authors declare that there is no conflict of interest.

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