



# Lutein as a functional ingredient in sheep milk yogurt: development, characterization and extraction recovery

Raphael Ferreira de BARROS<sup>1\*</sup> , Fernanda Romano TORRES<sup>1</sup>, Paulo Henrique Fonseca da SILVA<sup>2</sup>, Paulo Cesar STRINGHETA<sup>3</sup>, João Pablo Fortes PEREIRA<sup>2</sup>, Junio Cesar Jacinto de PAULA<sup>4</sup>, Camila Sampaio CUTRIM<sup>5</sup>, Marco Antonio Sloboda CORTEZ<sup>1</sup>

## Abstract

The research aimed to evaluate the behavior of different concentrations of lutein added in sheep milk yogurt. The work verified the effect on acidity, pH, color, lutein degradation, and recovery during storage. Different added lutein concentration into yogurt were compared: 0.00 mg; 3.0 mg; 3.45 mg; and 3.9 mg ( $\cdot 100 \text{ mL}^{-1}$ ). Analyzes were performed on days 1<sup>st</sup>, 15<sup>th</sup>, 30<sup>th</sup>, and 45<sup>th</sup> of refrigerated storage (5 °C). Lutein did not influence fermentation patters, but post acidification was observed, mainly in groups with the highest lutein concentrations. The amount of lutein recovered was different between groups ( $P < 0.05$ ) due to treatment. Some differences ( $P < 0.05$ ) in the same treatment occurred over time, tending to decrease lutein recovery. For all treatments, up to the end of storage, the final amount of lutein characterized the product as a nutritional source of this element. However, lutein recovery in G4 has reached the minimum daily intake recommended by researchers for health benefits. Recovery ranged from  $81.9 \pm 0.76 \cdot 100^{-1} (\text{w} \cdot \text{w}^{-1})$  to  $76.31 \pm 1.07 \cdot 100^{-1} (\text{w} \cdot \text{w}^{-1})$  on the storage period.  $L^*$  has no difference ( $P > 0.05$ ) between groups with different added lutein concentrations. In contrast,  $a^*$ ,  $b^*$ ,  $c^*$ , and YI (yellowness index) were different ( $P < 0.05$ ).

**Keywords:** carotenoids; antioxidant; yogurt; sheep milk; fermentation.

**Practical Application:** Sheep milk yogurt with lutein provides nutritional and functional appeals.

## 1 Introduction

The dairy industry is concerned about developing new high nutritive products to meet the consumers' demand for food enriched in carotenoids and other vitamins and provitamins (Fusaro et al., 2019; Renes et al., 2018). Most consumers much appreciate yogurt over other dairy products because of their sensory properties and higher digestibility than milk (Cruz et al., 2017a).

Furthermore, nutritional value and sensory quality of yogurt are also influenced by milk chemical composition (Kaminarides et al., 2007; Yildiz-Akgul, 2018; Yildiz & Ozcan, 2019). Yogurt manufacture using sheep milk is an exciting approach because of the improvement in nutritional value between other species (Balthazar et al., 2017; Park et al., 2007).

Ovine milk has higher contents of protein, lipids, minerals, and vitamins essential to human health compared with caprine and bovine milk (Barlowska et al., 2011; Kaminarides et al., 2007; Park et al., 2007). Moreover, the structure of sheep milk proteins has smaller micelles and with different partition micellar types than those of cow milk. This fact suggests that both casein and whey proteins in sheep milk can be a useful, convenient alternative for those who are allergic to cow milk (Masoodi & Shafi, 2010).

Kaminarides et al. (2007) and Park et al. (2007) highlight that, beyond the proteins, the sheep milk is more abundant than cow and goat milk in fat, ashes, calcium, iron, manganese, phosphorus, zinc, medium-chain fatty acids, monounsaturated fatty acids, linolenic acid (CLA), all essential amino acids and most of the vitamins. For this reason, sheep milk has high biological value high digestibility, alkalinity, and is hypoallergenic. Thus, yogurt made with sheep milk consequently presents, from a nutritional point of view, beneficial health effects.

Lutein (LT) is a yellow-pigmented macular carotenoid which is an excellent antioxidant that prevents tissues from free radical damages. It interferes with the free radical formation and kidnaps reactive oxygen species (Alves Rodrigues & Shao, 2004; Johnson, 2014; Shami & Moreira, 2004). Also, it protects photoreceptors by filtering out the incidence of high energy blue light, harmful to the macula. A decrease of visual sensitivity in older people with low macular pigment density in the eyes tissues could be the precursor of some eye diseases, including Age-Related Macular Degeneration (AMD) (Johnson, 2014; Madaan et al., 2017; Stringheta et al., 2006). In addition to evidence of a reduced risk of AMD developing, among many beneficial effects can be highlighted the protection against atherosclerosis, cataracts,

Received 30 Dec., 2019

Accepted 27 Jan., 2020

<sup>1</sup>Departamento de Alimentos, Faculdade de Veterinária, Universidade Federal Fluminense – UFF, Niterói, RJ, Brasil

<sup>2</sup>Departamento de Nutrição, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora – UFJF, Juiz de Fora, MG, Brasil

<sup>3</sup>Departamento de Tecnologia de Alimentos, Centro de Ciências Exatas e Tecnológicas, Universidade Federal de Viçosa – UFV, Viçosa, MG, Brasil

<sup>4</sup>Empresa de Pesquisa Agropecuária de Minas Gerais – EPAMIG, Juiz de Fora, MG, Brasil

<sup>5</sup>Núcleo Avançado de Tecnologia de Alimentos e Gestão de Cooperativismo – NATA, São Gonçalo, RJ, Brasil

\*Corresponding author: [raphaelbarros@veterinario.med.br](mailto:raphaelbarros@veterinario.med.br)

cancer and other diseases (Alves Rodrigues & Shao, 2004; Chasan-Taber et al., 1999; Deli et al., 2004; Krinsky & Johnson, 2005; Tokusoglu, 2013; Madaan et al., 2017; Stringheta et al., 2006; Sumantran et al., 2000).

Hammond et al. (1997) demonstrate through their studies that an increased intake of LT generally responds with higher concentrations of these carotenoids in blood serum and maculae. Lutein is found in green leafy vegetables, especially dark leafy such as spinach, kale, watercress, and broccoli (Rodriguez-Amaya, 2001). Since the human body cannot synthesize carotenoids, a balanced diet with the consumption of foods rich in these compounds is required (Stringheta et al., 2006). In addition Wang et al. (2018) demonstrate that lutein content of milk was affected by dietary supplementation of the cows' diet with lutein and antioxidant as Vitamin E, tea polyphenols and ethoxyquin..

The research aimed to evaluate the behavior of different concentrations of lutein added in yogurt manufactured from sheep milk. The study also assessed the effect of lutein on acidity, pH, color, lutein carotenoid degradation, and recovery by spectrophotometer during the storage period, as well as sheep milk characterization.

## 2 Materials and methods

All experiment was repeated three times, besides each analysis has been done at least three times as well. All laboratory analyses were carried out with artificial lights off, in low natural light, to avoid lutein degradation.

### 2.1 Sheep milk as raw material

Sheep milk was collected from the dairy farm and transported at a temperature between 4 °C and 5 °C, to be processed in a pilot-scale dairy factory.

Milk composition and physicochemical properties were analyzed according to Association of Official Analytical Chemists (2012). It was performed triplicate analysis of acidity by titrimetric method (AOAC Official Method 947.05); pH by using a pH probe (pH-meter PG1800, CapLab, São Paulo, Brazil) (AOAC Official Method 973.41); specific gravity at 15.0 °C by termohigrometer method (AOAC Official Method 925.22); solids (total) (AOAC Official Method 925.23); total nitrogen, obtaining the total protein percentage by multiplying the nitrogen percentage by 6.38 (AOAC Official Method 991.20); nonprotein nitrogen (AOAC Official Method 991.21); lipids (AOAC Official Method 2000.18) and ashes (AOAC Official Method 945.46).

Furthermore, non-fat solids were calculated subtracting the total fat (w/w) from total solids (w/w), and the approximately total carbohydrates were calculated subtracting all components from total solids (w/w).

### 2.2 Yogurt culture

Thermophilic lactic acid cultures DVS YC-X11 (Chr. Hansen Laboratories, Denmark), were used at the dosage of 50U in 500L of milk, following the supplier's instructions. Still, the culture was

diluted to 500 mL sterile milk in controlled aseptic conditions, to obtain  $10^{11}$  CFU per gram of inoculum.

### 2.3 Yogurt and samples preparation

Yogurts were made applying the traditional method (Barros et al., 2019; Tamine & Robinson, 2007). The process was modified, as described in Figure 1.

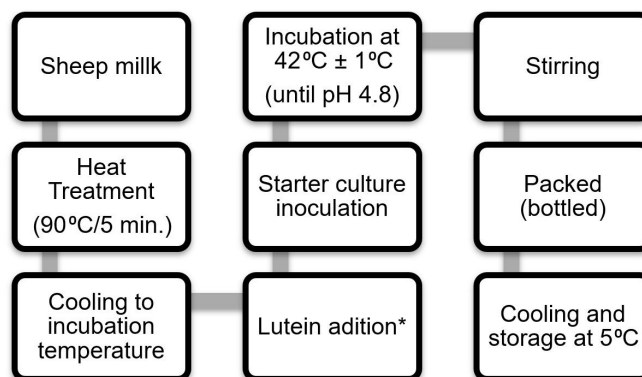
Initially, raw sheep milk was filtered and heat-treated at  $90\text{ °C} \pm 2\text{ °C}$  for five minutes in a Stainless steel double jacketed boiling vat with stirring, followed by cooling to  $42\text{ °C} \pm 1\text{ °C}$ .

Treated milk was divided into four different stainless steel fermentation vats, corresponding to the four experimental groups to prepare the yogurt. First, lutein (LT) was added individually in each vat and homogenized. After that, following the same precautions, one milliliter of the inoculum was added for each 1000 mL of the mixture, reaching a count of  $10^8$  CFU·mL<sup>-1</sup> in the product to initiate the milk fermentation process at  $42\text{ °C} \pm 1\text{ °C}$ , until fermented milk reached pH 4.8 (after approximately a 4 hours incubation).

Upon to achieve the stopping point (ph 4.8), yogurt was stirred (after the fermentation period), and the samples were bottled into opaque 200 mL polyethylene bottles and refrigerated at 5 °C. Four groups (treatments) were formed with different added FloraGLO® Lutein 5% CWS/S-TG (E161b\*) (DSMNutritional Products, Switzerland) concentrations: group 1 (G1): no FloraGLO® added in milk; group 2 (G2): 0.6 mg of FloraGLO® per mL of milk; group 3 (G3): 0.69 mg of FloraGLO® per mL of milk; and group 4 (G4) 0.78 mg of FloraGLO® per mL of milk. The final concentration of the inoculum in the final product was: 0.00 mg of LT·100 mL<sup>-1</sup>; 3.0 mg of LT·100 mL<sup>-1</sup>; 3.45 mg of LT·100 mL<sup>-1</sup>; and 3.9 mg of LT·100 mL<sup>-1</sup>, respectively (considering FloraGLO® Lutein five percent purity).

### 2.4 Physicochemical analyses of yogurt

After twenty-four hours of cooling (day 1), post-manufacture, yogurt composition, and physicochemical properties were determined in triplicate according to Association of Official



**Figure 1.** Flow chart of yogurt production from sheep milk with different lutein contents. The experiment was performed in triplicate. \*G1: 0.00 mg·100 mL<sup>-1</sup> of lutein added; G2: 3.0 mg·100 mL<sup>-1</sup> of lutein added; G3: 3.45 mg·100 mL<sup>-1</sup> of lutein added; G4: 3.9 mg·100 mL<sup>-1</sup> of lutein added.

Analytical Chemists (2012) (acidity, pH, specific gravity, total solids, total nitrogen, nonprotein nitrogen, and ashes). Furthermore, solids not-fat were calculated subtracting fat ( $w \cdot w^{-1}$ ) from total solids ( $w \cdot w^{-1}$ ). The approximately total carbohydrates were calculated subtracting all components from total solids ( $w \cdot w^{-1}$ ). Acidity and pH analysis were also assessed in triplicate during the product shelf life, on days 1<sup>st</sup>, 15<sup>th</sup>, 30<sup>th</sup> and 45<sup>th</sup>, of storage at refrigeration temperature.

## 2.5 Color determination

Color analyses were carried out using a portable measurement instrument CR-400 Croma Meter (Konica-Minolta, USA), previously calibrated with  $Y = 94.2$ ;  $x = 0.3160$ ;  $y = 0.3326$  standard white plate. Color measurements were determined based on the CIELAB coordinates ( $L^*$ ,  $a^*$ ,  $b^*$ ), assessing two-color coordinates,  $a^*$  and  $b^*$ , and the psychometric index of lightness,  $L^*$ . The parameter  $a^*$  displays positive values for reddish colors, and negative values for the greenish ones. The parameter  $b^*$  displays positive values for yellowish colors, and negative values for the bluish ones.  $L^*$  is an approximate measurement of the luminosity. Hue ( $h^*$ ) and saturation ( $c^*$ ) parameters were calculated from  $a^*$  and  $b^*$  values, according to Equations 1 and 2, respectively. Yellowness Index (YI) parameter was calculated according to Equation 3, respectively (Pathare et al., 2013; Vargas et al., 2008). Three readings for each sample were recorded. The equipment was inserted one centimeter into the yogurt sample to perform the analyses.

$$h^* = \tan^{-1} \left( \frac{b^*}{a^*} \right) \quad (1)$$

$$c^* = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

$$YI = \frac{142.86 b^*}{L^*} \quad (3)$$

## 2.6 Extraction and quantification of lutein

The extraction of the lutein was performed in triplicate on the days 1<sup>st</sup>, 15<sup>th</sup>, 30<sup>th</sup> and 45<sup>th</sup>, of storage at refrigeration temperature. The protocol was adapted from Rocha et al. (2017), due to the specific characteristics of the sheep milk. Five milliliters of Ethanol 95·100<sup>-1</sup> ( $v \cdot v^{-1}$ ) (Synth) were added to 5 g of each sample and then centrifuged at 2500 g-force for five minutes. The supernatant was reserved, and another 5 mL of ethanol 95·100<sup>-1</sup> ( $v \cdot v^{-1}$ ) was added to the precipitate and centrifuged. This procedure was repeated four more times. Then, the same procedure was repeated once with 5 mL of Acetone (Synth), and the supernatant also reserved. The extraction was performed by adding 50 mL of acetone to the total supernatant, stirring for one minute, and transferring to a separatory funnel containing 60 mL of Petroleum Ether (Synth). The mixture of ethanol, acetone, LT, and petroleum ether was washed successively with distilled water. The mixture of LT and ether obtained by separatory funnel was collected in a 250 mL round-bottom flask and dried on vacuum rotary evaporator (Tecnal TE-210, Brazil) at 40 °C, then re-suspended in 25 mL (G1 samples) and 75 mL (G2, G3, and G4 samples) of 99.8·100<sup>-1</sup> Absolute Ethanol (Synth). Carotenoid content was determined by Thermo Scientific™ Evolution 60S UV-Visible spectrophotometer (Thermo Scientific) at 445nm, corresponding to

the wavelength of maximum absorption for LT diluted in absolute ethanol, measured in a 1 cm cuvette (Rodriguez-Amaya, 2001). The molar absorption coefficient of 2550·cm<sup>-1</sup>·g<sup>-1</sup> was used, and the total carotenoid content was calculated according to the Equation 4 and 5, expressed as milligrams (mg) of LT per 100 g of yogurt.

$$x(\text{mg}) = \frac{A \cdot y(\text{mL}) \cdot 10^3}{A_{1\text{cm}}^{1\%} \cdot 100} \quad (4)$$

$$x(\text{mg} / 100\text{g}) = \frac{x(\text{mg})}{\text{sample}(\text{g})} \cdot 100 \quad (5)$$

$x$  = lutein content (mg);  $A$  = absorbance at 445 nm;  $A_{1\text{cm}}^{1\%}$  = molar absorptivity coefficient in absolute ethanol;  $y(\text{mL})$  = volumetric flask volume.

Carotenoid content was determined by spectrophotometer at 445nm, corresponding to the wavelength of maximum absorption for LT diluted in absolute ethanol. However, the wavelength for  $\beta$ -carotene diluted in absolute ethanol varies between 425 nm and 478nm. The maximum absorption was at a wavelength of 450 nm (Rodriguez-Amaya, 2001).

## 2.7 Statistical analysis

One-way ANOVA with three replications and three repeated measures of analytical methods was used to evaluate the differences between treatments and their interactions (product composition, pH, titratable acidity, LT content, and recovery), as well as Tukey's test. Statistical significance was set at a 95·100<sup>-1</sup> significance level. All analyses were performed using commercially available statistical package XLSTAT 2013.2.03 (Addinsoft, Paris, France).

## 3 Results and discussion

### 3.1 Sheep milk characterization

Table 1 displays the average results of physicochemical properties, and centesimal analyses of the main components of sheep milk used as experiment raw material. The results of our research agree with other studies (Cruz et al., 2017b; Park et al., 2007).

**Table 1.** Average of main nutrients composition and physicochemical properties of sheep milk used in the experiment.

Composition/Properties	Sheep milk
<b>Total dry extract</b> ( $\cdot 100 \text{ g}^{-1}$ )	18.45 $\pm$ 0.38
<b>Protein</b> ( $\cdot 100 \text{ g}^{-1}$ )	4.00 $\pm$ 0.09
<b>Non-protein nitrogen</b> ( $\cdot 100 \text{ g}^{-1}$ )	0.10 $\pm$ 0.00
<b>Fat</b> ( $\cdot 100 \text{ g}^{-1}$ )	8.8 $\pm$ 0.00
<b>Ash</b> ( $\cdot 100 \text{ g}^{-1}$ )	0.93 $\pm$ 0.02
<b>Carbs**</b> ( $\cdot 100 \text{ g}^{-1}$ )	4.62 $\pm$ 0.43
<b>Density</b> ( $\text{g} \cdot \text{mL}^{-1}$ )	1.037 $\pm$ 0.00
<b>Titratable acidity</b> ( $\text{g lactic acid} \cdot 100^{-1}$ )	0.25 $\pm$ 0.01
<b>pH</b>	6.67 $\pm$ 0.01

Values represent the average of triplicate analysis of milk from three different days  $\pm$  standard deviation. \*\*Carbohydrates were calculated subtracting all components from the total solids.

### 3.2 Centesimal composition of yogurt

Table 2 displays the composition of sheep milk yogurt obtained in the experiment. Based on our results, it is possible to state that the use of different LT concentrations did not affect the composition of the final product since there was no significant difference in composition among the treatments ( $P > 0.05$ ).

### 3.3 Fermentation process and lutein influence

The fermentation process was stopped when pH values reached 4.8. All treatments required the same time to reach the required pH: four hours. However, a slight post-acidification ( $P > 0.05$ ) occurred within the first twenty-four hours of cooling, as verified by the difference between the pH value defined as a stopping point and the pH observed in the following day. Yogurt culture uses lactose as energy source and converts lactose into lactic acid, lowering the pH values and elevating the titrable acidity (TA) values, which allows gel formation. Even at cooling temperatures, this process continues slowly (De Ancos et al., 2000; Vianna et al., 2019). Table 3 shows that one day after the conclusion of the fermenting stage of production, no difference of pH or TA was present among the treatments ( $P > 0.05$ ). This datum points out how LT did not affect sheep milk fermentation until this period, neither positively nor negatively. Vianna et al. (2019) studied yogurts produced with milk of different species. The authors observed that sheep milk yogurt had a higher TA. The high buffering effect of milk from ewe explains the

higher TA values, thus justifying the greater impact on TA results than pH values obtained in the present research.

### 3.4 Colorimetric determinations

Table 3 displays the colorimetric determinations based on the CIELAB coordinates ( $L^*$ ,  $a^*$ ,  $b^*$ ). G1 Lightness ( $L^*$ ) was higher than in the other treatments ( $P < 0.05$ ), confirming lighter color on treatment with no LT added.  $L^*$  values decreased in G2, G3, and G4 gradually, as LT concentration increased (Table 3). However, differences were non-significant. In contrast, there was a significant effect of LT content on  $a^*$  and  $b^*$  parameters. The values increased among treatments ( $P < 0.05$ ), following the increase in the LT level. Values were positive for  $a^*$ , tending to reddish rather than greenish, and for  $b^*$ , tending to yellowish rather than bluish. Studies performed on Prato cheese (ripened Brazilian cheese) added with different LT concentrations displayed similar results: higher  $a^*$  and  $b^*$  values at higher LT concentrations, while  $L^*$  did not change significantly (Kubo et al., 2013; Sobral et al., 2016). Aryana et al. (2006) also report that LT did not affect  $L^*$  in their research with LT in strawberry yogurt, as well as  $a^*$  value increase with the amount of LT. However,  $b^*$  was not significantly affected, probably influenced and hidden by the strawberry color.

The effect of LT addition on yogurt may be evidenced by the tonality, which is represented by the hue parameter (h). An angle of  $0^\circ$  or  $360^\circ$  represents red hue, while angles of  $90^\circ$  represent yellow hues, respectively (Pathare et al., 2013). Our hue values

**Table 2.** Centesimal composition of yogurt manufactured with different added lutein percentage.

Characteristics ( $\cdot 100^{-1}$ ; w·w <sup>-1</sup> )	Treatments			
	G1	G2	G3	G4
Total dry extract	18.58 ± 0.46 <sup>a</sup>	18.75 ± 0.51 <sup>a</sup>	18.38 ± 0.35 <sup>a</sup>	18.24 ± 0.27 <sup>a</sup>
Protein	4.05 ± 0.26 <sup>a</sup>	3.93 ± 0.16 <sup>a</sup>	4.14 ± 0.25 <sup>a</sup>	3.95 ± 0.18 <sup>a</sup>
Non-Protein nitrogen	0.1 ± 0.00 <sup>a</sup>	0.1 ± 0.00 <sup>a</sup>	0.1 ± 0.00 <sup>a</sup>	0.1 ± 0.00 <sup>a</sup>
Fat	8.73 ± 0.09 <sup>a</sup>	8.73 ± 0.09 <sup>a</sup>	8.73 ± 0.09 <sup>a</sup>	8.73 ± 0.09 <sup>a</sup>
Ash	0.92 ± 0.03 <sup>a</sup>	0.92 ± 0.02 <sup>a</sup>	0.92 ± 0.02 <sup>a</sup>	0.91 ± 0.02 <sup>a</sup>
Carbs*	4.79 ± 0.50 <sup>a</sup>	5.08 ± 0.46 <sup>a</sup>	4.50 ± 0.39 <sup>a</sup>	4.55 ± 0.34 <sup>a</sup>

<sup>a-d</sup>Lowercase letters in rows indicate no significant differences among the different treatments,  $P > 0.05$ . Experiments were performed in triplicate with the following triplicate analysis of each yogurt. Values represent the average of nine analyses performed ± standard deviation. G1: 0.00 mg·100 mL<sup>-1</sup> of lutein added; G2: 3.0 mg·100 mL<sup>-1</sup> of lutein added; G3: 3.45 mg·100 mL<sup>-1</sup> of lutein added; G4: 3.9 mg·100 mL<sup>-1</sup> of lutein added. \*Carbohydrates were calculated subtracting all components from the total solids.

**Table 3.** Physicochemical and carotenoids recovery results at first-day post-fermentation (sheep milk yogurt).

Physicochemical properties	G1	G2	G3	G4
<b>pH</b>	4.78 ± 0.07 <sup>a</sup>	4.77 ± 0.07 <sup>a</sup>	4.74 ± 0.02 <sup>a</sup>	4.74 ± 0.04 <sup>a</sup>
<b>Acidity (lactic acid g·100<sup>-1</sup>)</b>	0.99 ± 0.06 <sup>a</sup>	1.01 ± 0.03 <sup>a</sup>	1.06 ± 0.04 <sup>a</sup>	1.05 ± 0.10 <sup>a</sup>
<b>L*</b>	111.82 ± 2.85 <sup>a</sup>	100.05 ± 1.02 <sup>b</sup>	100.00 ± 2.51 <sup>b</sup>	99.04 ± 1.45 <sup>b</sup>
<b>a*</b>	-0.09 ± 0.08 <sup>d</sup>	4.85 ± 0.14 <sup>c</sup>	5.33 ± 0.27 <sup>b</sup>	5.65 ± 0.15 <sup>a</sup>
<b>b*</b>	9.64 ± 0.41 <sup>d</sup>	28.55 ± 0.30 <sup>c</sup>	29.80 ± 0.95 <sup>b</sup>	30.94 ± 0.52 <sup>a</sup>
<b>C*</b>	9.63 ± 0.34 <sup>d</sup>	33.98 ± 0.27 <sup>c</sup>	35.82 ± 0.91 <sup>b</sup>	37.75 ± 0.49 <sup>a</sup>
<b>h*</b>	90.51 ± 0.42 <sup>a</sup>	82.14 ± 0.14 <sup>b</sup>	81.82 ± 0.16 <sup>c</sup>	81.74 ± 0.12 <sup>c</sup>
<b>Yellowness index (YI)</b>	12.31 ± 0.25 <sup>d</sup>	40.77 ± 0.11 <sup>c</sup>	42.56 ± 0.30 <sup>b</sup>	44.63 ± 0.21 <sup>a</sup>
<b>Carotenoids / lutein (mg·100 g<sup>-1</sup>)*</b>	0.02 ± 0.01 <sup>d</sup>	2.45 ± 0.08 <sup>c</sup>	2.86 ± 0.06 <sup>b</sup>	3.16 ± 0.06 <sup>a</sup>

<sup>a-d</sup>Lowercase letters in rows indicate significant differences among the different treatments,  $P < 0.05$ . \*Total carotenoid content was expressed in mg of lutein in 100 g of yogurt. The experiment was performed three times with triplicate analysis of yogurt each. Values represent the average of nine analyses performed ± standard deviation. G1: 0.00 mg·100 mL<sup>-1</sup> of lutein added; G2: 3.0 mg·100 mL<sup>-1</sup> of lutein added; G3: 3.45 mg·100 mL<sup>-1</sup> of lutein added; G4: 3.9 mg·100 mL<sup>-1</sup> of lutein added.



results were equal or very close to 90°. Since from yellow hue in G1 ( $h = 90.51 \pm 0.42$ ), the treatments slight tended towards red hue in G2 and G3 ( $P < 0.05$ ), according to the increase of LT in the product. The latter was  $P > 0.05$  compared to G4 ( $h = 81.74 \pm 0.12$ ). The values of  $C^*$ , which represent color saturation degree and intensity, increased as LT content increased, evidencing the interference of LT in this parameter. Color intensity decreased among the treatments, from G4 to G1, with significant alterations from one treatment to the other ( $P < 0.05$ ). Kubo et al. (2013) and Sobral et al. (2016) are in agreement with our results and describe that the hue values tend to decrease with the LT concentration, starting from yellow representative values to orange representative values. On the other hand, the opposite occurred with saturation ( $C^*$ ), also corroborating to our results, in which values increased with increasing LT levels in Prato cheese.

Finally, the Yellowness Index (YI) indicates the degree of yellowness and is associated with  $b^*$  values. As  $b^*$  increased proportionally with the increase of LT in the product ( $P < 0.05$ ), YI also presented the same behavior ( $P < 0.05$ ).

### 3.5 Lutein content and recovery

According to Park et al. (2007), Sheep milk has high concentrations of Vitamin A compared to other species, due to the higher conversion of beta carotene into vitamin A. The recovery of carotenoid traces (Table 3) in the control treatment (G1) is mainly due to this compound.

Table 3 displays the average values of total carotenoids on the first day of storage (after fermentation) in G2, G3, and G4. The amount of lutein of each treatment agreed with its inclusion. LT values displayed significant differences among the treatments ( $P < 0.05$ ). The average recovery to all treatments with lutein added was  $81.90 \pm 0.76 \cdot 100^{-1}$ .

### 3.6 Titrable acidity, pH and lutein recovery during the storage period

Table 4 and Table 5 show pH and TA values, respectively. In general, pH decreased during storage ( $P < 0.05$ ). At the end of storage, yogurt with higher LT content displayed lower pH values than yogurt with lower LT content ( $P < 0.05$ ). Our data show a possible influence of LT on pH decrease. Further studies under the same conditions and the same lactic matrix are necessary for suspicion to be adequately confirmed.

As the acidity is less affected by the buffering effect, TA values increased during storage ( $P < 0.05$ ) in all treatments, but yogurt with higher LT contents displayed higher acidification than the other samples. Therefore, higher TA values were obtained comparing to yogurt with lower LT contents ( $P < 0.05$ ). For this reason, the same tendency of LT to influence post acidification was observed. Nevertheless, LT did not affect the characteristics of the product, suggesting the possibility to include it as a functional ingredient in the yogurt formulation.

Vianna et al. (2019) observed a significant difference in lactic acid production in sheep milk yogurt during the storage period. However, the pH values remained constant ( $P > 0.05$ ) while the TA values increased ( $P < 0.05$ ), which also justifies our pH and TA results, most likely due to the buffering effect of sheep milk. On the other hand, Aryana et al. (2006) found a pH reduction in strawberry yogurt during storage. Still, the research was based on cow's milk, which may have contributed to this inverse behavior. The authors also found no influence of LT on post acidification during storage, which is contrary to our results despite the particularities of the dairy matrix.

Mora-Gutierrez et al. (2018) researched the interactions between LT and the caseins isolated from bovine and caprine milk. They found LT concentrations higher in the LT-enriched emulsions prepared with caprine caseins compared with the

**Table 4.** pH determination values during 45 days of storage period.

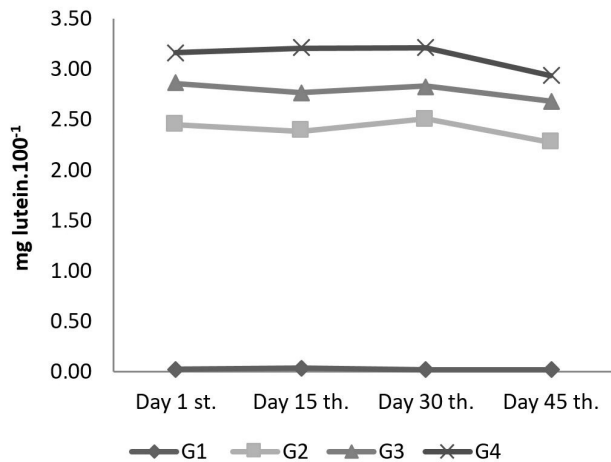
Treatments	Day 1st.	Day 15th.	Day 30th.	Day 45th
G1	$4.78 \pm 0.07^{Aa}$	$4.51 \pm 0.01^{Ab}$	$4.43 \pm 0.01^{Ac}$	$4.32 \pm 0.05^{Bd}$
G2	$4.77 \pm 0.07^{Aa}$	$4.50 \pm 0.03^{Ab}$	$4.44 \pm 0.02^{Abc}$	$4.38 \pm 0.05^{Ac}$
G3	$4.74 \pm 0.01^{Aa}$	$4.41 \pm 0.02^{Bb}$	$4.39 \pm 0.04^{Bb}$	$4.35 \pm 0.02^{ABb}$
G4	$4.74 \pm 0.06^{Aa}$	$4.36 \pm 0.04^{Bb}$	$4.30 \pm 0.03^{Cc}$	$4.23 \pm 0.03^{Cd}$

<sup>a-d</sup>Lowercase letters in rows indicate significant differences among different days in the treatment,  $P < 0.05$ . <sup>A-D</sup>Capital letters in the columns indicate significant differences among the different treatments,  $P < 0.05$ . The experiment was performed three times with triplicate analysis of yogurt each. Values represent the average of nine analyses performed  $\pm$  standard deviation. G1: 0.00 mg  $\cdot 100 \text{ mL}^{-1}$  of lutein added; G2: 3.0 mg  $\cdot 100 \text{ mL}^{-1}$  of lutein added; G3: 3.45 mg  $\cdot 100 \text{ mL}^{-1}$  of lutein added; G4: 3.9 mg  $\cdot 100 \text{ mL}^{-1}$  of lutein added.

**Table 5.** Titrable acidity measured values during 45 days of storage period.

Treatments	Day 1st. (mg.lactic acid $\cdot 100^{-1}$ )	Day 15th. (mg.lactic acid $\cdot 100^{-1}$ )	Day 30th. (mg.lactic acid $\cdot 100^{-1}$ )	Day 45th (mg.lactic acid $\cdot 100^{-1}$ )
G1	$0.99 \pm 0.10^{Ab}$	$1.08 \pm 0.11^{Ab}$	$1.21 \pm 0.07^{Ba}$	$1.27 \pm 0.01^{Ba}$
G2	$1.01 \pm 0.08^{Ab}$	$1.09 \pm 0.13^{Ab}$	$1.23 \pm 0.07^{ABa}$	$1.28 \pm 0.01^{Ba}$
G3	$1.06 \pm 0.08^{Ac}$	$1.16 \pm 0.13^{Abc}$	$1.24 \pm 0.08^{ABab}$	$1.30 \pm 0.01^{ABa}$
G4	$1.05 \pm 0.11^{Ac}$	$1.21 \pm 0.09^{Ab}$	$1.32 \pm 0.07^{Aab}$	$1.63 \pm 0.07^{Aa}$

<sup>a-d</sup>Lowercase letters in rows indicate significant differences among different days in the treatment,  $P < 0.05$ . <sup>A-D</sup>Capital letters in the columns indicate significant differences among the different treatments,  $P < 0.05$ . The experiment was performed three times with triplicate analysis of yogurt each. Values represent the average of nine analyses performed  $\pm$  standard deviation. G1: 0.00 mg  $\cdot 100 \text{ mL}^{-1}$  of lutein added; G2: 3.0 mg  $\cdot 100 \text{ mL}^{-1}$  of lutein added; G3: 3.45 mg  $\cdot 100 \text{ mL}^{-1}$  of lutein added; G4: 3.9 mg  $\cdot 100 \text{ mL}^{-1}$  of lutein added.



**Figure 2.** Treatments behavior of total carotenoids (lutein) recovery during 45 days of yogurt storage period. The experiment was performed three times with triplicate analysis of yogurt each. Values represent the average of nine analyses performed. G1: 0.00 mg · 100 mL<sup>-1</sup> (m · v<sup>-1</sup>) of lutein added; G2: 3.0 mg · 100 mL<sup>-1</sup> (m · v<sup>-1</sup>) of lutein added; G3: 3.45 mg · 100 mL<sup>-1</sup> (m · v<sup>-1</sup>) of lutein added; G4: 3.9 mg · 100 mL<sup>-1</sup> (m · v<sup>-1</sup>) of lutein added.

bovine casein, even during storage. Caprine and sheep milk are similar in most characteristics (Masoodi & Shafi, 2010; Park et al., 2007). Thus, this significant interaction should also occur with sheep milk in order to contribute to previously unknown behaviors, since protein is directly related to buffer effect (Vianna et al., 2019) and also acidity (Rocha et al., 2017).

The behavior of LT recovery, as main carotenoids in sheep milk yogurt, during storage is shown in Figure 2. Except in G1 that only traces of carotenoids were recovered, probably  $\beta$ -carotene residues (Rodríguez-Amaya, 2001), all following treatments had recovery ranging from  $81.9 \pm 0.76 \cdot 100^{-1}$  to  $76.31 \pm 1.07 \cdot 100^{-1}$  throughout storage. At the beginning of storage, the recovery was higher compared to its end ( $P > 0.05$ ), showing a tendency of recovery decline over the days ( $P > 0.05$ ). At the end of storage, LT content was different ( $P < 0.05$ ) between each treatment. The amounts recovery were  $2.3 \pm 0.01$  mg LT · 100<sup>-1</sup> mL of yogurt in G2,  $2.7 \pm 0.08$  mg LT · 100<sup>-1</sup> mL of yogurt in G3 and  $3.0 \pm 0.06$  mg LT · 100<sup>-1</sup> mL of yogurt in G4, which the last one reached the minimum daily intake recommended by researchers for health benefits, considering 200 mL per portion.

There is no official Recommended Dietary Allowance (RDA) or Recommended Daily Intake (RDI) of LT; however, the amount added was based on data indicating a 20 mg limit as safe for daily LT consumption (Agência Nacional de Vigilância Sanitária, 2019; Shao & Hathcock, 2006), and through studies showing the biological effects of this bioactive compound when consumed at minimal concentration of 6 mg per day (Abdel-Aal et al., 2013; Seddon et al., 1994).

According to Aryana et al. (2006), who evaluated LT behavior into yogurt, in all treatments with different initial LT content, there was a small yet steady decline in LT content over the storage time of yogurt. A small amount of LT breakdown may explain this slight decline in LT over storage over storage time.

LT has been incorporated into many food matrices. Even the same, as LT is sensitive to heat, light, and other oxidative stressors, it is not always assured that carotenoid molecule remains intact after food processing due to the low stability outside their natural tissue. Therefore, some loss of LT in products is to be expected (Martínez-Delgado et al., 2017).

Despite all the arguments regarding the reduction of LT content recovered during storage, the amount in all treatments of the present research is considered as a functional food, aiming to increase carotenoid consumption for health benefit effects. A lutein ingredient as a functional ingredient is important to the lacteal industry as it is an option to make products commercially appealing, however further studies should be conducted focusing on additional sensory methods such as projective tests (Judacewski et al., 2019; Pinto et al., 2018) and sensory properties (Chetachukwu et al., 2019).

## 4 Conclusions

Lutein did not influence fermentation patters, but post acidification was observed, mainly in groups with the highest lutein concentrations. Lightness was not influenced by lutein concentration. However, the two-color coordinates,  $a^*$  and  $b^*$ , as well as hue angle, saturation, and yellowness index, were affected by different concentrations of lutein. For all treatments, up to the end of storage, the final amount of lutein characterized the product as a nutritional source of this element. However, greater emphasis should be given to the product with 7.8 mg of LT per portion (200 mL), which has reached the minimum daily intake recommended by many researchers for health benefits. Sheep's milk was an excellent product for lutein incorporation, providing a product with nutritional and functional appeal, as well as adequate visual characteristics.

## Acknowledgements

Authors are grateful to the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior” (CAPES) - this study was financed in part by the CAPES - Brasil Finance Code 001, “Pró Reitoria de Pesquisa, Pós Graduação e Inovação” of Universidade Federal Fluminense (PROPPI-UFF) and veterinary medicine post-graduate program (Higiene Veterinária e Processamento Tecnológico de Produtos de Origem Animal) for financial support of this research; the “Instituto de Laticínios Candido Tostes” for allowing the manufacture of the samples in dairy pilot-scale dairy factory; the DSM Nutritional Products for supplying the commercial lutein; the Cabanha Vida farm for supplying the sheep milk; and AG Plast for supplying the polypropylene packaging.

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