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Milk biofortification may promote health benefits in institutionalized older people

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ABSTRACT

The aim of this study was to measure the changes caused by biofortified milk enriched with selenium, vitamin E and/or improved fatty acid profile in biochemical parameters of institutionalized older people. 132 institutionalized older persons were divided into four groups who received the following treatments: C= Control milk with 3.98 µmol/l vitamin E, 0.37 µmol/l selenium, 2.24 mmol/l CLA; A= Biofortified milk with 5.05 µmol/l vitamin E, 3.63 µmol/l selenium, 6.57 mmol/l CLA; O= Biofortified milk with 4.28 µmol/l vitamin E, 0.20 µmol/l selenium, 5.71 mmol/l CLA and AO=5.21 µmol/l vitamin E, 4.42 µmol/l selenium, 6.85 mmol/l CLA for 12 weeks. Consumption of AO or O milk decreased total cholesterol in 6.8 % (p=0.0429) and LDL in 10.6 % (p=0.0292). Participants who consumed O milk had a higher HDL (42.6±1.2 mg/dl) than those who consumed A (37.7±1.2 mg/dl, p=0.0047). The consumption of biofortified milk with selenium, vitamin E and CLA, resulted in a better lipid profile and higher plasma antioxidant levels in institutionalized elderly.

Keywords: aging, nutrients, milk, dietary biofortication

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Introduction

Healthy eating is a challenge to modern man and a concern for a large part of the world population. The main study about food trends have identified a number of healthy foods [1]. Recent studies have demonstrated that the consumption of milk and dairy products is associated with better cardiovascular health, reduced metabolic syndrome in obese individuals [2,3], weight loss with preservation of muscle mass, and reduced incidence of some cancers [4,5,6].

Studies in older people have reported marginal nutrient intake, which increases the risk of malnutrition and subclinical nutrient deficiencies that affect life functions and quality of life [7,8]. Institutionalized older people, in particular, are at high risk for undernutrition, due to immune dysregulation, loss of fat-free mass, increased incidence of chronic diseases, social isolation, physical inactivity, changes in diet, low appetite, low intake and monotone diet [9,10]. In addition, changes in metabolism typical of advanced age, including lower energy requirements, loss of lean body mass and lower physical activity contribute to these nutritional changes. In this context, milk provides high amounts of nutrients in proportion to energy and is easy to be consumed.

The present study aimed to verify the effect of milk biofortificated with selenium, vitamin E and conjugated linoleic acid [CLA] on selected serum biochemical markers of health in institutionalized older adults. Milk biofortification through changes in the cows' diet, brings advantages not only from the market perspective, but also from the public health standpoint, given the importance of milk and its excellent nutritional value [11].

4. Methods

The study was approved by the Human Research Ethics Committee respectively [*Comissão de Ética Humana do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto* - HCFMRP/USP – number 4921/2011]. This trial was registered at ClinicalTrials.gov as NCT02980094.

4.1 Study Design

All participants and their family members were contacted in presentations made in the *Sociedade Espírita Cinco de Setembro* [*Casa do Vovô* and *Casa da Amizade*] in Ribeirão Preto, Brazil, when the purposes and methods of the study were presented and residents were invited to participate.

A total of 132 participants aged 60 years or over of both sexes were recruited in August 2012, and followed up from September to November, [12 weeks] [12-13]. All participants had previously undergone clinical and biochemical examinations after they consented to participate in the study. Table 1 shows the general characteristics of the groups and there were no significant differences between them. The participants were divided into four groups to receive one of the biofortified milk.

The milk was offered three times a day, at 7:30 h [200 mL], 15:00 [200 mL] and 20:00 h [200 mL], comprising 20%-30% of daily energy consumption [14]. The milk for each group had the following nutritional characteristics: C = Control milk with 3.98 µmol/l vitamin E, 0.37 µmol/l selenium, 2.24 mmol/l CLA; A = Biofortified milk with 5.05 µmol/l vitamin E, 3.63 µmol/l selenium, 6.57 mmol/l CLA; O = Biofortified milk with 4.28 µmol/l vitamin E, 0.20 µmol/l selenium, 5.71 mmol/l CLA and AO = 5.21 µmol/l vitamin E, 4.42 µmol/l selenium, 6.85 mmol/l CLA.

The biofortified milk used in the human experiment originated from an animal science study conducted simultaneously at the Experimental Farm of Animal Science Institute [IZ] in Ribeirao Preto, Brazil. The cow's study was described previously [Salles et al, 2019] [15]. Nutritional composition of pasteurized milk produced by cows fed diets with sunflower oil, selenium and vitamin E and offered to the elderly during the experiment is shown in Table 2.

4.2 Selection of Participants

Inclusion criteria were individuals aged 60 years or over, living in one of two homes for the aged – *Sociedade Espírita Cinco de Setembro* [*Casa*

do Vovô and *Casa da Amizade*] in Ribeirão Preto, Brazil, who agreed to participate in the study. When there was cognitive deficit, caregivers were asked to allow participation. Exclusion criteria included inability to drink, lactose intolerance, use of enteral diets, decompensated endocrine disorders [diabetes, hypothyroidism], autoimmune disorders, infections, allergies or inflammation in the two weeks prior to blood collection.

4.3 Data Analysis

Blood samples were collected at baseline [week 0], and at week 12. Baseline was considered the third week of consumption of the control milk by all the participants [adaptation period].

4.4 Biochemical analysis

Biochemical analyses of blood samples were performed for total cholesterol and its fractions, triglycerides, and uric acid using the enzymatic colorimetric method described by Henry [1995] [16]; glucose and creatinine by the automated kinetic Jaffe method; urea and protein-C reactive by the automated, enzymatic UV method by scatter laser light as described by Henry [1995] [16].

4.5 Analysis of vitamin E

Separation of tocopherols in blood serum was performed by high-performance liquid chromatography [HPLC] using a HPLC System 110 [Hewlett Packard / Agilent Technologies, Germany] in the Food Analysis Laboratory of the School of Food Engineering, UNICAMP, Campinas, Brazil. The analysis was performed as described by Pinheiro-Sant'Ana et al. [2011] [17]. Blood plasma samples were prepared according to the method validated by Korchazhkina et al., 2006 [18], and Arnaud et al, 1991 [19], respectively.

4.6 Analysis of selenium

Selenium in the milk was analyzed by wet digestion with nitric-perchloric mixture and subsequent fluorometric reading in the Mineral Laboratory of the FZEA / USP, according to the methodology proposed by Olson et al. [1975] [20]. Serum concentrations of selenium and standard reference [21] were measured using graphite furnace atomic absorption spectrometry [AA 6200 atomic absorption spectrometer, Shimadzu, Japan]. The analysis was performed at the Mass Spectrometry Laboratory of the Ribeirao Preto Medical School, University of Sao Paulo [FMRP-USP].

4.7 Analysis of fatty acids

Blood serum samples were analyzed in triplicate. Total lipids were extracted from milk by the Folch method [22], and lipid extraction by direct transesterification as described by Lepage and Roy [1984] [23]. Analyses were performed in the mass spectrometry laboratory of FMRP-USP. Fatty acid concentrations are expressed in $mmol.L^{-1}$. The nutritional quality of the milk lipid fraction was assessed using the atherogenicity index, thrombogenicity index and the ratio of hypocholesterolemic to hypercholesterolemic fatty acids [15].

After extraction, total fatty acids were determined by gas chromatography [SHIMADZU, GC-2010] equipped with an AOC-20i automated injector, capillary column [SF.S.CAP.SP -2560, reference number 24056, lot number 190166D].

4.8 Analysis of milk samples

Four milk samples of each treatment were analyzed for vitamin E concentration as described in section 4.6. Three milk samples of each treatment were analyzed for selenium concentration as described in section 4.6 and for fatty acids concentration as described in section 4.7 [15]. All the milk samples were analyzed in triplicate [15].

5.0 Statistical analysis

The categorical variables, expressed in number and percentage of the participants, were analyzed by the chi-square test. For the variable age, mean and standard deviation was analyzed by the ANOVA model. The other data were expressed in estimate mean and standard error.

Variables [before and after] were analyzed by the ANOVA model including fixed effects of

Antioxidant, Oil, Antioxidant*Oil. The other variables were analyzed with the application of a model with treatments in a 2x2 factorial scheme [Antioxidant: With or Without; Sunflower Oil: With or Without] in a completely randomized design, with two repeated measures [before, after] in the same individuals. The ANOVA model included fixed effects of Antioxidant, Oil, Antioxidant*Oil, Time, Antioxidant*Time, Oil*Time and Antioxidant*Oil*Time. In the results tables the name O and oil effect were used to represent the biofortified milks with CLA and nutritional quality of the lipid fraction of milk from cows supplemented with sunflower oil [all biofortified milk had better AI, TI, and h / H values than control milk]. All the assumptions of the model were tested using the SAS/LAB module [version SAS 9.3]. When necessary, variable transformations were performed to guarantee the normality of the data, and all withdrawals from outliers indicated by the module were evaluated. The statistical analyses were then performed using the proc mixed [SAS 9.3] with an unstructured covariance matrix [R = UN]. A p-value < 0.05 was considered as statistically significant and a trend when 0.05 > P ≤ 0.10.

Table 1. General characteristics of the groups of elderly participants studied by treatment.

	Treatment[1]				
Categorical variables	**AO**	**O**	**A**	**C**	***p-value[2]***
Number of participants	24	25	25	26	
Diabetes					
Yes	4 [17.0%]	5 [20.0%]	5 [19.2%]	5 [19.2%]	0.237
Smoker					
Yes	4 [17.0%]	4 [16.0%]	4 [15.4%]	3 [12.0%]	0.106
Systemic Arterial Hypertension					
Yes	14 [58.3%]	15 [60.0%]	11 [42.3%]	9 [34.6%]	0.211
Gender					
Women	13 [54.0%]	15 [60.0%]	13 [52.0%]	17 [65.0%]	0.177
Men	11 [46.0%]	10 [40.0%]	12 [48.0%]	9 [35.0%]	
Continuous variable	mean [SD][3]	mean [SD]	mean [SD]	mean [SD]	
Age [years]	76.0 [9.4]	81.0 [9.7]	79.0 [10.4]	78.0 [9.7]	0.277

[1] AO: milk biofortified with antioxidants [vitamin E and selenium] plus CLA; O: milk biofortified with CLA; A: milk biofortified with antioxidants [vitamin E and selenium]; C: control milk; [2] Anova test used for variable age and the others in chi-square test; [3] SD: standard deviation

Table 2. Nutritional composition of the pasteurized milk produced by cows fed diets with sunflower oil, selenium and vitamin E and offered to the elderly during the experiment.

	Biofortificated milks[2]			**Control Milk[2]**
Nutrients[1]	**AO**	**A**	**O**	**C**
Selenium [umol/l, n=3]	4.42±0.20	3.63±0.34	0.20±0.03	0.37±0.08
Vitamin E [umol/l, n=4]	5.21±0.50	5.05±0.14	4.28±2.88	3.98±0.20
Fatty acids [mmol/l, n=3]				
9c, 11t-18:2	6.85±0.10	6.57±2.01	5.71±1.72	2.24±0.01
18:0	35.28±6.62	33.27±8.23	27.50±11.70	15.09±3.99
9c-18:1	63.60±5.48	64.22±7.75	49.69±5.48	33.36±7.75
Saturated [mmol/l]	27.71±1.67	26.50±3.97	27.21±6.52	17.62±6.12
Mono [mmol/l]	11.72±0.18	10.72±1.34	8.32±3.19	6.96±1.55
Poly [mmol/l]	3.17±0.39	2.47±0.56	2.19±0.71	2.79±0.32
Nutritional quality of the lipid fraction of milk				
AI	2.87±0.27	3.57±0.09	3.22±0.28	3.94±0.68
TI	3.76±0.15	4.62±0.16	4.23±0.28	4.76±1.14
h/H	0.79±0.07	0.63±0.00	0.69±0.05	0.62±0.11

[1] 9c, 11t-18:2: conjugated linoleic acid [CLA]; 18:0: stearic acid; 9c-18:1: oleic acid; Mono: monounsaturated fatty acids; Poly: polyunsaturated fatty acid; AI = atherogenicity index; TI = thrombogenicity index; h/H = ratio of hypocholesterolemic to hypercholesterolemic fatty acids; [2] AO: milk biofortified with antioxidants [vitamin E and selenium] plus CLA; O: milk biofortified with CLA; A: milk biofortified with antioxidants [vitamin E and selenium]; C: control milk

Table 3. Serum lipid profile and biochemical parameters in the elderly who consumed control and biofortified milk during the experimental period.

Items	Treatments[2]					Collection time			*p-value*[4]			
[mg/dl]	AO	O	A	C	SEM[3]	Basal	12 weeks	SEM	A	O	A x O	Time
TC	165.75	159.80	174.93	174.42	5.92	173.91	163.54	3.10	0.5799	0.0429	0.6402	<.0001
HDL	42.60	38.61	37.75	40.45	1.20	43.04	36.66	0.72	0.5901	0.2069	0.0060	<.0001
LDL	97.51	96.32	108.75	108.02	5.29	106.07	99.23	2.82	0.8537	0.0292	0.9641	0.0035
TGL	136.45	128.81	144.82	133.10	9.50	135.30	136.29	5.44	0.3160	0.5116	0.8323	0.8184
VLDL	26.24	24.71	27.76	25.57	1.66	24.84	27.31	1.00	0.3160	0.5200	0.8560	0.0058
Glucose	91.09	85.68	96.67	94.09	6.15	95.02	88.75	3.24	0.5103	0.2498	0.8149	0.0305
Uric acid	4.05	4.40	4.71	4.38	0.27	4.49	4.28	0.14	0.9821	0.2414	0.2233	0.0408
Urea	41.31	45.46	47.93	45.23	3.62	43.73	46.23	2.22	0.8387	0.3691	0.3368	0.1952
Creatinine	0.98	1.03	1.07	1.00	0.07	1.02	1.02	0.03	0.8637	0.6590	0.3287	0.8403
PTNc	10.63	7.14	7.80	10.31	2.86	6.31	11.63	2.32	0.8501	0.7966	0.6984	0.0015

[1]TC: total cholesterol; HDL: high density lipoprotein cholesterol; LDL: low density lipoprotein cholesterol; TGL: triglycerides; VLDL: very low-density lipoprotein cholesterol; PTNc: protein-C reactive; [2] AO: milk biofortified with antioxidants [vitamin E and selenium] plus CLA; O: milk biofortified with CLA; A: milk biofortified with antioxidants [vitamin E and selenium]; C: control milk; [3] SEM: mean standard error; [4] A: antioxidant effect, O: effect of the fatty acid profile of milk, AxO: interaction effect antioxidant and fatty acid profile of milk; Time: time effect

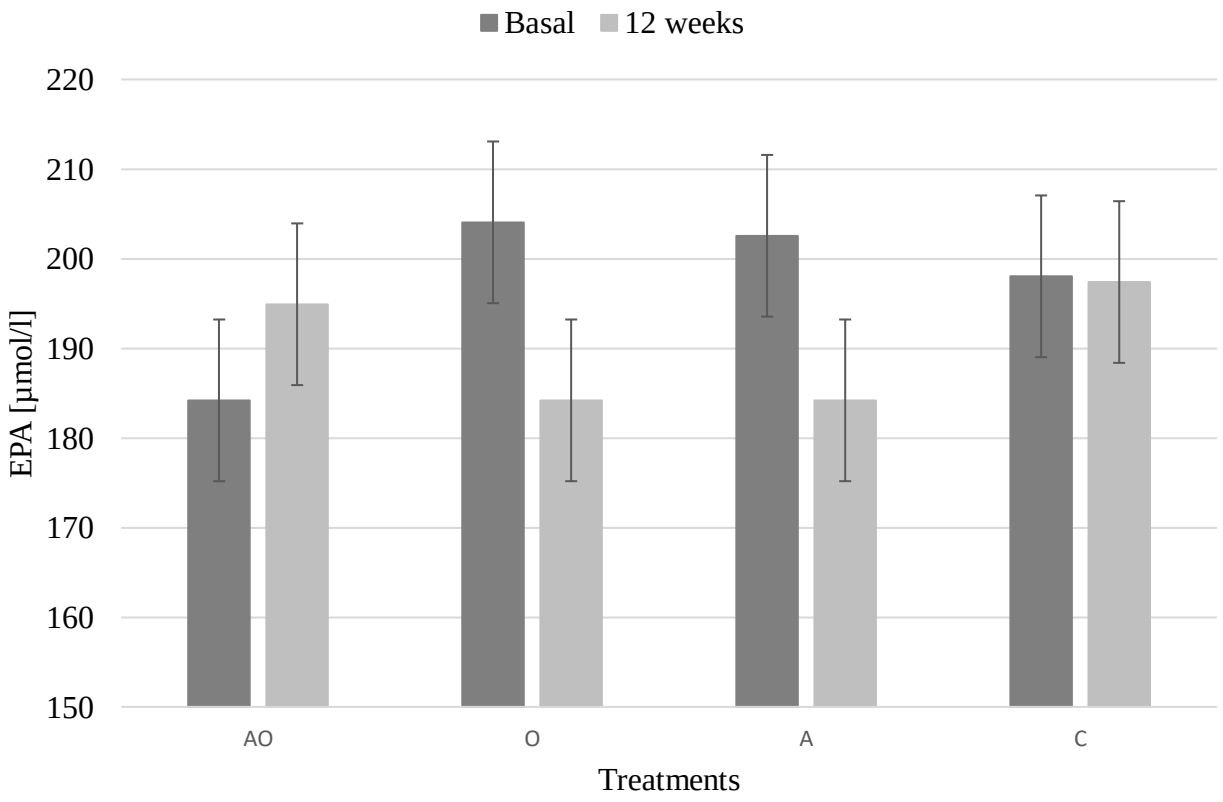

Figure 1. Eicosapentaenoic acid in serum [antioxidante vs oil vs time interation effect, p = 0.048, SEM = 9.02, AO: milk biofortified with antioxidants [vitamin E and selenium] plus CLA; O: milk biofortified with CLA; A: milk biofortified with antioxidants [vitamin E and selenium]; C: control milk] of the elderly supplemented with biofortified or not biofortified milk.

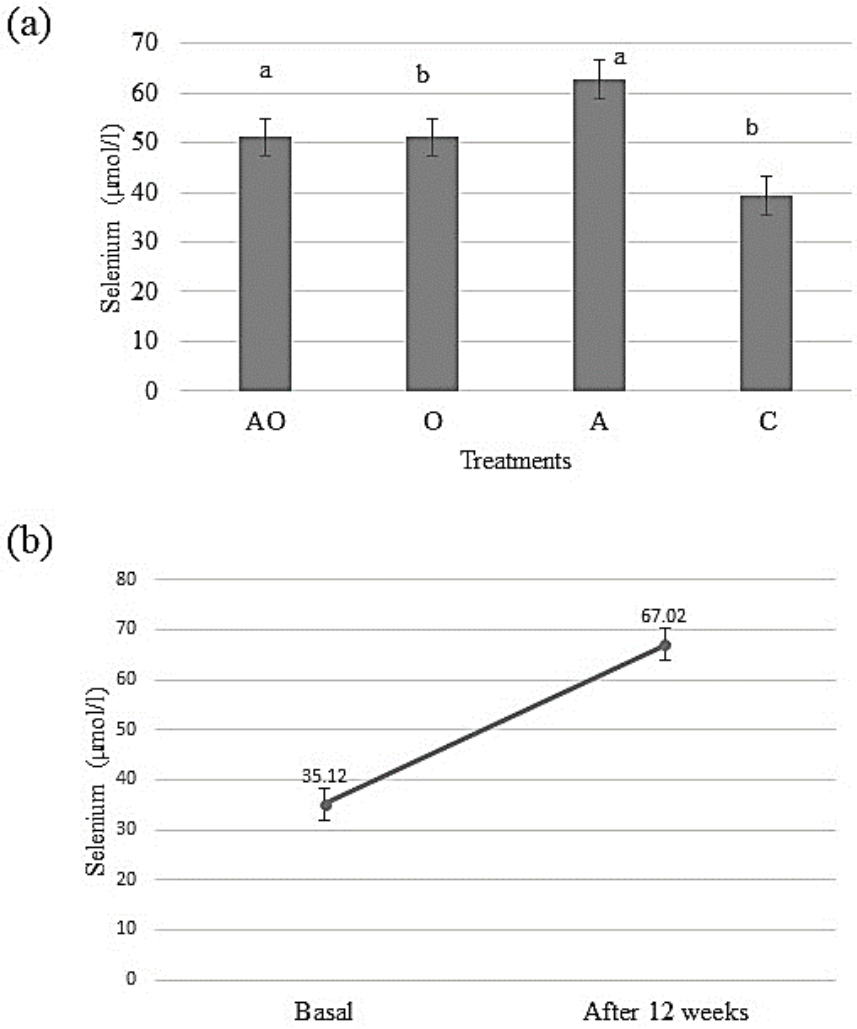

Figure 2: Selenium in serum [[a], antioxidante effect, p <.001, SEM = 3.77, AO: milk biofortified with antioxidants [vitamin E and selenium] plus CLA; O: milk biofortified with CLA; A: milk biofortified with antioxidants [vitamin E and selenium]; C: control milk]; [b], time effect, p <.001, SEM = 3.30] of the elderly supplemented with biofortified or not biofortified milk.

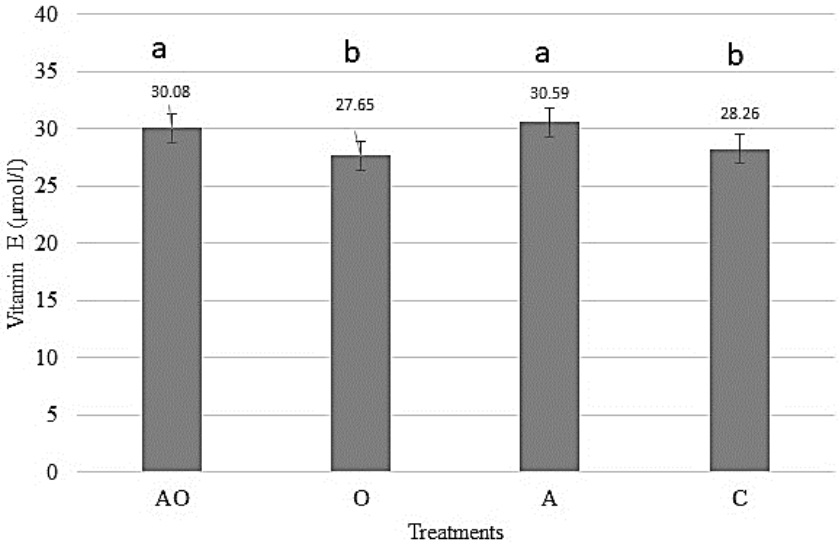

Figure 3: Vitamin E in serum [tendency of antioxidant effect, p = 0.064, SEM = 1.25, AO: milk biofortified with antioxidants [vitamin E and selenium] plus CLA; O: milk biofortified with CLA; A: milk biofortified with antioxidants [vitamin E and selenium]; C: control milk] of the elderly supplemented with biofortified or not biofortified milk.

6. Results

Blood serum biochemical analysis

The participants who consumed the O biofortificated milk had lower values of total cholesterol [*p* = 0.042] and LDL [*p* =0.029] in serum [table 3]. There was effect of the interaction Antioxidant and Oil over the HDL level [*p* = 0.0060], it is observed that the participants of the AO [42.60 mg/dl] group had a higher HDL than those of A [37.75 mg/dl] and O [38.61 mg/dl] treatments [table 3].

The concentrations of total cholesterol [*p* <.0001], HDL [*p* <.0001], LDL [*p* =0.003], VLDL [*p* =0.005], glucose [*p* = 0.030] and uric acid [*p* =0.040] decreased after 12 weeks of consumption, while the values of reactive protein-C increased [*p* = 0.0015, table 3].

Blood serum fatty acids

Regarding the analysis of fatty acids, when the time is compared [before and after supplementation], only polyunsaturated fatty acid decreased [p-value=0.0204] in all groups after biofortification [3.36 μmol/l at basal time, and 3.21 μmol/l after 12 weeks SEM = 0.09].

When fatty acids were analyzed separately, oleic acid, a monounsaturated fatty acid, decreased along time [926.60 μmol/l at basal time, and 885.88 μmol/l after 12 weeks, SEM = 19.73, p = 0.016]. EPA had a triple significant interaction [A*O*Time, p-value=0.045], the EPA values of AO increased after consumption for 12 weeks, whereas for A and O these values decreased, and the control group remained similar [figure 1].

Blood serum selenium and vitamin E

The participants who consumed biofortified milk with antioxidant nutrients had an increase in blood selenium levels [*p* <.001, figure 2a], and a tendency to have higher vitamin E values [*p* = 0.064, figure 3]. Blood selenium levels increased after 12 weeks of the experiment [*p* <.001, figure 2b].

7.Discussion

Institutionalized older people are older and have a high number of comorbidities, with higher nutritional risk [10, 14]. The selection of participants in this research was carefully conducted to prevent the influence of the variables [diabetes, smoking, sex and age] on the study outcome and there were no differences in the prevalence of these conditions when groups were compared [table 1].

Although milk consumption contributed to 20-30% of daily energy intake in our study groups, the participants maintained their usual diet, offered by the institutions. Our objective was to offer extra nutrients to the usual intake, not to supplement according to recommendations for age [table 2].

Biofortified milk produced by cows showed different lipid profiles from a milk commonly produced by animals [Salles et al, 2019], and consumption of this milk resulted in changes in serum cholesterol, LDL, HDL, glycemia, uric acid, protein-C reactive, vitamin E and selenium in institutionalized older people. Although the increase values of VLDL and protein-C reactive and decrease values of uric acid showed significant differences in time, the mean of biofortified and the control group were within the normal levels.

Milk is among the top ten foods that provide energy and saturated fat in human diet. Milk consumption has been associated with increased LDL-cholesterol and an increased risk of cardiovascular diseases [24]. However, a systematic review showed that there are no consistent data associating a high intake of saturated fat with cardiovascular disease and diabetes in the USA, Europe and Australia [25, 26]. The consumption of milk-based oils may be associated with lower cardiovascular risk factors [27].

The consumption of CLA-rich foods such as milk and dairy products can influence the lipid profile, increasing [Wanders et al, 2010, Sofi et al, 2010, Santurino et a, 2020] or reducing the lipoproteins [Khanal, 2004]. The effects of CLAs present in food or supplements on the lipid profile are not yet well established [Hartighet al, 2019]. In this

study, lower values were observed in the atherogenicity index, in the thrombogenicity index and in the proportion of hypocholesterolemic to hypercholesterolemic fatty acids in the milks of treatments O and AO.

In the long-term, this western dietary pattern causes an increase in the prevalence of obesity, cardiovascular and other chronic diseases [33,34]. This study aimed to produce milk with a lower proportion of saturated fat [15], which would represent a valuable source of C18 mono- and polyunsaturated fatty acids, and contribute to a decrease in LDL-cholesterol [35]. Also, serum levels of both total cholesterol and LDL-cholesterol decreased by 6·8 and 10·6%, respectively, in the group of participants receiving the O and AO milks. It is of note that such a decrease is higher than the effect of smoking cessation on LDL-cholesterol levels [5%] and within the 5-10% reduction range in LDL levels in response to physical activity [36].

A strength of this study is that it places the food chain into perspective; this has been addressed by few studies. The term biofortification is quite new. Witkowska et al. [2015] [37] biofortified the diet of goats with zinc, copper, iron and manganese. The goats produced milk and cheese with higher amounts of those nutrients, but those were not offered to humans [37]. In our study, the increase of vitamin E in milk [15] provided a tendency to increase of vitamin E concentration in the serum of the participants who consumed these biofortified milks. Although the amount of vitamin E in the milk [5.05±1·6μmol/L in milk A and 5.21±1·6μmol/L in milk AO] was lower than the DRI for the nutrient and age range, these findings indicate that the biofortified milk may have played an important role in vitamin E intake. The concentration of selenium in the milk was significantly increased by the addition of the nutrient to the cows's diet, and the consumption of the milk significantly increased selenium serum concentrations in the participants. Also, daily intake of this nutrient was within the recommended values, indicating the role of the modified milk in selenium supplementation. The presence of complex diseases and adverse situations in the older population may increase the demand for nutrients, and differently affect the other outcome variables.

According to Faraji et al. [2019] [38] the role of oxidative stress and free radicals in the aging process is one of the latest theories in which there is an increase in oxidative stress levels with the aging process. The elderly has mild to moderate inflammation, which always accompanies the aging process, and with the aging process there is an imbalance between antioxidant defense and reactive oxygen species [ROS] [39]. Supplementation of antioxidant nutrients such as selenium and vitamin E becomes necessary for the maintenance of health for the elderly. For older individuals, selenium supplementation could be necessary because this nutrient is part of protective enzymes and as an antioxidant it acts to reduce ROS-mediated inflammation [39]. A meta-analysis study was performed to verify the effect of supplementing omega-3 fatty acids with vitamin E on people's oxidative stress and found that co-supplementation increased levels of total antioxidant capacity, while malonaldehyde levels decreased relative to the placebo [40]. A study with patients with knee osteoarthritis was conducted to assess the antioxidant and anti-inflammatory effects of vitamin E on plasma oxidative stress, and vitamin E was reported to act as an effective antioxidant in improving clinical symptoms and reducing stress conditions. oxidative stress in patients with advanced stage of this disease [41].

This study has some limitations that should be considered. All volunteers were recruited from a unique institution which guarantees that all subjects received the same meals. The duration of the intervention [12 weeks] was not long enough to allow the detection of changes in the lipid profile in the longer term. Institutionalized older people are susceptible to health risks, usually associated with chronic diseases, altered nutritional status and clinical situations

that affect nutrient absorption. Nevertheless, in this study biofortificated milk impacted the concentration of the studied nutrients and biochemical parameters after 12 weeks.

In conclusion, our study demonstrated that biofortification of the milk changes important biochemical parameters associated with health maintenance in this population. This study also places into evidence the importance of the food chain in researches in the area of biofortification, and the use of animals as a mediator of nutrient supplementation in human health. New studies are needed to verify if the addition of the elements instead of biofortification would lead to the same biological effects.

Author Contributions: K.P., M.S.V.S., L.C.R.J., H.V., A.S.N., M.A.Z., E.Z.M. conceived and designed the experiments; D.T., M.AZ., R.F.M., J.KS.H., V.M.M.S., H.T.G., A.D.M. performed the experiment and analyzed the data; K.P., E.F., M.S.V.S., L.C.R.J. wrote the paper. In addition, M. Meirelles contributed to the analysis tools and C. G Lima contributed to the analyzed the data and wrote the results.

Conflicts of Interest: The authors declare no conflict of interest.

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