The objectives were to investigate the lutein content of bovine milk as affected by the cows’ dietary lutein supplementation in the presence or absence of antioxidants, vitamin E (Vit E), tea polyphenols (TP) and ethoxyquin (EQ), and to assess the impact of subsequent pasteurisation on the lutein content and sensory quality of the bovine milk. Among the dietary supplement formulations studied, lutein plus Vit E was the most effective, lutein plus TP was moderately effective, and lutein plus EQ was adversely effective at improving the milk’s lutein content by 53–88%, relative to the basal diet. Co-supplementation with antioxidant also mitigated the lutein loss caused by the milk’s processing and storage. This study showed that supplementation of bovine feed with Vit E plus lutein resulted in milk with high lutein content, and a superior ability to resist heat-induced lutein loss and sensory changes.

**Keywords** Bovine feed, Lutein, Antioxidant, Heat process, Vitamin E, Sensory evaluation, Milk.

**INTRODUCTION**

Lutein, a naturally occurring fat-soluble oxy-carotenoid (β, ε-carotene-3, 3’-diol), belongs to the family of the xanthophyll carotenoids. Like all carotenoids, lutein cannot be synthesised in the human body and must come from the diet. Along with zeaxanthin (an isomer), lutein is found in human serum and selectively accumulates in the macula of the retina (Landrum and Bone 2001) and lenses (Yeum et al. 1995).

Lutein is known for its ability to reduce or prevent ocular diseases including age-related macular degeneration (AMD) (Landrum and Bone 2001), cataracts (Kijlstra et al. 2012) and diabetic retinopathy (Muriach et al. 2006). It has been suggested (Krisisky et al. 2003) that the protective mechanism of lutein is probably due to its shielding effect against potentially harmful and short-wavelength radiation, which can cause photo-induced damage in the membrane system (Landrum and Bone 2001). Although no recommended dietary allowance currently exists for lutein, positive effects on health issues such as AMD have been reported at dietary intake levels of 6–12 mg/day (Seddon et al. 1994).

Lutein exists mainly in leafy greens such as spinach, collard greens, turnip greens and kale, which contain as much as 40 mg/100 g (Sommerburg et al. 1998). Depending on the breed and diet of the cow, milk can be an excellent resource of lutein with exceptional bioaccessibility and bioavailability because of the favourable lipid-soluble state within the milk fat globules (Schweiggert and Carle 2015). A previous study (Calderon et al. 2007) showed that the lutein content of milk was rapidly increased by shifting feed from a hay diet to one with high levels of carotenoids and vitamin E. Our recent work (Xu et al. 2014) found that dietary lutein supplementation (up to 4 g/day per head) significantly improved both the milk yield (from 34.6 to 37.4 kg/day) and the lutein content of the milk, from 0.59 to 1.50 g/100 mL. In addition to the improved plasma antioxidative activity, such dietary lutein supplementation did not alter the protein and fat contents significantly.
Because of the high degree of unsaturation in its chemical structure, (a total eleven conjugated double bonds), lutein is highly sensitive to light, heat and oxygen. It can easily become unstable and decomposes when exposed to these adverse conditions (Subagio et al. 1999). Additionally, its poor solubility in aqueous media, hence its low bioavailability, also imposes severe limitations on its applications by the food and pharmaceutical industries (Madaan et al. 2017).

Vitamin E (Vit E) is a mixture of tocopherol and tocotrienol compounds. These potent lipid-soluble antioxidants are located in cell membranes and can prevent the propagation of free radical reactions (Herrera and Barbas 2001). Extensive research (Schneider 2005) has yielded a significant amount of information on the action, effects and metabolism of Vit E. In addition, it has also been shown that Vit E plays a protective role in bone, inflammatory, cardiovascular, eye, neurological and neurological disease, and cancer (Peh et al. 2016). As a major vitamin in bovine milk, Vit E acts as radical scavengers in the lipid phase (Lindmark-Mansson and Åkesson 2007). It has been shown (Focant et al. 1998) that Vit E supplementation in feed significantly increased the concentrations of Vit E in the milk and plasma, as well as resistance to milk fat oxidation in dairy cows.

Laboratory and epidemiological studies have suggested that green tea polyphenols (TP) have preventive effects against chronic diseases including obesity, heart disease, diabetes, neurodegenerative disease and cancer (Lambert and Elias 2010). Although the details of the protective mechanisms remain to be fully understood, strong evidence exists demonstrating the antioxidative properties of the catechins, which can quench free radicals and chelate transition metals.

Due to its low production cost and high efficiency in extending shelf life, ethoxyquin (EQ; 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline), a synthetic quinoline-based compound with powerful antioxidative activity, is widely used as a preservative in animal foods such as fish meals and pet foods (Błaszczyk et al. 2013). Despite its usefulness, some adverse health effects relating to the consumption of EQ by animals have been reported (Dzanis 1991). Both the European Union (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2015) and the United States (Code of Federal Regulations (CFR), Title 21, Parts 573.380 & 573.400) have established concentration limits on the use of EQ in animal feeds.

To take advantage of milk as a natural delivery vehicle for many bioactive compounds (Livney 2010) including lutein (Yi et al. 2016), fortification of lutein in liquid milk (Matsumoto et al. 2014) and infant formula (Capeding et al. 2010) has been reported. Lutein has also been added to other dairy products such as fermented milk (Granado-Lorencio et al. 2010), cream cheese (Tokuşoğlu 2013) and yoghurt (Domingos et al. 2014). Some limited data (Odorissi Xavier et al. 2014) have suggested that the bioaccessibility of lutein was lower in reconstituted products compared to liquid milk and yoghurt. Studies on the bioavailability of lutein have revealed (Kullen et al. 2007) that serum lutein concentrations were much higher in breastfed infants than those fed with fortified formula. Recent work by Kelly et al. (2014) demonstrated that serum lutein levels in healthy adults were increased by the daily consumption of lutein or zeaxanthin enriched eggs and an egg yolk-based buttermilk beverage. The scientific literature is, however, insufficient regarding increasing the lutein content of milk or other dairy-related products through dietary supplementation of cows’ feed.

To the best of our knowledge, increasing the lutein content of milk by cosupplementation of lutein with potent antioxidants including Vit E, TP and EQ in cows’ feed has remained unexplored. In this work, we aimed to investigate the effects of the antioxidants, of subsequent processing treatment [that is high-temperature short-time (HTST) and ultra-high temperature (UHT) pasteurisation], as well as storage time on the lutein content of milk. The lutein and antioxidant contents of food are expected to affect the sensory quality. Therefore, the sensory traits of the milk, as a result of the dietary supplementation with lutein and antioxidants, were also evaluated.

MATERIALS AND METHODS

Materials
AbsoLUTEIN, vitamin E and tea polyphenols were supplied by Kemin Industries, Inc. (Zhuhai, China). Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) was provided by Litian Science and Technology Inc. (Jiangsu, China). AbsoLUTEIN contained 15% oleoresin extracted from marigolds and was used with silica and rice bran as carriers. The lutein content was about 2% in this product. The purity of vitamin E (Vit E), tea polyphenols (TP) and ethoxyquin (EQ) was 90%, 75% and 93% respectively. The following abbreviations are used throughout the paper: Vit E, vitamin E; TP, tea polyphenols; and EQ, ethoxyquin.

Sample preparation
The milk samples used in this study were obtained from a local dairy farm, Qiaoshi Dairy Farm, Hangzhou, Zhejiang Province. Sixty multiparous Holstein cows in mid-lactation were selected and divided randomly into five groups. Group A, the control, was fed basal diet only (Table 1). The remaining four groups, B, C, D and E, received a basal diet containing a supply of AbsoLUTEIN at 200 g/day per head (an effective dose of 4 g/day per head). The feed for group B contained lutein only. The diet for groups C and D was supplemented with vitamin E (Vit E) and tea polyphenols (TP) at 1.2 g/day per head, respectively. Group E was given ethoxyquin (EQ) at 2 g/day per head. The dose of each antioxidant was based on the recommendations of the manufacturer. Feeds were formulated according to the nutrient
requirements for lactating Holstein cows weighing 600 kg and producing 30 kg/day of milk on average. All ingredients were mixed in a total mixed ration (TMR) and offered to each cow three times per day. The lutein and antioxidant preparations were top-dressed on the TMR based on each cow three times per day. The lutein and antioxidant preparations were mixed in a total mixed ration (TMR) and offered to each cow three times per day. The lutein content of the milk from each experimental group that was collected and pooled at 8 weeks of feeding was analysed (Odorissi Xavier et al. 2014). Briefly, one gram of milk sample was weighed and placed into a test tube. Ten millilitres of the extraction solution (hexane:disopropyl ether, 75:25 v/v) were added to each tube, which was then shaken for 5 min. Deionised water (20 mL) was added, and the tube was capped, shaken well and centrifuged for 10 min at 2000 rpm. An aliquot of 1 mL of the upper solvent layer was transferred to an HPLC vial, evaporated (by a SpeedVac) and reconstituted with 1 mL mobile phase.

Lutein content determination

The lutein content of the milk from each experimental group that was collected and pooled at 8 weeks of feeding was analysed using the HPLC according to previously published work (Gill and Indyk 2008). Sample preparation was adapted from the method used for the rapid analysis of carotenoids in bovine milk (Odorissi Xavier et al. 2014). Briefly, one gram of milk sample was weighed and placed into a test tube. Ten millilitres of the extraction solution (hexane:disopropyl ether, 75:25 v/v) were added to each tube, which was then shaken for 5 min. Deionised water (20 mL) was added, and the tube was capped, shaken well and centrifuged for 10 min at 2000 g. An aliquot of 1 mL of the upper solvent layer was transferred to an HPLC vial, evaporated (by a SpeedVac) and reconstituted with 1 mL mobile phase.

Lutein and lutein esters were separated on an Agilent HPLC unit, 1200, (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a diode array and multi-wavelength detector. A 20 µL sample of the milk extract was injected into a polymeric reversed-phase ZORBAX C18 column (250 × 4.6 mm, 5 µm particle size, Agilent Technologies). A pre-column (12.5 × 4.6 mm, 4/PK) was used to protect the column. The
separation was isocratic using methyl tertiary-butyl ether (TBME): methanol (5:95, v/v) as the mobile phase with a total separation time of 30 min. The flow rate was 1.0 mL/min, and the detection wavelength was set at 450 nm.

All HPLC analyses were repeated three times for each sample representing each experimental group.

**Sensory evaluation**

The sensory attributes of the HTST and UHT pasteurised milk samples were evaluated using the quantitative response scales method (Granado-Lorencio et al. 2010). A trained sensory panel consisted of 12 people (six men and six women) with prior experience in sensory evaluation. The panellists were trained following the international standards on sensory profiles, the sniffing technique, the use of scales and the intensity rating procedure (Tokusoglu 2013). A refreshing sensory training was conducted just prior to the actual sensory evaluation of the experimental samples.

Ten millilitres of the milk samples were weighed into a 50-mL glass bottle with a screw cap and kept in a 25 °C water bath for 30 min before evaluation. Each sample was coded and presented in random order. The panellists were instructed to remove the cap of the bottle, take three short sniffs, taste and then rate the overall intensity of the samples.

**Statistical analysis**

All data obtained in this work were expressed as the means ± standard deviations of triplicate determinations. One-way analysis of variance (one-way ANOVA) was applied to the experimental data (n > 3) using the general linear model and the SPSS statistical software package (17.0). Tukey’s range tests were performed to compare all possible pairs of means across all groups of samples within the same measured quantity. All differences were considered statistically significant when P < 0.05.

**RESULTS AND DISCUSSION**

**Effect of dietary antioxidant supplementation on lutein content of the milk**

Table 2 lists the lutein content (µg/L) of the milk samples as affected by various dietary supplementation after 2 months of feeding. It is evident that including lutein (at an effective dose of 4 g/day per head) in the feed significantly increased (P < 0.05) the lutein content of the milk, by ~3.8 µg/L. The addition of the antioxidants, Vit E, TP and EQ, to the diet, also improved the lutein content compared to the control (basal diet). The extent of this improvement, however, depended on the type of antioxidant given. The feed containing lutein combined with Vit E yielded the highest lutein content, ~12.11 µg/L in the milk, which was nearly twice as high as that of the control (~6.45 µg/L). The diet of TP plus lutein also increased the milk’s lutein content significantly (P < 0.05), to 10.72 µg/L. We speculate that molecular properties, such as the structure and water or fat solubility of the antioxidants, played an important role in imposing a positive, or lack of, effect on the milk’s resulting lutein content. Vitamin E and lutein, both only fat-soluble, possess more similarity in their molecular structures than lutein versus TP and EQ, and may act synergistically resulting in the considerably increased level of lutein in the milk, as observed. Further research is needed to confirm this hypothesis as such results and interpretation have not previously been reported.

The milk resulting from the diet supplemented with lutein plus EQ contained 9.88 µg/L lutein, the lowest among the four experimental groups, and even below the level of lutein-only group. Such negative result of EQ on the lutein content of the milk may be explained, in part, by some of its known adverse effects on animals’ health (Domingos et al. 2014) such as weight loss (rats, dogs, mice), urinary bladder (rats), lethargy (rabbits). The effects of EQ on the health of large animals such as dairy cows as well as milk production remain unknown. The inclusion of EQ in this work was comparative, and its dose was used as per the manufacturer’s instructions.

### Table 2 Lutein content1 (µg/L) of milk as affected by dietary supplementation with lutein and antioxidant, and subsequent processing.

<table>
<thead>
<tr>
<th></th>
<th>Control Lutein</th>
<th>Lutein + Vit E</th>
<th>Lutein + TP</th>
<th>Lutein + EQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>6.45 ± 0.1324E</td>
<td>10.25 ± 0.051D</td>
<td>12.11 ± 0.104AA</td>
<td>10.72 ± 0.144EC</td>
</tr>
<tr>
<td>HTST</td>
<td>6.21 ± 0.0941GH</td>
<td>10.03 ± 0.224D</td>
<td>11.87 ± 0.255AB</td>
<td>10.57 ± 0.115cC</td>
</tr>
<tr>
<td>UHT</td>
<td>6.05 ± 0.044H</td>
<td>9.44 ± 0.144F</td>
<td>11.72 ± 0.214EF</td>
<td>10.13 ± 0.134D</td>
</tr>
</tbody>
</table>

EQ, ethoxyquin; HTST, high-temperature short-time; TP, tea polyphenols; UHT, ultra-high temperature; Vit E, vitamin E.

Data sharing the same superscript lowercase letter were not significantly different (P < 0.05) within the same row.

Data sharing the same superscript uppercase letter were not significantly different (P < 0.05) within the same column.

1Values were reported as averages of a set of triplicate measurements at weeks 8 and 9 milking, n = 6.

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Effect of processing and storage on lutein content of the milk

Table 2 also shows the lutein content of the milk after subsequent HTST and UHT processing. The HTST pasteurisation did not cause significant lutein loss \((P < 0.05)\) in the milk of all five experimental groups (including the control) compared to the raw milk.

The UHT-treated milk, however, showed a noticeable reduction in the lutein content across all samples. At 0.81 \(\mu g/L\), which corresponded to \(-8.0\%\) of the lutein level in raw milk, the lutein-only group (in the absence of antioxidant) suffered the most loss of lutein content. In contrast, the cosupplementation of antioxidant Vit E, TP or EQ with lutein in the feed considerably mitigated the lutein loss in the milk caused by ultra-high temperature treatment. Among the various supplementations studied, the lutein plus Vit E supplementation was particularly effective in protecting the milk’s lutein content, keeping the loss as low as \(-3.0\%\) relative to raw milk. Less effective, the cosupplementations of the feed with TP and EQ with lutein also lowered the milk’s heat-induced lutein reduction to \(-5.0\%\) of the level of raw milk. The exact protective mechanism of Vit E on lutein remains to be fully understood. It was reasonable to speculate, however, that the fat-soluble nature of both Vit E and lutein may contribute to such a combined ability to resist lutein loss during UHT processing.

Table 2 demonstrated that the differences in lutein content were small in the case of the lutein and lutein plus TP diets between the HTST- and UHT-treated milk, and essentially insignificant \((P < 0.05)\) as in the cases of the control, the lutein plus Vit E and the lutein plus EQ supplementations.

Figure 1(a,b) shows that the lutein content changed as a function of storage time in HTST and UHT pasteurised milk, respectively. It is clear that the lutein content underwent a slow and continuous decline in all the HTST processed samples during the experimental period of 6 days at 4 °C. A linear regression analysis of these data could predict the lutein loss in each sample as time progresses, beyond the experimental duration performed in this work. Table 3 shows the calculated total net loss of lutein content over the entire storage time relative to that of the raw milk (no storage). The amount of decrease was \(-4.0\%,\) virtually indistinguishable among all the milk samples produced from lutein supplementation. The control (basal diet) was the only milk sample with slightly more significant lutein loss, \(-5.6\%,\) demonstrating the protective role that lutein and the antioxidants played in the feed against the milk’s lutein loss.

The lutein content of the UHT-processed samples was monitored over a period of 5 months (Figure 1b) at room temperature (20 °C). Nonlinear regression analysis suggested that the milk’s lutein content followed a trend of steeper decline in all the samples compared to those of the HTST-treated samples. The total lutein loss relative to the raw milk over time was the greatest for the lutein-only milk sample, \(-41\%.\) Vitamin E and TP were the most effective dietary supplements in preventing such a loss in the milk, down to 21% and 27%, respectively. Similar to the control, with \(-30\%\) lutein loss over time, the EQ plus lutein diet apparently produced milk that was the least capable of resisting storage-related lutein decline post-UHT treatment.

It is well documented that antioxidants such as lutein, Vit E, TP and EQ can eliminate active oxygen free radical activity and delay cell damage. However, more research is needed to provide a comprehensive understanding on the remarkable ability of Vit E (which EQ lacked) to stabilise lutein in milk (against UHT and then storage) when co-supplemented with lutein, as observed in this work.

Effect of dietary lutein and antioxidant supplementation on the sensory quality of milk

Figure 2(a) gives the average scores from the sensory evaluation of the HTST pasteurised milk produced by dietary
lutein and antioxidant supplementation. It is clear that both the lutein-only and lutein plus Vit E diets produced milk that scored higher than that from the control diet. This was especially true with regard to the texture, flavour, aroma and global aspect of the milk. Appearance was the only sensory attribute for the lutein-only supplementation to be perceived slightly unfavourably compared to the control. The vivid orange-red colour of lutein probably contributed to such a change in appearance. Adding Vit E to the cows’ diet, however, seemed to have averted this problem. The cosupplementation of lutein and TP in the diet also improved the texture and global aspect of the milk without compromising its appearance, aroma and flavour relative to the control. The milk produced from the lutein plus EQ diet, on the other hand, received poor grades for its appearance and global aspect, and an even worse score for flavour. Texture and aroma were not affected greatly.

Figure 2(b) compares the average sensory scores of the UHT-treated milk samples from feed supplemented with lutein and antioxidants. Unlike the HTST samples, the aroma and the global aspect of the UHT milk samples were rather insensitive to the effects of the various diets. The appearance of the milk produced from the lutein-only supplementation earned higher marks than that of other diets, including the control. The lutein plus Vit E and lutein plus TP milk showed similar scores as the control in their appearance. The lutein plus EQ diet, however, produced milk with highly unfavourable scores for appearance. The texture of the milk from the experimental groups was preferable to that of the control. The milk produced by the lutein with and without Vit E supplementation displayed improved flavour attributes compared to the other diets.

It is expected that the lutein content of the food is closely related to flavour and appearance because of the strong orange-red colour and sour taste of lutein. Results from this work suggested that dietary lutein supplementation with or without antioxidant impacted the sensory properties of the milk post-HTST and post-UHT treatments. The lutein-only and lutein plus Vit E diets affected the milk’s appearance and flavour positively in both cases. The lutein plus TP diet did not alter these sensory aspects of the milk significantly.

### Table 3 Calculated total net lutein loss (µg/L) in milk caused by storage relative to raw milk.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Lutein</th>
<th>Lutein + Vit E</th>
<th>Lutein + TP</th>
<th>Lutein + EQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTST storage</td>
<td>0.36 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.47 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.45 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.48 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Storage loss, %</td>
<td>5.6</td>
<td>4.6</td>
<td>4.5</td>
<td>4.2</td>
<td>4.8</td>
</tr>
<tr>
<td>UHT storage</td>
<td>2.09 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.24 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.56 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.87 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.35 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Storage loss, %</td>
<td>32.4</td>
<td>41.4</td>
<td>21.2</td>
<td>26.7</td>
<td>33.9</td>
</tr>
</tbody>
</table>

EQ, ethoxyquin; HTST, High-temperature short-time; TP, tea polyphenols; UHT, Ultra-high temperature; Vit E, vitamin E.

Data within a row with the same superscript letter were not significantly different (<i>P</i> < 0.05).

<sup>1</sup>Values were reported as average of a set of triplicate measurements at weeks 8 and 9 milking (<i>n</i> = 6), and the pooled standard deviations were estimated based on the IUPAC Gold Book (1997).

<sup>2</sup>The total HTST storage period was 6 days at 4 °C.

<sup>3</sup>The total UHT storage period was 5 months at room temperature (20 °C).
compared to the control diet. The lutein plus EQ diet, on the hand, yielded milk with notably reduced quality with regard to appearance and flavour.

CONCLUSIONS

The lutein content of milk was greatly increased by dietary supplementation of the cows’ diet with and without commonly used antioxidants, that is Vit E, TP and EQ. Subsequent HTST treatment and storage (up to 6 days) caused little change in the milk’s lutein content. The UHT processing, on the other hand, reduced the milk’s lutein content considerably and storage for up to 5 months caused further lutein loss (>30%) in the absence of the antioxidant. The feed formulation containing lutein plus Vit E was highly effective in delaying such a loss in the milk, down to ~18%. The lutein plus TP diet was slightly effective, whereas the lutein plus EQ diet was ineffective in preventing lutein loss in the milk during storage. The sensory quality of the HTST and UHT milk was also similarly affected, and milk from the lutein only and lutein plus Vit E diets received favourable scores. The milk from the lutein plus TP diet was rated neutral, and milk from the lutein plus EQ diet had the least desirable sensory attributes compared to the control milk (basal diet).

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CONFLICT OF INTEREST/COMPLIANCE WITH ETHICS REQUIREMENT

The authors declare that they have no conflicts of interest.

REFERENCES


