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ORIGINAL ARTICLE

The evaluation of *Zataria multiflora* Boiss. essential oil effect on biogenic amines formation and microbiological profile in Gouda cheese

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Significance and Impact of the Study: The presence of biogenic amines in cheese has a serious impact on public health. Besides, there is growing concern about the use of chemical preservatives and the food industry is looking for new natural preservation methods. *Zataria multiflora* Boiss. essential oil is well known for its antimicrobial effects, and we attempted to reduce biogenic amines formation in Gouda cheese using *Z. multiflora* Boiss. essential oil as a natural additive. Furthermore, the desirable organoleptic qualities such as flavour, odour, texture and colour were achieved by adding *Z. multiflora* Boiss. to cheese.

Keywords
biogenic amines, essential oils, Gouda cheese, histamine, microbiological profile, tyramine, *Zataria multiflora* Boiss.

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Abstract

The effect of *Zataria multiflora* Boiss. (*Z. multiflora*) essential oils (EO) on biogenic amines (BAs) production and microbial counts in Gouda cheese has been investigated. *Zataria multiflora* was added to milk in different concentrations (0·05, 0·1, 0·2 and 0·4% (v/v)). The BAs (tyramine and histamine) were measured by RP-HPLC, following extraction from the cheese. Various microbiological analyses (aerobic mesophilic bacteria, enterococci, mesophilic lactobacilli, Enterobacteriaceae, lactococci and yeasts) were performed during ripening using the viable plate count method on specific culture media. The overall acceptability of cheeses was investigated by seven panellists. All the samples containing different concentrations of EO were acceptable to the panellists. Also, Gouda cheeses with 0·2% *Z. multiflora* EO showed the highest acceptability among all the samples. At the end of maturation period, 0·1, 0·2 and 0·4% *Z. multiflora* EO reduced tyramine and histamine significantly to 5%, 22% and 44% for tyramine and 14%, 29% and 46% for histamine, respectively, when compared to the control group. The increase of *Z. multiflora* EO concentrations led to further decrease in BAs content and microbial counts. The maximum microbiological reduction was observed in yeasts, and minimum microbiological reduction was seen in Enterobacteriaceae counts. *Zataria multiflora* EO could be used for reduction of BAs and also as a flavouring agent in Gouda cheese and could contribute to consumers' health.

Introduction

BAs are low molecular weight organic bases that can be formed and degraded during the normal metabolism in animals, plants and micro-organisms. BAs are mainly produced through the decarboxylation of certain free amino acids and play an important role in human physiological functions such as immune response, brain activity and gastric acid secretion (Shalaby 1996). The presence of BAs in food is undesirable and could cause health damage, especially when they are in abundance (Martuscelli *et al.* 2005). Excessive oral intake of BAs may

lead to vasoactive and psychoactive implications like headaches, nausea, rashes and alterations of the blood pressure (Ladero *et al.* 2010). In the presence of nitrites, BAs can be converted into nitrosamines which are potential carcinogens (Kim *et al.* 2009). In addition, BAs toxicity increases by consuming food containing BAs, drinking alcoholic beverages, or in genetically inadequate detoxification and the inhibitory effects of pharmacological treatment (mono amine oxidase inhibitor (MAOI)) (Bodmer *et al.* 1999). The interactions among BAs should also be considered. The presence of putrescine and cadaverine, for example, increases the toxicity of histamine at the same concentration (Stratton *et al.* 1991; Hernández-Jover *et al.* 1997; Emborg and Dalgaard 2006). Hence, recognition of toxic levels of BAs appears to be extremely difficult, and there are no legal limits concerning BAs concentrations in most foodstuffs. The only toxicity level available for histamine (one of the most physiologically effective BAs) is in fresh fish at the value of 200 mg Kg^{-1} (Commission regulation 2005). Nevertheless, BAs synthesis and accumulation in foods should be controlled due to their toxicological and hazardous effects.

Cheese is the second most frequently implicated foodstuff associated with histamine poisoning after fish (Stratton *et al.* 1991), and the term 'cheese reaction' refers to tyramine intoxication from cheese (Ten Brink *et al.* 1990). Some kind of cheeses, like hard and semi-hard cheeses, represent an ideal kind of food for accumulation of BAs due to a longer ripening period. In general, the increase in ripening time leads to an increase in BAs (Schneller *et al.* 1997; Valsamaki *et al.* 2000; Komprda *et al.* 2008; Ladero *et al.* 2008).The concentrations of these components can even increase up to 2000 mg Kg^{-1} (Roig-Sagués *et al.* 2002; Fernández *et al.* 2007). There are several factors such as milk pasteurization, bacterial density, level of proteolysis, general hygienic conditions, ripening and storage temperatures, synergistic effects between micro-organisms, time of ripening, pH, salt-in-moisture level that influence the BA contents in cheese (Edwards and Sandine 1981; Joosten 1988; Stratton *et al.* 1991; Schneller *et al.* 1997; Novella-Rodríguez *et al.* 2003; Komprda *et al.* 2007). It appears that the presence of micro-organisms with decarboxylase activity is the main factor affecting BA production in cheese, and this characteristic can be found in starter culture, nonstarter lactic acid bacteria and adventitious micro-organisms (Fernández-García *et al.* 2000; Roig-Sagués *et al.* 2002). However, it seems micro-organisms that produce BA are spontaneous rather than part of the starter culture population (Voigt and Eitenmiller 1977; Joosten 1988). Also, some gram negative bacteria (especially Enterobacteriaceae) found in milk have the ability to form histamine, putrescine, and cadaverine (Ten Brink *et al.* 1990; Marino *et al.* 2000).

Various methods have been suggested to reduce BAs in foodstuffs such as food additives, high hydrostatic pressure, irradiation, packaging and starter cultures. Food additives are divided into synthetic and natural. The latter has proved to be more popular with consumers thanks to their being natural and FDA safety approval. Essential oils that fall into the category of natural food additives possess antibacterial and good health-improvement qualities. *Zataria multiflora* is a plant of *Laminaceae* family that grows only in Iran, Afghanistan and Pakistan (Hosseinzadeh *et al.* 2000; Shaiq Ali *et al.* 2000). Therefore, it was decided that *Z. multiflora* EO would be used for the first time to reduce BAs in Gouda cheese. Gouda is a yellow Dutch cheese made from cow's milk. It is one of the most popular cheeses in the world, and many biochemical and microbiological changes occur during the ripening period which lead to the production of BAs.

Results and discussion

Composition of *Zataria multiflora* Boiss. essential oil

The essential oil of air-dried aerial parts of the representative sample of *Z. multiflora* yielded 1·66% (v/w). The percentages of various components in essential oil are shown in Table 1. Gas chromatography–mass spectrometry (GC–MS) analysis reveals 12 identified components comprising 91·9% of the oil. Carvacrol was the main component (71·1%), and other important compounds were gamma-terpinene (7·34%), alpha-pinene (4·26%) and eucalyptol (3·37%).

Table 1 Composition of *Zataria multiflora* Boiss. (*Z. multiflora*) essential oil (EO) determined by gas chromatography–mass spectrometry (GC–MS)

Compound	Retention index*	Percentage
Alpha-pinene	937	4·26
Thujene	930	0·19
Beta-myrcene	985	0·85
Beta-pinene	976	0·43
Gamma-terpinene	1055	7·34
Eucalyptol	1024	3·37
Carvacrol methyl ether	1243	0·46
Carvacrol	1299	71·12
Thymol methyl ether	1236	0·47
Linalool	1090	0·68
Globulol	1582	2·32
Trans-caryophyllene	1418	0·41
Total		91·90

*Retention index on DB5 column.

Sensory evaluation

The mean acceptability scores of cheese samples containing *Z. multiflora* EO and the control group have been shown in Table 2. The samples treated with *Z. multiflora* EO at 2% were the most preferred samples. All the samples scored above the acceptability point.

Biogenic amines

BAs (tyramine and histamine) contents in Gouda cheese in control and *Z. multiflora* EO-treated groups are demonstrated in Table 3. In all the samples, tyramine increased substantially during the first 30 days of storage. As shown in Table 3, tyramine continues to increase less intensely in the control group than in samples with 0·05 and 0·1% of EO. In the control group, tyramine amounts increased from 8·93 mg Kg^{-1} on day 0 up to 172·40 mg Kg^{-1} on day 90 of ripening. Histamine production like tyramine followed a similar pattern with a slightly lower slope in the early phase of ripening, and at the end of ageing, the mean concentration of 6·31 mg Kg^{-1} reached 41·57 mg Kg^{-1}. According to Schirone *et al.*'s (2011) results, mean concentration of tyramine was 418 mg Kg^{-1} and higher than our findings, while mean concentration of histamine was 5·18 mg kg^{-1} and lower than our results after 90 days of storage. Totally, there is extreme variation in BAs contents, and different factors such as quality of milk, type of cheese, manufacturing process, duration and temperature of ripening, type of starter culture, micro-organisms present and chemical characterization make it difficult to compare BA contents in different studies (Ordoñez *et al.* 1998; Gardini *et al.* 2001).

As shown in Table 3, increasing the concentration of *Z. multiflora* results in lower concentrations of tyramine and histamine. At the end of ageing, amounts of tyramine reduction at 0·1, 0·2 and 0·4% of *Z. multiflora* EO concentrations were 5, 22 and 44%, respectively, compared to the control group. In addition, histamine reductions in the above-mentioned concentrations were 14, 29 and 46%, respectively. In 0·05% *Z. multiflora* samples, a significant decrease was not seen in tyramine and histamine content in comparison with the control group.

Histamine formation draws the general attention of food safety researches because it is the most prevalent food borne intoxication associated with BAs. However, histamine content in the control group is lower than the proposed toxicological limit (100 mg kg^{-1})(Shalaby 1996), but these amounts would be considered riskier in groups like MAOI consuming patients, allergic people, and the allergic people and individuals that consume

Table 2 Mean scores for the acceptability of Gouda cheese samples with different concentrations of *Zataria multiflora* Boiss. (*Z. multiflora*) essential oil (EO)

Z. multiflora EO (% (v/v))	Mean scores ± SD
0	$7·12 \pm 0·14^{a}$*
0·05	$6·75 \pm 0·35^{a}$
0·1	$7·64 \pm 0·25^{b}$
0·2	$8·11 \pm 0·19^{c}$
0·4	$7·20 \pm 0·41^{ab}$

*Means in columns without common letters are significantly different ($P < 0·001$).

Table 3 Effect of *Zataria multiflora* Boiss. (*Z. multiflora*) essential oil (EO) on tyramine and histamine contents (Mean ± SD) in Gouda cheese during ripening

Components (mg kg^{-1})	EO concentrations (%(v/v))	Days of analysis					
		0	15	30	45	60	90
Tyramine	0	$8·93 \pm 0·40^{a}$*	$84·90 \pm 1·87^{a}$	$144·03 \pm 2·57^{a}$	$156·37 \pm 1·82^{a}$	$161·83 \pm 1·70^{a}$	$172·40 \pm 2·63^{a}$
	0·05	$9·15 \pm 0·62^{a}$	$83·4 \pm 1·08^{a}$	$139·4 \pm 3·36^{ab}$	$161·30 \pm 2·79^{a}$	$160·97 \pm 3·59^{a}$	$171·60 \pm 2·33^{a}$
	0·1	$8·33 \pm 0·50^{a}$	$76·43 \pm 1·33^{ab}$	$137·97 \pm 2·07^{b}$	$159·23 \pm 2·71^{ab}$	$151·63 \pm 2·17^{b}$	$163·70 \pm 1·30^{b}$
	0·2	$8·03 \pm 0·87^{a}$	$68·33 \pm 1·90^{c}$	$121·17 \pm 1·82^{c}$	$128·20 \pm 2·62^{c}$	$132·67 \pm 0·83^{c}$	$134·57 \pm 1·51^{c}$
	0·4	$7·71 \pm 0·72^{a}$	$53·97 \pm 1·32^{d}$	$118·67 \pm 1·97^{c}$	$117·93 \pm 1·31^{d}$	$122·30 \pm 1·80^{d}$	$113·10 \pm 1·21^{d}$
	P value	>0·05	<0·001	<0·001	<0·001	<0·001	<0·001
Histamine	0	$6·31 \pm 0·94^{a}$	$19·13 \pm 2·27^{a}$	$28·27 \pm 2·19^{a}$	$33·30 \pm 1·63^{a}$	$37·61 \pm 1·59^{a}$	$41·57 \pm 2·74^{a}$
	0·05	$5·93 \pm 0·68^{a}$	$19·20 \pm 1·45^{a}$	$27·53 \pm 2·40^{a}$	$32·60 \pm 0·78^{a}$	$37·00 \pm 1·60^{a}$	$41·00 \pm 1·10^{a}$
	0·1	$4·33 \pm 1·40^{a}$	$15·97 \pm 1·21^{ab}$	$22·76 \pm 0·71^{b}$	$26·22 \pm 1·52^{a}$	$31·52 \pm 1·24^{b}$	$35·75 \pm 0·93^{b}$
	0·2	$4·41 \pm 0·75^{a}$	$14·61 \pm 1·40^{bc}$	$19·91 \pm 1·55^{bc}$	$23·68 \pm 1·60^{b}$	$28·47 \pm 1·20^{b}$	$29·26 \pm 1·75^{c}$
	0·4	$4·80 \pm 0·60^{a}$	$11·95 \pm 1·35^{c}$	$17·60 \pm 2·65^{cd}$	$18·55 \pm 2·02^{c}$	$23·87 \pm 1·15^{c}$	$22·20 \pm 1·11^{d}$
	P value	>0·05	<0·01	<0·001	<0·001	<0·001	<0·001

**P* value, obtained from ANOVA is listed. Means in columns without common letters are significantly different ($P < 0·05$).

more cheese. According to different studies, *Z. multiflora* has antimicrobial effects (Mansour *et al.* 2010; Noori *et al.* 2012; Sajed *et al.* 2013) and could lead to the reduction of micro-organisms, some of which are positive decarboxylase and responsible for producing BAs. It has been confirmed that the antimicrobial activity of most essential oils is attributed to their content on phenolic monoterpenes, in particular, thymol and carvacrol (Saei-Dehkordi *et al.* 2010). *Zataria multiflora* EO has a high percentage of carvacrol and possesses notable antimicrobial properties. Electron micrographs revealed that carvacrol, which is lipophilic in nature, acts on cell membrane and causes significant morphological damage which leads to permeability changes and release of cellular contents (Moosavy *et al.* 2008).

In the present study, increasing concentrations of *Z. multiflora* EO result in decreasing BAs content and at a concentration of 0·4%, the decrease is approximately 45%, so it is safer for the consumer.

Tyramine reduced in *Z. multiflora* EO-treated groups, but it was still more than the stated limit (100 mg kg^{-1}) by Shalaby (1996). According to the results, higher concentrations of 0·4% *Z. multiflora* showed adverse effects on organoleptic properties. Therefore, the simultaneous use of *Z. multiflora* EO and other essential oils or application of *Z. multiflora* EO with other BA controlling measures would be recommended.

It is noteworthy that NSLABs (nonstarter lactic acid bacteria) and contamination by micro-organisms are more responsible for the production of BAs. To inhibit BAs formation in hard and semi-hard cheeses (Gouda), using a negative amino acid-decarboxylase starter like most *lactobacillus* is effective.

Microbiological composition

Enterococci

The number of different microbial groups in Gouda cheese in control and *Z. multiflora* EO-treated samples during 90 days of ripening is shown in Table 4. In the control group, the amounts of enterococci bacteria from 3·44 log CFU g^{-1} (on the first day) reached 8·36 log CFU g^{-1} (on the 90th day of ripening). The highest growth was observed in the first 30 days of ripening, increasing more than 3 log CFU g^{-1}. This remarkable increase could be due to lactose present at the early phases of ripening.

Then, enterococci counts decreased compared to the first 30 days of ripening period. This may be closely related to depletion in lactose content.

Schneller *et al.* (1997) reported 6·93 log CFU g^{-1} of enterococci in semi-soft ripening cheese and also pointed out that after 2 months of ripening, enterococci counts in cheese produced from raw milk are similar to cheese produced from pasteurized milk. Some enterococci are resistant to pasteurization process and are able to grow after heat treatment (Ladero *et al.* 2011b). As there are different sources for enterococci, it is difficult to locate the exact ones. Enterococci are considered as major micro-organisms in semi-hard and fully ripened cheese due to milk contamination, hygienic condition of cheese production and their resistance to salt and heat (Galgano *et al.* 2001; Sarantinopoulos *et al.* 2001). The probable explanation for ineffectiveness of *Z. multiflora* EO at the concentration of 0·05% could be attributed to the high content of proteins and fat in cheese because it is commonly assumed that the high values of fat or protein in food play a protective role for bacteria against essential oils (Aureli *et al.* 1992; Pandit and Shelef 1994).

An almost similar increment pattern between enterococci counts and tyramine and histamine contents during ripening caused enterococci to have the highest correlation coefficient with BA production (correlation coefficient = 0·96 and 0·98 for tyramine and histamine, respectively).

Tyramine-producing micro-organisms were expected to be present within the groups of enterococci, Enterobacteriaceae and LAB as reported by Martuscelli *et al.* (2005). Although enterococci are a subgroup of LAB, they are described in BAs-producing suspected micro-organisms separately. Among LAB, enterococci are the most controversial due to their various characteristics, some of which play an effective role during ripening such as proteolysis, citrate breakdown, lipolysis, being applied as probiotics, producing bacteriocins, while some others are related to cases of human infections and are suspected of being a tyramine producer in cheese (Roig-Sagués *et al.* 2002; Foulquié Moreno *et al.* 2006).

Mesophilic lactobacilli

As shown in Table 4, mesophilic lactobacilli are the dominant bacterial group in initial days of ripening due to their presence in the starter culture. In general, an extreme increase is observed during the first 15 days. Then, counts of mesophilic lactobacilli decrease gradually and reach 7·20 log CFU g^{-1} after 90 days of ripening. Mesophilic lactobacilli counts reported by Marino *et al.*(2008) in the starter-added group were approximately similar and in natural milk culture had lower CFU g^{-1} than our findings on the first day of ripening. Although the pattern of mesophilic lactobacilli changes during ripening was different, they increased up to 7·8 log CFU g^{-1} after 90 days of ripening, which was in agreement with our experiment. As the lactobacilli are saccharolytic, the depletion of lactose at early phases of ripening could be the main reason for lactobacilli reduction.

Table 4 Effect of *Zataria multiflora* Boiss. (*Z. multiflora*) essential oil (EO) on microbiological counts (Mean ± SD) in Gouda cheese during ripening

Microbiological viability(Log CFU g^{-1} cheese)	EO concentrations (% (v/v))	Days of analysis					
		0	15	30	45	60	90
Enterococci	0	3·44 ± 0·19^{a*}	5·07 ± 0·25^{a}	6·63 ± 0·18^{a}	7·24 ± 0·12^{a}	8·20 ± 0·14^{a}	8·36 ± 0·16^{a}
	0·05	3·40 ± 0·15^{a}	5·11 ± 0·11^{a}	6·57 ± 0·12^{a}	7·31 ± 0·14^{a}	8·14 ± 0·23^{a}	8·16 ± 0·13^{a}
	0·1	3·42 ± 0·26^{a}	4·87 ± 0·15^{a}	6·45 ± 0·21^{ab}	7·10 ± 0·21^{a}	8·08 ± 0·18^{a}	7·93 ± 0·24^{ab}
	0·2	3·31 ± 0·11^{a}	4·77 ± 0·16^{a}	6·04 ± 0·29^{b}	6·69 ± 0·14^{b}	7·48 ± 0·34^{ab}	7·74 ± 0·14^{b}
	0·4	3·13 ± 0·10^{a}	4·42 ± 0·13^{b}	5·23 ± 0·17^{c}	6·20 ± 0·09^{c}	7·00 ± 0·35^{bc}	7·21 ± 0·11^{c}
	P value	>0·05	<0·01	<0·001	<0·001	<0·01	<0·01
Mesophilic lactobacilli	0	5·48 ± 0·32^{a}	8·54 ± 0·11^{a}	8·16 ± 0·21^{a}	7·83 ± 0·16^{a}	7·64 ± 0·07^{a}	7·20 ± 0·38^{a}
	0·05	5·34 ± 0·20^{a}	8·39 ± 0·11^{ab}	8·17 ± 0·09^{a}	7·93 ± 0·28^{a}	7·58 ± 0·26^{a}	7·37 ± 0·18^{ab}
	0·1	5·41 ± 0·33^{a}	7·98 ± 0·21^{b}	7·95 ± 0·14^{ab}	7·54 ± 0·08^{ab}	7·31 ± 0·08^{ab}	6·99 ± 0·15^{ab}
	0·2	5·22 ± 0·12^{a}	7·83 ± 0·23^{bc}	7·59 ± 0·17^{bc}	7·07 ± 0·22^{bc}	6·84 ± 0·19^{bc}	6·63 ± 0·27^{b}
	0·4	5·02 ± 0·36^{a}	7·02 ± 0·12^{d}	7·08 ± 0·18^{d}	6·40 ± 0·18^{d}	6·43 ± 0·38^{cd}	5·75 ± 0·20^{c}
	P value	>0·05	<0·001	<0·001	<0·001	<0·05	<0·001
Aerobic mesophilic bacteria	0	4·57 ± 0·21^{a}	5·33 ± 0·35^{a}	5·25 ± 0·28^{a}	6·96 ± 0·18^{a}	8·08 ± 0·14^{a}	7·74 ± 0·24^{a}
	0·05	4·51 ± 0·17^{a}	5·22 ± 0·13^{a}	5·12 ± 0·10^{ab}	6·77 ± 0·37^{a}	8·20 ± 0·28^{ab}	7·65 ± 0·14^{a}
	0·1	4·56 ± 0·40^{a}	5·13 ± 0·38^{ab}	5·08 ± 0·31^{ab}	6·56 ± 0·15^{ab}	8·04 ± 0·09^{ab}	7·17 ± 0·13^{b}
	0·2	4·46 ± 0·15^{a}	4·58 ± 0·17^{b}	4·91 ± 0·09^{ab}	6·04 ± 0·19^{b}	7·68 ± 0·07^{b}	6·97 ± 0·12^{b}
	0·4	4·34 ± 0·18^{a}	4·37 ± 0·12^{bc}	4·60 ± 0·15^{b}	5·54 ± 0·20^{bc}	7·47 ± 0·18^{bc}	6·17 ± 0·16^{c}
	P value	>0·05	<0·05	<0·05	<0·001	<0·01	<0·01
Enterobacteriaceae	0	1·23 ± 0·09^{a}	1·78 ± 0·09^{a}	2·30 ± 0·17^{a}	2·73 ± 0·24^{a}	2·78 ± 0·15^{a}	2·96 ± 0·11^{a}
	0·05	1·15 ± 0·16^{a}	1·65 ± 0·16^{ab}	2·31 ± 0·06^{a}	2·66 ± 0·15^{ab}	2·84 ± 0·09^{ab}	2·76 ± 0·18^{a}
	0·1	1·28 ± 0·11^{a}	1·61 ± 0·12^{ab}	2·21 ± 0·16^{a}	2·56 ± 0·17^{ab}	2·76 ± 0·14^{ab}	2·70 ± 0·20^{a}
	0·2	1·19 ± 0·12^{a}	1·58 ± 0·11^{ab}	1·91 ± 0·19^{ab}	2·26 ± 0·09^{b}	2·48 ± 0·14^{b}	2·28 ± 0·12^{b}
	0·4	1·15 ± 0·18^{a}	1·39 ± 0·18^{b}	1·73 ± 0·25^{b}	1·90 ± 0·13^{bc}	2·11 ± 0·08^{c}	2·21 ± 0·09^{c}
	P value	>0·05	<0·05	<0·01	<0·01	<0·001	<0·001
Lactococci	0	3·48 ± 0·19^{a}	4·55 ± 0·18^{a}	5·13 ± 0·21^{a}	6·21 ± 0·24^{a}	6·55 ± 0·16^{a}	7·42 ± 0·11^{a}
	0·05	3·36 ± 0·11^{a}	4·48 ± 0·12^{a}	5·08 ± 0·17^{a}	6·43 ± 0·45^{a}	6·44 ± 0·08^{a}	7·31 ± 0·11^{ab}
	0·1	3·41 ± 0·15^{a}	4·30 ± 0·19^{a}	4·73 ± 0·18^{ab}	5·92 ± 0·18^{ab}	6·12 ± 0·10^{b}	7·15 ± 0·04^{b}
	0·2	3·44 ± 0·08^{a}	4·19 ± 0·16^{ab}	4·55 ± 0·19^{b}	5·56 ± 0·13^{b}	6·05 ± 0·11^{b}	6·84 ± 0·14^{c}
	0·4	3·20 ± 0·14^{a}	3·89 ± 0·14^{b}	4·20 ± 0·20^{bc}	5·25 ± 0·16^{bc}	5·62 ± 0·09^{c}	6·11 ± 0·10^{d}
	P value	>0·05	<0·01	<0·01	<0·01	<0·01	<0·001
Yeasts	0	2·98 ± 0·18^{a}	3·31 ± 0/16^{a}	3·52 ± 0·17^{a}	4·85 ± 0·14^{a}	5·33 ± 0·12^{a}	6·31 ± 0·11^{a}
	0/5	2·96 ± 0·27^{a}	3·19 ± 0/11^{ab}	3·36 ± 0·06^{ab}	4·72 ± 0·11^{a}	5·31 ± 0·07^{a}	6·22 ± 0·06^{a}
	1	2·81 ± 0·16^{a}	3·07 ± 0/11^{ab}	3·27 ± 0·23^{ab}	4·62 ± 0·16^{a}	4·94 ± 0·05^{b}	5·73 ± 0·12^{b}
	2	2·85 ± 0·18^{a}	2·92 ± 0/20^{b}	3·20 ± 0·28^{ab}	3·86 ± 0·28^{b}	4·41 ± 0·14^{c}	5·47 ± 0·18^{b}
	4	2·89 ± 0·10^{a}	2·59 ± 0/14^{c}	2·95 ± 0·13^{b}	3·15 ± 0·09^{c}	3·74 ± 0·28^{d}	4·29 ± 0·15^{c}
	P value	>0·05	<0·01	<0·05	<0·001	<0·001	<0·001

*P value, obtained from ANOVA is listed. Means in columns without common letters are significantly different ($P < 0·05$).

On the first day, counts of mesophilic lactobacilli in 0·4% *Z. multifora* concentration were 0·05 log CFU g^{-1}, lower than control group but not significantly. On the 15th day, approximately 05, 1·2 and 1·5 log CFU g^{-1} reductions of mesophilic lactobacilli were seen in 0·1, 0·2 and 0·4% *Z. multifora* EO-treated samples compared to the control group, respectively ($P < 0·05$).

Wilkinson *et al.* (1994) reported a positive correlation between the release of amino acids in cheese and cell lysis of the starter cultures. Moreover, according to Visser (1993), free amino acid release is associated with action of microbiological peptidase. Tyramine and histamine contents during 15 days of ripening, with the concentration of 0·1% *Z. multifora*, did not significantly decrease, whereas counts of mesophilic lactobacilli reduced significantly ($P < 0·05$). The probable explanation could be over the cell lysis, and lower BAs formation may have occurred, but amino peptidase releasing and increasing of free amino acid facilitated production of BAs. In higher concentrations of EO (0·2 and 0·4%), the inhibitory effects of essences prevent BAs production.

Correlation coefficient of mesophilic lactobacilli resulted in the least value among microbial groups examined, and tyramine and histamine were 0·61 and 0·47, respectively. Even though mesophilic lactobacilli increased remarkably during the first 15 days of ripening like

histamine and especially tyramine, they started to decrease unlike tyramine and histamine. Once BAs are produced, it is difficult to degrade them (Shalaby 1996). Hence, it is probable that mesophilic lactobacilli play a role in BA production, especially during the early phase of ripening.

Schneller *et al.* (1997) mentioned Enterobacteriaceae and enterococci as tyramine producers and that lactobacilli do not seem important in semi-soft ripening cheeses. Starter bacteria affect the protein breakdown (Lane and Fox 1996; Lynch *et al.* 1997), whereas the NSLAB are contributed to peptidolysis and the release of free amino acids (Muehlenkamp-Ulate and Warthesen 1999). Marino *et al.* (2008) showed BAs content in the cheese produced by commercial starter culture was much lower than the cheese produced by natural milk culture. Furthermore, Marino *et al.*(2000) reported lactobacilli should be in high amount (10^7 CFU g^{-1}) for a long period (at least 6 months) in cheese to produce high concentrations of histamine and tyramine. It appears that lactobacilli do not play the main role in production of BAs but could predispose the production condition.

Enterobacteriaceae

Enterobacteriaceae counts in the control group, from 1·23 log CFU g^{-1} (on the first day) reached 2·96 log CFU g^{-1} at a constant trend (after 90 days of ripening), that is higher than values reported by Komprda *et al.* (2008) and lower than those of Marino *et al.* (2000). At the end of ripening, Enterobacteriaceae counts reduced by 0·68 and 0·75 log CFU g^{-1} in concentration of 0·2 and 0·4% of *Z. multiflora* compared to the control group. Among all micro-organisms examined, Enterobacteriaceae resulted in the least reduction. It could be due to hydrophilic characteristic of impermeable outer membrane to lipophilic component that leads to more resistance of gram negative bacteria compared to gram positive bacteria (Ozturk and Ercisli 2006; Sandri *et al.* 2007; Saei-Dehkordi *et al.* 2010).

Enterobacteriaceae are destroyed through pasteurization but poor hygienic milk-handling and manufacturing practices could contaminate cheese (Schirone *et al.* 2011). Enterococci and Enterobacteriaceae are adventurous contaminating micro-organisms and the main producers of BA in cheese (Joosten 1988; Martuscelli *et al.* 2005). Therefore, the presence of BAs could be an indicator of bad manufacturing practices or poor quality.

Lactococci

Lactococci counts increase from 3·48 log CFU g^{-1} up to 7·35 log CFU g^{-1} in the control group. Several peptidases with different qualities have been identified in lactococci which can degrade milk casein (Salminen and Wright 2004). Despite lactose depletion, lactococci count increase constantly during ripening period in all the samples. In 0·1, 0·2 and 0·4% of *Z. multiflora* EO-added groups, lactococci counts significantly reduced by 0·27, 0·58 and 1·31 log CFU g^{-1} at the end of ageing compared to the control group.

High amounts of lactococci on the first day of ripening could be attributed to resistance to pasteurization or poor hygiene during manufacturing. It was shown that NSLAB and Enterobacteriaceae contribute to the accumulation of BA in Gouda cheese (Joosten 1988). Lactococci constitute part of NSLAB and include some strains that have been described as BA producers (Ladero *et al.* 2011a,b).

Aerobic mesophilic Bacteria

In the control group, the initial loads of aerobic mesophilic bacteria were 4·57 log CFU g^{-1} and increased up to 8·08 log CFU g^{-1} after 60 days of ripening. Then, they decreased to 7·64 log CFU g^{-1} at the end of ageing. Total aerobic mesophilic counts on the first day of ripening are consistent with Marino *et al.* (2008), but it is approx. 1 log CFU g^{-1} lower than the present study after 90 days of ripening. In concentration of 0·1, 0·2 and 0·4% of *Z. multiflora*, the amounts of aerobic mesophilic 0·57, 0·77 and 1·57 log CFU g^{-1}, respectively, significantly reduced.

Yeast

Initial counts of yeasts were approximately 2·98 log CFU g^{-1} which reached 6·31 log CFU g^{-1} at the end of ageing. These amounts are higher than that reported by Gardini *et al.* (2006) and Schirone *et al.* (2011). In concentration of 0·1, 0·2 and 0·4% of *Z. multiflora* EO-treated groups, 0·58, 0·84 and 2·2 log CFU g^{-1} yeast reductions were observed after 90 days of ripening. Yeasts counted in the control group, 0·05 and 0·1% *Z. multiflora* EO treatments, were higher than the report by Marino *et al.* (2008), whereas in 0·2 and 0·4% *Z. multiflora* EO-treated samples, the counts were lower after 90 days of ripening. In our experiment, yeasts were the most susceptible microbial group to *Z. multiflora* EO and this is in agreement with Saei-Dehkordi *et al.*(2010).

In general, high amounts of yeast were observed in our study. This could be because of the characteristics such as high tolerance towards reduced water, low pH, high salt concentration and the ability to grow at low storage temperature that enables yeast to survive and grow in ripening cheese (Ferreira and Viljoen 2003). Several yeast species have proved potential for BAs production. For example, *Yarrowiali polytica* and *Debaryomyces hansenii* isolated from cheese seem to be able to build up tyramine and histamine, respectively (Gardini *et al.* 2006).

It should be noted that BAs-producing capacity is generally a strain-level feature which is spread among various microbial species (Novella-Rodríguez *et al.* 2002).

Thereby, the use of nonspecific method for identification of BAs-producing micro-organisms could not be appropriate and would decrease the reliability of the findings.

The effect of *Z. multiflora* EO on BAs production in Gouda cheese was evaluated. *Zataria multiflora* EO significantly reduced BAs (tyramine and histamine) contents and microbial counts compared to the control group. This desirable reduction is a practical method that could be used in different cheeses, especially those with higher BAs content. Besides, the combination of *Z. multiflora* EO with other essential oils or the application of *Z. multiflora* with other BAs controlling measures would be suggested.

Methods and material

Preparation of *Zataria multiflora* essential oil Boiss

Zataria multiflora was acquired from the province of Fars in Iran and characterized by the Institute of Medicinal Plants, Tehran University of Medical Sciences, Iran. The Clevenger-type apparatus was used for hydro distillation of air-dried aerial parts of the plant for 2 h. The essential oil obtained from the air-dried material was analysed by gas chromatography (GC) (THERMOQUEST 2000, UK). The chromatograph is comprised of DB5 capillary column (30 × 0·25 mm ID × 0·25 lm film thicknesses), and the data were collected as follows: primary temperature 50°C; programme rate 2·5°C; final temperature 265°C; and injector temperature 250°C. Helium was the carrier gas, and the split ratio was 120. The *Z. multiflora* EO was also examined by gas chromatography–mass spectrometry (GC–MS) (THERMOQUEST FINNIGAN, Manchester, UK), and the same capillary column and aforementioned analytical conditions were used. The MS was run in the electron ionization mode, using ionization energy of 70 eV.

Gouda cheese production and sampling

Gouda cheese was produced at a commercial dairy factory according to the following procedure: pasteurized (75°C, 16 s) cow's milk was poured into a stainless steel double-boiler vat. After reaching the optimal temperature, mesophilic starter culture (*Lactobacillus casei* and *Leuconostoc mesenteroides*), calcium chloride and Annatto colorant (optional) were added and mixed thoroughly with a whisk to make the milk uniform throughout. Then, the curd was covered and allowed to ripen. Forty minutes later, 2 ml of fungal rennet was added. The milk was supplemented with *Z. multiflora* in different concentrations 0·05, 0·1, 0·2 and 0·4% and the control group (without EO). Forty minutes after the rennet addition, the curd was cut into 0·5–1·25 cm cubes and, thereafter, transferred to moulds and the whey allowed to drain. Then, the cheese was placed in saturated brined solution for 3–4 h. The brine was removed, and the cheese pieces were placed onto a dry mat at 12–14°C and 85–90% relative humidity. After a few days, the cheese moulds were waxed and stored for 90 days. For each concentration of *Z. multiflora* EO, two batches of Gouda cheese were prepared. Microbiological enumeration and BAs analyses were performed in triplicate for each concentration at 0, 15, 30, 45, 60 and 90 days of ripening.

Microbiological analyses

Ten grams of the cheese was taken aseptically, homogenized in the stomacher Lab-Blender 400 (Steward Medical, London, UK) with 90 ml of sodium citrate (2% w/v) solution, and serial decimal dilutions were prepared in sterile peptone water (0·1% w/v) in duplicate. The following groups of micro-organisms were enumerated: aerobic mesophilic bacteria were grown on Plate Count Agar (PCA; Biolife, Milan, Italy) at 30°C for 2 days; mesophilic lactobacilli on MRS agar (Biolife), with pH adjusted to 5·5, at 30°C for 2 days in anaerobic conditions via placing into the Gas-Pack anaerobic system (Biolife); Enterobacteriaceae on Violet Red Bile Glucose Agar (VRBGA; Biolife) at 37°C for 24 h; Coliforms on Violet Red Bile Agar (VRBA; Bioife); Yeasts on Peptone Dextrose Agar (PDA, Bioife) at 20°C for 5 days; Enterococci on Kenner Fecal agar (Biolife) at 37°C for 48 h.

Biogenic amine determination

The extraction method was according to Eerola *et al.*(1993) as reported by Lanciotti *et al.*(2007).

Sensory evaluation

Sensory properties were examined by acceptance test in duplicate. Seven panellists were chosen among the staff of Tehran University of Medical Sciences and trained according to ISO standards (ISO 8586-1:1993). Each sample was scored using a 9-point scale in which 9 = like extremely, 1 = dislike extremely, and acceptability point was 5 = neither like nor dislike. Using repeated measures ANOVA, the means of the scores were compared and Bonferroni correction was employed to show the significance levels in pairwise comparison. Data were analysed using SPSS 18.0 statistical software (SPSS 18.0 for Windows; SPSS Inc., Chicago, IL).

Statistical analysis

The repeated measures one-way ANOVA was used to evaluate the effect of different concentrations of *Z. multiflora*

essential oils on tyramine, histamine and microbiological profiles during ripening. The interaction effect between time and different concentrations of *Z. multiflora* was analysed. If the interaction effect between the aforementioned factors was significant, the mean comparison would be conducted for different concentrations of *Z. multiflora* at each time separately. Also, to identify which group of micro-organisms was more responsible for histamine and tyramine production, Pearson correlation coefficient was utilized. The level of P value $<0{\cdot}05$ was considered significant.

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Conflict of Interest

The authors declare no conflict of interest.

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