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Polyphenols as Antioxidants in Sheep Cheese Measured by Superoxide Anion

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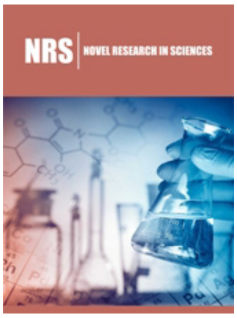

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Abstract

The world, regarding COVID-19, recorded 693,666,294 cases and 6,908,586 deaths according to Worldometer. Due to the high incidence of obesity in Mexico (75%), Mexicans are more prone to COVID-19 due to obesity and being overweight leading to chronic degenerative diseases, with a total of 7,633,355 cases of COVID-19, and 334,366 deaths in a population of 131,562,772 inhabitants -2,541 deaths per 1 million- ranking 18th in the world (as of August 2023), compared to the United States that came first with 107,797,340 cases, and 1,172,433 demises, 502 deaths per million, in a population of 333,805,269. Of the diseased, 76% showed chronic degenerative diseases that could have been prevented with foods rich in antioxidants. Sheep cheese in Mexico comes mainly from animals in full confinement fed a high degree of concentrates (45%) of their diet, but a small percentage also comes from grazing. We studied 10 farms from seven different Mexican states that used both feeding methods. The objective of the present work was to compare polyphenol quantities in both feeding systems. It's known that cheese can provide antioxidants that could help to prevent diseases associated with cellular oxidation (favoring deterrence of COVID-19 infections for instance). Subsequently, the goal was to determine the degree of antioxidant protection in ewes cheese, based on the superoxide anion contents, or differences among cholesterol and alpha-tocopherol including samples from zero grazing. Polyphenols are expressed as a molar ratio between antioxidant compounds and an oxidation target, distinguishing products from grazing and zero-grazing animals, divided into two different production systems: grazing (G), and confinement (FC). In the G treatment, cholesterol concentration lowered during the month of September (high-grazing season) and remained constant when ewes pastured. The concentration of alpha-tocopherol in milk increased from 270.09 to 279.04µg/100g for grazing animals (G) and, decreased from 215.25 to 205.45µg/100g when the grasses have begun to mature, increasing their fibrositis compared with ewes fed diets rich in concentrates, more than 45% of the daily intake (25.22mg/100g) with 12.8 to 11.7µg//100g. The level of alpha-tocopherol in milk was higher ($p<0.05$) in grazing milk 279.04µg/100g, compared to FC: 77.74µg/100g the highest recorded among both systems. The degree of antioxidant protection (DAP) increased significantly between treatments, with the highest value being observed in G (11.17) compared with the FC (3.42) group. It's concluded that there is an effect of the feeding system that directly influences the degree of antioxidant protection in milk and therefore cheese. This effect is greater in milk that comes from grazing sheep, directly measured by the superoxide anion or indirectly by cholesterol versus alpha-tocopherol.

Keywords: Cardiovascular diseases; oxidative stress; COVID-19

Introduction

The concept of a biological antioxidant has been self-defined as any compound that, when present at a concentration lower than that of an oxidizable substrate, is capable of delaying or preventing the oxidation of the substrate (Halliwell et al., 1992; Godic et al., 2014). The antioxidant functions involve the reduction of oxidative stress - protection of DNA against malignant transformations, as well as other cell damage. Epidemiological studies have demonstrated the ability of antioxidants to contain the effects of reactive oxygen species activity, therefore decreasing the incidence of cancers and other chronic degenerative diseases (Cheesman., Slater, 1993).

It has been verified that, under physiological conditions, the balance between oxidant and antioxidant compounds moderately favors oxidants, thus producing a slight oxidative stress, overcoming the intervention of the endogenous antioxidant systems of the organism (Droge, 2002). This problem of oxidative stress becomes more acute with age when endogenous antioxidants and repair systems can´t effectively counteract it. Thus, antioxidant supplementation is convenient, and it can be obtained from polyphenols, alpha-tocopherol, and beta-carotene; gotten from grazing products such as cheese (Galina, et al., 2016; 2017; 2023).

Antioxidants, such as vitamins D, E, carotenoids, phenolic acids, benzoic and hydroxybenzoic acids, cinnamic and hydroxycinnamic acid derivatives, and flavonoids, flavanols, flavans, flavanones, flavones, and anthocyanidins such as anthocyanin aglycones), known as polyphenols, currently considered to have an antioxidant protection degree values higher than 7.0×10^{-3}, were found in samples from grazing animals. In addition, values lower than 7.0×10^{-3} in samples from zero grazing goats, both in milk and cheese, were recorded, which means that cholesterol was highly protected against oxidative reactions when grass was the only food or was dominant in the ewe's diet, the same effect that we have measured in goats, (Galina et al., 2023). Cholesterol antioxidants seemed more effective when grazing (Galina et al., 2003). The DAP index was able to distinguish dairy products when cropped grass in the diet exceeded 15%. This has led to measuring the Degree of Antioxidant Protection (DPA) of food. The efficacy of this parameter to distinguish milk and cheese from animals under grazing and complete confinement was discussed by Pizzoferrato et al., (2007), providing a useful methodology to determine the origin of dairy products based on ruminant grazing.

Oxidative stress

In milk, the intrinsic properties of Reactive Oxygen Species (ROS) are the mechanisms for biological oxidation, therefore regulating many signal transduction pathways by directly reacting and modifying the structure of proteins, transcription factors, and genes. ROS are involved in regulating the activity of enzymes, mediating inflammation by stimulating cytokine production, signaling cell growth, differentiation, and eliminating pathogens plus removing foreign particles. ROS mainly includes superoxide ions (O_2-), single oxygen ($1O_2$) hydrogen peroxide (H_2O_2) and per oxynitrate ($ONOO^-$) all are signaling molecules as inflammatory mediators. A moderate increase in ROS can facilitate cell metabolism and differentiation, whereas the persistent generation of excess ROS might result in molecular oxidative damage, including lipid peroxidation, protein degradation, and DNA fragmentation, progressing in cells and organs, eventually producing various diseases, such neurodegenerative illness, atherosclerosis, diabetes, cancer, and aging (Liochev, 2013). 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 (Pearson et., al. 2014; Maulik et al., 2013; Lopez-Alarcona., De Nicola, 2013).

Previously, 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-19 infections and/or death (Halliwell, et al., 1992; Gutteridge, 1993; Galina et al., 2019). 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 defense functions, promoting disease occurrence and reducing life span (Maulik et al., 2013).

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. Therefore, oxidative stress is the repercussion of a production increase of free radicals, but also of a reduction in the antioxidant defense system (Poljsak et al., 2013). 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 (López-Alarcona., De Nicola, 2013).

Oxidative stress is due to free radical production such as: hydrogen peroxide (H_2O_2), superoxide (O_2), free oxygen (1/2 O_2), and hydroxyl radicals (OH); with some acquired exogenously, and others from metabolic processes such as: cellular respiration, exposure to microbial infections producing 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 (Poljsak et al. al., 2013).

Previous works show how the substances most susceptible to oxidation are polyunsaturated fats especially arachnoid acid and docosahexaenoic acid, which produce malondialdehyde and 4-hydroxynonenal, recognized markers of lipid oxidation decline (Godic et al., 2014). The lipid oxidation also produces aldehydes that affect proteins and can impede their functions (Poljsak et al., 2013). 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 are related to the appearance of senile plaque or inflammation (Pisoschi., Pop, 2015).

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 (Halliwell et al., 1992; Godic et al., 2014). 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 (Cheesman., Slater, 1993).

Previously, 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 (Droge, 2002). Therefore, oxidative stress becomes more acute with age when the endogenous antioxidants and repair systems cannot effectively counteract it. Thus, supplementation of antioxidants into a diet could be convenient, and can be achieved in the form of alpha-tocopherols, and beta-carotenes of poyphenols, which can be obtained from grazing ewe´s milk (Galina, et al., 2016; 2017).

Antioxidants, like vitamin D and E, carotenoids and polyphenols are currently being considered as the main exogenous antioxidants. Clinical studies have shown a diet rich in fruits, vegetables, and whole grains; and their abundant presence in plants and grass makes food for grazing goats an important source of these beneficial compounds for health (Pizzoferrato et al., 2007).

Recent research work showed 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 (Claps et al., 2014). Its importance in relation to human health is documented, inhibiting arterial thrombosis (Leiber, et al., 2011). On the other hand, its antioxidant capacity, containing alpha-tocopherol, can be considered functional foods and/or as a source of nutraceutical compounds (Galina, et al., 2017). It becomes especially relevant when people currently have health problems associated with cellular oxidative stress, diseases such as Parkinson's, Alzheimer's, Huntington's diseases, emphysema, cardiovascular diseases, cancer, and diabetes (Psiochi., Pop, 2015).

Quantity degrees values of antioxidant protection greater than 7.0×10^{-3} were found in samples from grazing goats, and lower than 7.0×10^{-3} were observed in zero-grazing, 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's 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 eaten indoors by animals. The DAP index was able to distinguish dairy products when the grazed herbage in the goats' diet exceeded 15% (Pizzoferrato et al., 2007).

This has led to the development of techniques or equations to determine the Degree of a Food's Antioxidant Protection (DAP). This parameter's efficacy to distinguish milk and cheese from grazing and full confinement animals was discussed by Pizzoferrato et al., (2007), proving to be a useful methodology to determine the origin of dairy products in relation to grazing.

Material and Methods

The experiment was carried out on ewe's cheese 10 farms (total of 345 dairy sheep) in Mexico for two years: 2021 and 2022. The sampling was carried out on farms from Querétaro, Guanajuato, and México state in Mexico, in each farm births were grouped, thus, most ewes had the same milking days; The stages of lactation considered were from 30 to 60 days after delivery and 90 to 110 days postpartum. The farms were grouped according to their feeding system: Grazing (101 ewes), and ET (44.6%, 244 heads) grazed on Lolium perennial, *Cynodon niemfluensis, Muhlenbergia robusta, Brachiaria brizantha, B. decumbens and Echinochloa Polystachya*. The animals in G were permanently grazing while those in ET were supplemented with 400 g/d with commercial balanced concentrates (18% CP). The animals in the ET group were fed with corn silage, alfalfa hay and commercial balanced concentrate (16% CP; 5 to 7kg/head/day).

Analysis of fatty acids. Lyophilized samples of frozen sheep cheese were soxhlet extracted with petroleum ether/ethyl ether (1:1v/v) to characterize the fatty acid composition. Fatty acid methyl esters were separated using a gas chromatograph equipped with a flame ionization detector and a split injector (1:24). Separations were performed using a capillary column (25cm x 0.2mm i.d. x 0.3um). The fatty acid content was expressed as a percentage. After determination, the sum of saturated, monounsaturated, polyunsaturated fatty acids, as well as the content of mono-saturated fatty acids was calculated using Method 923.07 (AOAC, 2000). From each heard samples were processed in duplicate, one from each year

Milk samples (.200ml/ranch) were taken. These samples were transported in refrigerators with ice to later be frozen at -20 °C and stored until their saponification process. The superoxide anion was determined by spectrophotometry, and incubation of milk and 10μL of nitroblue tetrazolium in a 1mg/L solution, for 30 minutes in a dark environment, luPo, 50μl of dimethyl sulfoxide and 50μl of 2M sodium hydroxide were added. The absorption of the sample was measured at 600nm (Marchesino et al., 2017).

Degree of Antioxidant Protection (DAP) was calculated with Pizzaferrato's et al., 2000;2007 methodology.

Statistical analysis. Total milk or cheese production and milk or cheese composition were analyzed using a one-way ANOVA design. Data analysis was carried out using general linear model procedures (Statgraphics-Centurion).

Result

Results showed cholesterol levels, alpha-tocopherol, and the degree of antioxidant protection were mostly constant among the farms sampled inside the same system (Table 1-4). Ten samples from different farms were taken: 5 from 2021 and the others from 2022.

Table 1: Composition of a mixture of grasses and full confinement feeds.

	Grazing	Full Confinement
Dry matter	100	100
Humidity	0	0
Ether extract	2.5	2
Ashes	5.5	6
Crude Protein	14	16.5
Crude Fiber	66	25.1
Nitrogen-free extract	12	50.4

In Table 1 composition of both diets were shown, and protein was higher in full confinement compared to a more crude fiber when sheep were in pasture. In treatment G, the concentration of cholesterol was reduced during the month of May, unlike SG where the amount of this metabolite increased in the same period, and in FC treatment where it remained constant for three months.

Table 2: Levels of polyphenols with super oxidide ion, and degree of antioxidant protection (DAP) in milk from sheep from different grazing systems (P1, P2, etc.).

	F1	F2	F3	F4	F5	G1	G2	G3	G4	G5
CHOLESTEROL (µg/100g)	42.8	52.1	51.4	53.5	52.4	11.2	13	11.3	12.4	11.7
ALFATOCOPHEROL (µg/100g)	182.2	181	173.7	167.5	165,1	266.7	268.7	275.3	266.7	260.9
DAP	4	4.4	5.3	5.7	5.6	13.3	12.7	14	12.1	13.1

First five 2021, second five 2022.

F= Full confinement

P= Grazing

Table 3: Levels of cholesterol, alpha-tocopherol, and degree of Antioxidant Protection (DAP) in milk from sheep from different locations (FC1, FC2, etc.) under full confinement (FC).

	FC1	FC2	FC3	FC4	FC5	FC6	FC7	FC8	FC9	FC10
CHOLESTEROL (µg/100g)	127.1	121.3	123.4	118.4	122.8	128.9	137.4	129.4	128.1	114.3
ALFATOCOPHEROL (µg/100g)	312.9	301.3	295.6	284.7	283	282.6	283.4	279,0	290,2	285.2
DAP	2.4	2.4	2.5	2.4	2.3	2.2	2	2.16	2.2	2.5

Table 4: Levels of cholesterol, alpha-tocopherol, and degree of antioxidant protection (DAP) in milk from sheep from different locations (FC1, FC2, etc.) under grazing (G).

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
CHOLESTEROL (µg/100g)	27.1	21.3	23.4	18.4	22.8	28.9	27.4	29.4	28.1	27.4
ALFATOCOPHEROL (µg/100g)	282.9	293.3	284.6	284.7	277	272.6	283.4	279,0	290,2	285.2
DAP	10.4	13,9	12.3	15.7	12.5	9.7	10.4	9.6	10.3	10.5

Similarly, the cholesterol level in sheep milk in the humid tropics during the dry period was higher ($p<0.05$)

Regarding the results for alpha-tocopherol levels in milk, that were higher ($p<0.05$) in the milk whose origin was the grazing system (273.02µg/100g), compared to those which came from feeding that involved ingredients rich in starches (FC: 28.3.95µg/100g).

In the present study, the degree of antioxidant protection (DAP) had a statistically significant increase between treatments with the highest value being observed in grazing (13.9), and full confinement groups (2.92).

Discussion Forage intake was estimated based on forage mass, measured in 2 × 2m of pasture before and after grazing. The real consumption of grazed forage was 0 (control fed with commercial concentrate), 450, 600 and 1100 g of DM/d, and the contribution of grazing to the diet in each treatment was calculated as a percentage of the maximum real consumption of forage [from 0g/d (0% grazing) to 1100g/d (100% grazing), (Cabiddu., et al., 2017).

The linear equation ($y=a+bx$) fits the experimental points with a high correlation coefficient ($R2=0.97$) and could be utilized as an analytical calibration curve. 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 could be calculated. This value (15%) can be considered the limit of DAP detection if the same amounts of freshly cut pasture herbage that 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 (R2=cheese commercial quality), contributing to price setting and probably to the animal's 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; Esti et al., 2004).

In México, grazing showed the highest DAP when compared to the other management systems offered with different degrees of concentrate in the diet, DAP diminished when concentrate was administered (Priolo et al., 2004); results are in agreement with previous data obtained in another study carried out in goat cheese by Pizzoferrato et al., in Italy (2007), and Galina et al., (2023) where they reported data with the same trends, having in their work four treatments in which the DAP of sheep's cheese was evaluated, obtaining higher values in the treatments where neither grains nor commercial concentrates were offered: DAP were 11.3 on full grazing compared to 3.1 in FC were they were fed full commercial concentrates in confinement (Shingfi et al 2013).

On the other hand, Pizzoferrato et al., in 2007 reported that no significant differences were found in their samples in cholesterol levels in goat milk, with 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 ewe's milk were: 23.8 in grazing, and 125.2

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 ewe's milk, being similar to the results presented above.

The data obtained from alpha-tocopherol in this work demonstrated significant differences in the treatments where there is supplementation, having an average of 290.3 in grazing, and 83.96 in full confinement. Similar results to those reported by Pizzoferrato et al., in 2007 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 Pizzoferrato et al., in 2007, 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, a study by Cabiddu et. al., in 2017 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 (LH), and the second treatment HH (high herbage cover diet). Results were 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 DAP of their data, and a higher DAP was obtained in the second treatment 11.66 for HH and 11.97 for LH.

Conclusions: In this investigation, a decrease in DAP was demonstrated, which was used to differentiate the products of the grazing and zero grazing feeding systems, provided that the grazed grass exceeded 15% in the animals that were in the total diet group. In fact, the action of grazing, not just the consumption of forage cut from grazing, is a determinant of the quality of commercial milk and cheese, contributing to pricing and probably to animal welfare. In addition, it was found that the DAP parameter is related to oxidative reactions, the main determinant of food quality loss: the higher the DAP value, the greater the stability and safety of the product, considering the risk related to the intake of carbon oxides. cholesterol in humans.