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Research Article

Effect of Nutritional System on the Degree of Antioxidant Protection of Bovine Milk

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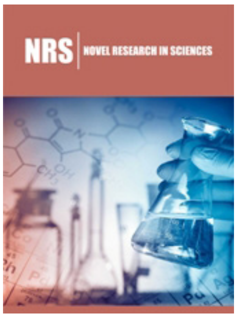

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Abstract

Due to a high obesity incidence within the Mexican population (75% of individuals), Mexican residents are prone to higher chronic degenerative diseases like COVID-19, with 6,448,447 total of reported cases-326,504 deaths in a population of 131,192,333 inhabitants, ranking as 16th on the world chart (June 2022). On the other hand, it's known that milk can provide antioxidants that help prevent diseases associated with cellular oxidation (that may well favor COVID infections); therefore, the main objective was to determine it was measured the degree of antioxidant protection in cow's milk, based on alpha-tocopherol and cholesterol innards including milk samples from grazing, partial grazing and zero grazing cows to study and identify tracing parameters correlated to the feeding system. Alpha-tocopherol and cholesterol were analyzed by HPLC on a normal phase column and were confronted to calculate the Degree of Antioxidant Protection (DAP). This parameter, expressed as a molar ratio between antioxidant compounds and an oxidation target, is useful for tracing and distinguishing products from grazing and zero-grazing animals, divided into three different production systems: (Grazing (G), Supplemented Grazing (SG) and Full Confinement (FC). In treatment G, cholesterol concentration was reduced during the month of May; unlike SG, where the amount of this metabolite increased during the same period; and in treatment FC, where it remained constant for three months. The concentration of alpha-tocopherol in milk increased from 170 to 179.04µg/100g in the month of May for grazing animals (G), decreased from 115.25 to 105.45µg/100g, and from 94.23 to 77.74µg/100g for SG and FC treatments respectively. The animals were kept in a humid tropical area, and the level of cholesterol in these cows' milk during the dry period was higher ($p<0.05$) than those animals fed with diets rich in concentrates (25.22mg/100g), compared to those that consumed a greater amount of forage (G: 15.63mg/100g, PS: 21.44mg/100g). The level of alpha-tocopherol in milk was higher ($p<0.05$) in grazing milk (173.02µg/100g), compared to SG: 109.83µg/100g and FC: 83.95µg/100g. The Degree of Antioxidant Protection (DAP) increased significantly between treatments, with the highest value being observed in G (11.17), followed by SG (5.13) and FC (3.42) groups. It is concluded that there is an effect of the feeding system that directly influences the degree of antioxidant protection in milk, and this effect being greater in milk that comes from grazing cows.

Keywords: Cardiovascular diseases; Oxidative stress; Enzyme system; Pesticides

Introduction

Oxidative stress

Oxidative stress has been defined as the imbalance between the presence of Reactive Oxygen/Nitrogen Species (ROS/RNS), and the body's ability to counteract their actions through the antioxidant protection system [1-3]. In scientific literature, oxidative stress has been described as the product of an increase of ROS/RNS with a decrease in antioxidant protection ability, characterized by a reduction in the capacity of endogenous systems to combat oxidative attacks directed at biomolecular targets. Its severity is associated with several pathologies such as: cardiovascular diseases, cancer, diabetes, atherosclerosis, etc., [3,4]. It has been proven that oxidative stress is related to more than 100 diseases, either as a primary cause or as an associated factor, producing immune deficiency and facilitating COVID infections and/or death [5-7]. It is an irreversible process of decay in the organism product of reactive oxygen species, which also expresses its negative influence on the physiology of aging, disabling physiological defense functions, and promoting disease occurrence and reducing life span [2]. Oxidative stress is understood as the production of an excessive amount of ROS/RNS in the organism, which is the product of an imbalance between the generation and destruction of ROS/RNS. As a consequence, oxidative stress is the repercussion of a production increase of free radicals, but also of a reduction in the antioxidant defense system [8]. Substances reactive to oxygen or nitrogen (ROS and RNS) should not only be seen as species that cause biomolecular damage affecting the enzyme system (chemical defense or detoxification), but also the cellular response system to molecular signals, and therefore modifying biosynthetic reactions [3]. Oxidative stress is due to free radical production such as: hydrogen peroxide (H_2O_2), superoxide (O_2), free oxygen ($1/2O_2$) and Hydroxyl Radicals (OH); with some acquired exogenously, and others from metabolic processes such as: cellular respiration, exposure to microbial infections that produce phagocytic activation, during intense physical activity; or by the action of polluting substances such as cigarette smoke, alcohol, ultraviolet ionization, pesticides, coronavirus infection and ozone [8]. Previous works show how the substances most susceptible to oxidation are polyunsaturated fats especially arachidonic acid and docosahexaenoic acid, which produce malondialdehyde and 4-hydroxynonenal, recognized markers of lipid oxidation decline [9]. The lipid oxidation also produces aldehydes that affect proteins and can impede their functions [8]. Oxidative damage to lipid membrane components has been related to mechanisms of neurodegeneration, cancer, cardiovascular or inflammatory diseases. It has been confirmed that the excessive production of reactive oxygen species can lead to the overexpression of oncogenic genes, or the formation of mutagenic compounds that can cause proatherogenic activity and is related to the appearance of senile plaque or inflammation [10].

Antioxidants in milk

The concept of biological antioxidants has been defined as any compound that, when present at a lower concentration compared to that of an oxidizable substrate, is capable of delaying or preventing oxidation of the substrate [5,9]. Antioxidant functions involve reducing oxidative stress-DNA protection against malignant transformations as well as other parameters of cell damage. Epidemiological studies have demonstrated the ability of antioxidants to contain the effects of reactive oxygen species activity and decrease the incidence of cancers and other degenerative diseases [11]. It has been confirmed that, under physiological conditions, the balance between oxidant and antioxidant compounds moderately favors the oxidants, thus producing a slight oxidative stress, overcoming the intervention of the organism's endogenous antioxidant systems [12]. This issue of oxidative stress becomes more acute with age when the endogenous antioxidants and repair systems cannot effectively counteract it. For this reason, supplementation of antioxidants into a diet is convenient, and it can be achieved in the form of alpha-tocopherol and beta-carotene, which can be obtained from grazing products such as milk [13,14]. Antioxidants, like vitamin D and E, carotenoids and poliphenolics (phenolic acids such as benzoic and hydroxybenzoic acids, cinnamic and hydroxycinnamic acid derivatives, and flavonoids-flavonols, flavans, flavanones, flavanols, flavones, and anthocyanidins such as anthocyanin aglycones (flavil or skeleton of 2-phenylchromenyl ions),and tannins, are currently being considered as the main exogenous antioxidants. Their abundant presence in plants and grass make food for grazing cows an important source of these beneficial compounds for health [15].

In recent research works, it has been shown that livestock production systems that are managed in grazing can have a positive impact on the health of the population, through the consumption of dairy products rich in omega 3, with an adequate balance of lower omega 6 at a ratio of 4:1 [7]. Its importance in relation to human health is documented, with a decrease in cholesterol [16]. On the other hand, its antioxidant capacity, containing alpha tocopherol, can be considered as functional foods and/or as a source of nutraceutical compounds [14]. It becomes especially relevant when people currently have health problems associated with cellular oxidative stress, diseases such as Parkinson's, Alzheimer's, Huntington's disease, emphysema, cardiovascular diseases, cancer, and diabetes [10]. Previously degree of antioxidant protection values greater than 7.0×10^{-3} were found in samples from grazing goats, and values lower than 7.0×10^{-3} were found in samples from zero-grazing goats, for both milk and cheese, meaning that cholesterol was highly protected against oxidative reactions, when herbage was the only feed or was dominant in the goat diet. The DAP reliability to measure the antioxidant protection of cholesterol appeared more effective when the feeding system was based on grazing, or when cut herbage was utilized indoors by animals [15]. The DAP index was able to distinguish dairy products when the grazed herbage in the goats' diet exceeded 15%. This has led to the development of techniques or equations to determine the degree of a food's antioxidant protection (GPA). This parameter's efficacy to distinguish milk and cheese from grazing and full confinement animals was discussed by [15], proving to be a useful methodology to determine the origin of dairy products in relation to grazing.

Methods

Milk sampling was carried out in 30 production units located in the municipalities of Balancán and Tenosique in the state of Tabasco, which were divided into units that established their feeding based on Grazing (G), grazing+concentrate (GS) and Full Confinement (FC); having 10 units with P, 10 units with PS and 10 with ES. Sampling was carried out during the dry season in Tabasco, which includes the months of March, April and May of 2019, taking samples every 28 days with three samples per unit. The animals were bovine crosses of Bos indicus x Bos Taurus, with different numbers of lactations, and in different periods of lactation. The animals were milked once a day. Milk samples (1.5

l/ranch) were taken from the general tank. These samples were transported in refrigerators with ice to later be frozen at -20 °C and stored until their saponification process. Alkaline saponification (Fletouris Method) of 0.2 grams per sample was taken and mixed with 5 milliliters of KOH (1M) dissolved in methanol. It was shaken thoroughly for 15 seconds in a vortex. It was heated from 60 to 80 °C for 15 minutes with stirring intervals every 5 minutes for 15 seconds. Afterwards, it was allowed to cool, and 1milliliter of distilled water and 5 milliliters of hexane were added. Hydrolyzed or saponified samples were shaken for 1 minute and centrifuged for 1 minute at 2000-3000rpm. The n-hexane fraction [17] was recovered and filtered through a Polyvinylidene Fluoride (PVDF) membrane with a pore diameter of 0.45μm and concentrated by means of a stream of nitrogen gas. Resuspending was performed in acetonitrile: 2-propanol (95:5v/v) used as mobile phase.

HPLC analysis

A High-Performance Liquid Chromatography (HPLC, Agilen) with UV detector and autosampler, equipped with a binary pump, and a C8 RP column (ZORBAX Eclipse, XDB C8, 4.6x150mm 5μm) was used. A volume of 20μL of sample was injected, the solvent flow rate and the oven temperature were 2mL/min and 50 °C, respectively, and a wavelength of 210nm. A calibration curve was prepared using cholesterol (Sigma-Aldrich) from 0 to 1000μg as standard, resuspended in the mobile phase and a running time of 5min. For α-tocopherol determination present in the samples, the methodology described above was applied, in this case the wavelength was to be 292nm and an oven temperature of 35 °C. A calibration curve of 0 to 1000μg of α-tocopherol was be used, which was be resuspended in the mobile phase and a running time of 6min. With cholesterol and alpha-tocopherol values, the equation proposed by [15]was carried out.

Degree of antioxidant protection (DAP)

$$DAP = \frac{\sum_{i=1}^{n}\left(MAn^{0}moli\right)_{i}}{\left(BOn^{0}moli\right)}$$

Where:

MA is the molar part of the antioxidant molecule (alpha tocopherol) BO is the molar part of the oxidizing molecule (cholesterol) [15] For instance, α-tocopherol, β-carotene, and polyphenols represent a natural and particularly efficient antioxidant system in extra virgin olive oil, whereas linoleic acid, an oxidizable unsaturated fatty acid, can be selected as the oxidation target. Their combination in the DAP parameter is useful as a predictive index of oil sensitivity to oxidation during storage [18]. In goat dairy products, only α-tocopherol was selected as the antioxidant because of the absence of detectable levels of β-carotene in goat's milk, and cholesterol was the oxidation target because of the low content of oxidizable unsaturated fatty acids in milk fat [19,20]. Moreover, even if less easily oxidizable than unsaturated fatty acids, cholesterol is a molecule usually charged, especially in the oxidized form; cholesterol oxides are responsible for heart disease in humans [21,22]. The effectiveness of the DAP parameter to distinguish milk and cheese from grazing and zero-grazing animals was evaluated in experiments 1, 2, and 3; DAP's relationship with actual herbage intake was evaluated in experiment 4.

Statistical analysis

The experimental model was carried out under a completely randomized design, using a one-factor analysis of variance test and its Tukey tests with the Excel 2019 program.

Results

Results showed the cholesterol levels, alpha-tocopherol, and the degree of antioxidant protection (DAP) were mostly constant among the farms sampled inside the same system (Tables 1-3). In (Table 2) it was summarized the cholesterol, alpha tocopherol, and degree of antioxidant protection in several samples from various ranches, in different nutritional offered, the grazing feeding system was the lowest in cholesterol and the highest in alpha tocopherol as demonstrated. In table it was summarized cholesterol, alpha tocopherol and degree of antioxidant protection among ranches, differences are showed among feeding system, significant cholesterol levels could be seined among feeding systems. In treatment G, the concentration of cholesterol was reduced in the month of May, unlike SG where the amount of this metabolite increased in the same period (Table 4), and in FC treatment where it remained constant for three months. In (Table 5) showed seasonal average alpha-tocopherol changes among in the three systems. Concentration of alpha-tocopherol in milk increased from 170 to 179.04μg/100g in the month of May for grazing animals (P), decreasing from 115.25 to 105.45μg/100g and from 94.23 to 77.74μg/100g for PS and E treatments respectively (Table 5). Similarly, the cholesterol level in cow milk in the humid tropics during the dry period was higher ($p<0.05$) in those animals fed under stable systems with diets rich in starches (25.22mg/100g), compared to with those who consumed a greater number of forages (Grazing (15.63mg/100g), Grazing+Supplement (21.44mg/100g) (Figure 1). Regarding the results for the levels of alpha-tocopherol in milk (Figure 2) was higher ($p<0.05$) in the milk whose origin was the grazing system (173.02μg/100g), compared to that which came from feeding that involved ingredients rich in starches (SG: 109.83μg/100g, E: 83.95μg/100g). In the present study, the degree of antioxidant protection (DAP) had a statistically significant increase between treatments (Figure 3), with the highest value being observed in Grazing (11.17), followed by the SG (5.13) and full confinement (3.42) groups.

Table 1: Levels of cholesterol, alpha-tocopherol, and degree of antioxidant protection (DAP) in milk from cows from different feeding systems (P1, P2, etc.) in the municipalities of Balancán and Tenosique in the state of Tabasco, Mexico.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Cholesterol (mg/100g)	14.8	18.1	14.4	14.5	16.4	13.2	15.0	15.3	17.4	16.7
Alfa tocopherol (µg/100g)	179.2	171	163.7	170.5	175.1	176.7	160.7	185.3	176.7	170.9
DAP	12.0	9.4	11.3	11.7	10.6	13.3	10.7	12.0	10.1	10.1

Table 2: Levels of cholesterol, alpha-tocopherol and degree of antioxidant protection (DAP) in milk from cows from different ranches (PS1, PS2, etc.) under supplemented grazing systems (SG).

	SG1	SG2	SG4	SG4	SG5	SG6	SG7	SG8	SG9	SG10
Cholesterol (mg/100g)	21.0	21.4	22.1	22.2	20.2	21.1	20.7	21.5	22.0	21.7
Alfa tocopherol (µg/100g)	109.3	109.2	108.9	109.7	111.8	109.8	108.1	108.8	109.7	112.6
DPA	5.1	5.0	4.9	4.9	5.5	5.2	5.2	5.0	4.9	5.1

Table 3: Levels of cholesterol, alpha-tocopherol and degree of antioxidant protection (DAP) in milk from cows from different location (FC1, FC2, etc.) under full confinement (FC).

	FC1	FC2	FC3	FC4	FC5	FC6	FC7	FC8	FC9	FC10
Cholesterol (mg/100g)	24.7	19.3	21.4	18.4	25.8	29.9	30.4	27.4	28.1	26.4
Alpha tocopherol (µg/100g)	82.9	82.3	84.6	84.7	83	82.6	83.4	84.6	85.9	85.2
DAP	3.3	4.2	3.9	4.6	3.2	2.7	2.7	3.0	3.0	3.2

Table 4: Average levels of cholesterol in cow's milk in March, April, and May (mg/100g).

	March	April	May	Average
Grazing	17.26±0.26	15.42±0.44	14.23±0.34	15.63
Supplemented grazing	20.45±0.24	21.45±0.32	22.44±0.25	21.44
Full confinement	25.48±0.76	25.43±0.77	24.76±0.75	25.22

Table 5: Average levels of alpha-tocopherol in cows' milk in March, April, and May (µg/100g).

	March	April	May	Average
Grazing	170.00±1.99	170.04±1.56	179.04±1.17	173.02
Supplemented grazing	115.25±0.60	108.80±0.54	105.45±0.67	109.83
Full confinement	94.23±0.64	79.90±0.43	77.74±0.27	83.95

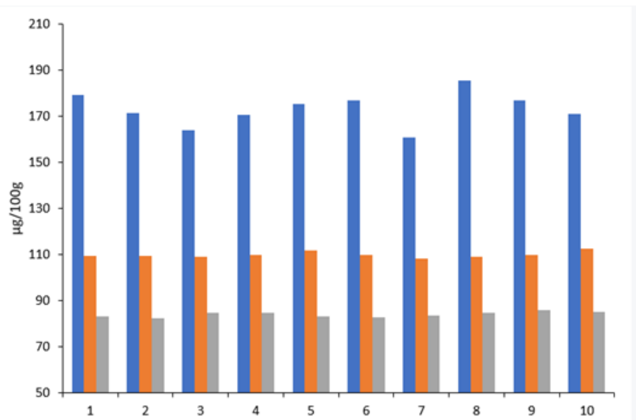

Figure 1: Cholesterol in samples from grazing, grazing plus and supplemented cows. Blue cholesterol supplemented; Brown grazing plus; Black total grazing.

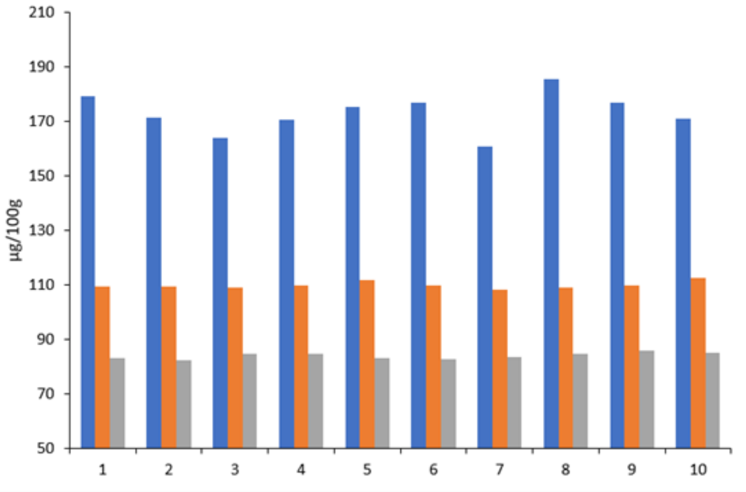

Figure 2: Alpha-tocopherol in samples from grazing, grazing plus and supplemented cows. Blue cholesterol supplemented; Brown grazing plus; Black total grazing.

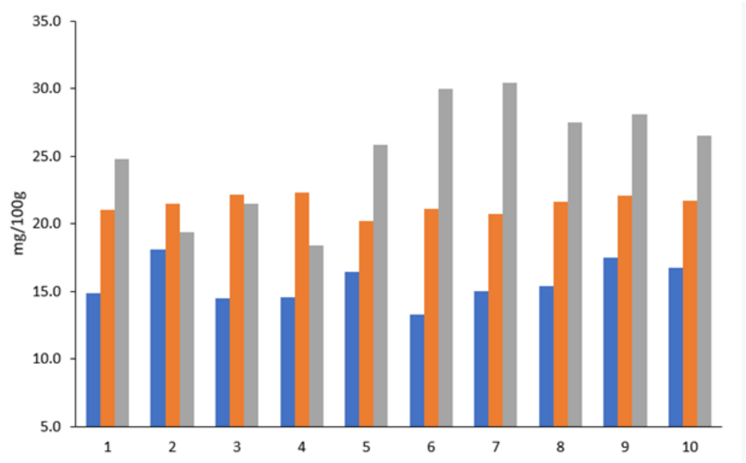

Figure 3: DAP, degree of antioxidant protection in samples from grazing, grazing plus and supplemented feeding systems cows. Blue cholesterol supplemented; Brown grazing plus; Black total grazing.

Discussion

Herbage intake was estimated on the basis of herbage mass, measured on 2×2m of pasture before and after grazing. The real intake of grazed herbage for each group was 0 (control fed on commercial concentrate), 450, 600, and 1,100g of DM/d, and the contribution of grazing to the diet in each treatment was calculated as a percentage of the maximum actual herbage intake [from 0g/d (0% grazing) to 1,100g/d (100% grazing), [23]. The linear equation (y=a+bx) fits the experimental points with a high correlation coefficient (R_2=0.97) and can be utilized as an analytical calibration curve [15]. Replacing the variable y with the DAP threshold value, able to differentiate "pasture" products from "stabled" ones (DAP=7.0 exp-3), the x value can be calculated. This value (15%) can be considered the limit of DAP detection if the same amounts of freshly cut pasture herbage were ingested indoors, and not grazed by animals, the linear fitting of DAP values vs. the amount of cut herbage intake would show a lower correlation value (R_2=cheese commercial quality), contributing to price setting and probably to animal well-being. Moreover, the DAP parameter was found to be related to oxidative reactions, the main determinant of quality loss in food: the higher the DAP value, the greater the product stability and safety, considering the risk related to the intake of cholesterol-oxides in humans as discussed by Pizzoferrato et al. (2017) and [18]. In Tabasco México, grazing showed the highest DAP when compared with two management systems as full confinement and supplemented grazing [24]; results are in agreement with previous data obtained in another study carried out in goat cheese by [21], where they reported data with the same trends, having in their work four treatments in which the GPA of goat's milk and cheese was evaluated, obtaining higher values in the treatments where neither grains nor commercial concentrates were offered: DAP were 11.3 on full grazing, 9.8 supplemented grazing, supplemented grazing with concentrate, 10 cows in each observation were feed full commercial concentrates in confinement [25]. Pizzoferrato

[15] reported that no significant differences were found in their samples in cholesterol levels in goat milk, with cholesterol values of 14.7 for grazing, 11.8 for supplemented grazing, 12.8 for grazing with commercial supplement and 12.8 in full confinement. In the present study, the cholesterol levels in cow's milk were: 15.63 in grazing, 21.44 in grazing+supplement and 25.22 in confinement, showing a slight increase in cholesterol between the different treatments being higher in full confinement, this difference could be due to the milk source, since those researchers carried out his study on goat's milk.

The data obtained from alpha-tocopherol in this work demonstrated significant differences in the treatments where there is supplementation, having an average of 173.03 in grazing, 109.83 in supplemented grazing and 83.96 in full confinement. Similar results to those reported by [15] in goat milk where the highest value of alpha-tocopherol is 154.9 in grazing, 118.9 in supplemented grazing, 125.2 in grazing with concentrate and 77.1 in full confinement. In the study by [15], samples were obtained for three years, and during these three years, the same trend was followed in the data for cholesterol, alpha-tocopherol and FPG. Similarly, in a study by Andrea [23] in Italy reported a higher amount of vitamin E, but also a higher amount of cholesterol in the milk of goats fed a diet high in forage. In this study, two feeding treatments were made: the first treatment determined a diet with low forage coverage, and I call it LH (low herbage cover), and the second treatment HH (high herbage cover) was a diet with high forage coverage and obtained results in HH of 3.66 and LH of 3.52 in vitamin E, showing that the feeding of goats with high concentrations of forage contained greater amounts of this vitamin. In the case of cholesterol, higher amounts were obtained in the HH treatment, with values of 314, and in the LH treatment 294 were obtained. They also performed the GPA of their data, and a higher GPA was obtained in the second treatment 11.66 for HH and 11.97 for LH The foregoing can be explained by the presence of forage, in their case they used hay forage, a process that alters the antioxidant profile due to the plant's desiccation, this being reported by [26] where they obtained greater amount of alpha-tocopherol in diets with more than 40% green forage: This coincides with the similar parameters from this work since the cows were grazed with fresh forage, thus obtaining the best conditions to produce milk with high concentrations of alpha-tocopherol, and therefore have a higher GPA (Degree of Antioxidant Protection) as discussed by [4]. Another study conducted by [27] studied the degree of antioxidant protection of organic milk, reporting that their indoor feeding data showed the lowest values compared to grass-fed groups, and the groups fed with grass+corn grain in the concentration of vitamin E, A and β-carotene. The Degree of Antioxidant Protection showed an increasing trend in organic milk. Grazing+maize feeding was associated with the highest level of FPG (9% higher than grass-only feeding and 79% higher than indoor feeding) demonstrating a very similar trend to that found by [23] in his study in Italy, and that we attribute to feeding the animals with hay forage; however, the prevalence follows in all reported cases where diets based on fresh forage denote a higher GPA. It is worth mentioning that, in the study by [27] their forage+corn feeding treatment was only supplemented with 4kg. of corn to balance the diet to the cow's energy needs, and no commercial concentrate was offered [28-30].

Conclusion

In this research we successfully developed the DAP parameter, which was used to differentiate products from grazing and zero-grazing feeding systems, provided that grazed herbage exceeded 15% in the animals that were in the total diet group. In fact, the grazing action, not just the herbage intake cut from pasture, is a quality determinant for commercial milk and cheese, contributing to price setting and probably to animal well-being. The present observation showed a direct effect of the feeding system on the cholesterol and alpha-tocopherol concentration in cow's milk, influencing the degree of antioxidant protection of the product, this effect was significant higher when animals were feed only in pasture where not supplementation was added, particularly when concentrates are offered in high volumes to the diet.

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