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Cane molasses and oligofructose in the diet of laying hens improves the mineral content of eggs and meat

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ABSTRACT

Poultry-based foods contribute to human health due to their high nutrient value. Previously, it was shown that short-chain fatty acids (SCFAs) produced by *in vitro* intestinal fermentation of a molasses and oligofructose mixture (M-O) stimulated iron and calcium transport through the colonic epithelium of laying hens. However, the real impact of including M-O mixture in the diet on the mineral content of poultry products had not yet been demonstrated. In this study, Hy-Line W-36 leghorn hens were assigned into two groups that either received a conventional diet or a diet supplemented with cane molasse and oligofructose, over a period of 42 days. The weight of the animals and their eggs, blood parameters and intestinal epithelium integrity were determined. Intestinal bacteria, their fermentation products, and the mineral content of eggs, bones and muscles were also assessed. The experimental diet proved to be safe, favored the proliferation of SCFA producing bacteria in the intestines, led to higher concentration of acids (mainly SCFA) in the digesta, and induced the elongation of microvilli at the apical tip of enterocytes. Mineral content of eggs and meat were improved after four weeks of feeding with the experimental diet compared to the conventional one. Higher iron content was observed in the edible portion of eggs and leg muscle, and higher calcium content was observed in the egg edible portion and shell in hens fed the supplemented diet. This feeding strategy could be useful to improve the mineral content of poultry products and therefore human nutrition, while diversifying molasses applications.

1. Introduction

In recent years, the demand for poultry meat and by-products of the poultry industry has increased notably. Among foods of animal origin, poultry meat and eggs provide high quality proteins, vitamins and minerals, at an affordable cost that has led to its increased consumption over other foods of animal origin. Moreover, poultry-based foods may contribute to improving health, especially in populations with nutritional deficiencies (Pal & Molnár, 2021). Currently, the poultry industry faces the challenge of developing new technologies to achieve a rapid and effective increase in production, guarantee the expected contribution of poultry products to human nutrition and also consider strategies to mitigate the impact of animal husbandry on the environment.

Feed greatly influences the quality of poultry products. This has led to studying the effects of the use of different feed additives or the partial replacement of some ingredients commonly used in poultry diets with more promising ones. Molasses is a liquid with a viscous consistency which is a sub-product of sugar extraction from sugar beets and sugar cane. In livestock production, molasses is considered an energetic feedstuff due to its content of easily fermentable sugars, but the main reason for using molasses in poultry diets is to enhance palatability and reduce dust in feeds (Mordenti, Giaretta, Campidonico, Parazza & Formigoni, 2021). Indeed, few studies have aimed to determine the nutritional value of animal-based foods obtained by using molasses. Expanding its use in feed production would prevent the disposal of an organic rich industrial waste.

In an *in vitro* study, Gultemirian, Corti, Perez Chaia and Apella (2014) reported that the addition of a mixture of cane molasses and oligofructose to the hens cecal content, is responsible for changes in bacterial populations and higher production of SCFA and lactic acid by

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fermentation. Mounting the colonic mucosa and submucosa of laying hens onto an Ussing Chamber, these authors demonstrated that pure SCFA in concentrations and proportions similar to those produced in the *in vitro* cecal fermentation, drive the iron and calcium transport through the intestinal epithelium. However, the inclusion of molasses and oligofructose in the hens' diet has not been demonstrated to be safe and improve the nutritional value of poultry products.

Considering the previous *in vitro* findings, the aim of the present study was to determine if the production of SCFAs by microbial fermentation in hens receiving a diet supplemented with cane molasse and oligofructose, is enough to improve the absorption of minerals from feed and increase its content in poultry products. The minimum term of feeding necessary to observe significant changes on minerals content was also assessed.

2. Materials and methods

All experiments described in this study were carried out at INTA-Pergamino (Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Pergamino, Buenos Aires, Argentina), according to regulation (decree 4238/68) of the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA)-Argentina (2010).

2.1. Birds and diets

Twenty-four leghorn laying hens (Hy-Line W-36) of similar weight and age (*ca.* 1350 g; 40 weeks), in the 20th week of posture, were selected and randomly distributed into two groups of twelve animals each (4 birds per cage; 3 replications) 2 weeks before the beginning of the trial for adaptation to the housing conditions. A light/darkness regime of 16 and 8 h, respectively was maintained throughout all the study. During the trial, they received one of the following isocaloric diets: conventional diet (Control group) and conventional diet with the addition of cane molasses and oligofructose, 10.0 g/kg of each (M-O group). Molasses and oligofructose were supplied by La Florida sugar industry (Tucumán, Argentina) and SAPORITI S.A. Orafti (Brasil), respectively, and manually blended with the solid basal diet for their administration. Minor modifications were made with respect to the conventional diet to maintain the energetic values of that of the Control group (Table 1). The birds received their assigned diet of solids and water *ad libitum* during 6 weeks.

Table 1
Conventional and modified diets compositions.

Ingredients	Conventional (g/kg)	M-O (g/kg)
Corn	622	587
Full fat soybean	171	171
Oyster shell	93.4	93.3
Meat meal	54.7	54.9
Soybean meal	49.9	55.6
Soybean oil	–	9.05
Salt	2.79	2.79
DL-metionine	2.03	2.07
Vitamin and trace mineral premix[1]	1.50	1.50
Treonine	0.93	0.92
Lysine	0.80	0.70
Choline	0.50	0.50
Molasses	–	10.0
Oligofructose	–	10.0

[1] Vitamin and mineral provide per kg of feed: vitamin A, 9000 IU; vitamin D3, 2700 IU; vitamin E, 15 mg; vitamin K3, 2 mg; vitamina B1, 1.5 mg; vitamin B2, 5 mg; vitamin B6, 1.5 mg; vitamin B12, 0.01 mg; niacin, 22.5 mg; pantothenic acid, 8 mg; folic acid, 0.5 mg; choline chloride, 90 mg; copper, 6 mg; iron, 37.5 mg; manganese, 60 mg; iodine, 0.75 mg; zinc, 52.5 mg; selenium, 0.225 mg.

2.2. Hens' weight and hematological parameters

The birds of Control and M-O groups were weighed at the beginning and the last day of each week of the trial. In the morning of days 1 and 28, after 10 to 12 h fasting, 4 mL of peripheral blood was taken from the wing vein of two hens per cage ($n = 6$ per group). An aliquot of 0.5 mL blood was placed in a tube with anticoagulant and used to determine red blood cells (RBC), hematocrit (HCT), hemoglobin (Hb), white blood cells (WBC) and to perform the WBC differential count. Plasma samples were used for calcium and iron quantification by flame atomic absorption spectrometry (FAAS) with a Perkin Elmer 3100 (USA) and a calibration curve was used for each mineral with certified standards solution (Merk CertiPur®, Germany).

2.3. Transmission electron microscopy

The intestinal tissue of hens from both groups of feeding at day 42 of the trial was evaluated using Transmission Electron Microscopy (TEM) at Centro Integral de Microscopía Electrónica (CIME-UNT-CONICET, Tucumán, Argentina). To this end, three birds of each group were sacrificed by cervical dislocation and their intestines were immediately carried at 4 °C to the laboratory. The ceca, livers and legs of these animals were conditioned for further studies as later describe in the fourth and sixth subsections.

The small bowel of each animal was opened longitudinally; the digesta was removed and tissue gently washed with 0.1 M phosphate buffered saline solution (PBS) pH 7.4. Clean portions of the duodenum were submerged in Karnovsky fixative (1965) for 3 h at 4 °C, and then thoroughly washed with the same buffer solution. The samples were post-fixed with a 1% (w/v) osmium tetroxide solution in PBS solution for 12 h at 4 °C and later dehydrated in successive alcohol and acetone baths of increasing graduation. Inclusion was carried out in Spurr resin. Ultrathin sections of 60 to 90 nm obtained from the blocks of tissue, were mounted on a copper grid, contrasted with uranyl acetate and lead citrate and examined by TEM (Zeiss EM 109, Carl Zeiss, Oberkochen, Germany). TEM images were used to assess enterocytes morphology and features. Mitochondria (M), microvilli (Mv), actin filaments (AF), cell membranes and the apical junction complex (AJC) including desmosomes (Ds) were observed. Six to ten microvilli were measured in each cell from the tip of the microvillus to its bound on the enterocyte membrane. A total of 2 cells per jejunum sample, and 3 samples (one sample from a hen of each replicate), were evaluated per feeding group. The height of microvilli was determined using the ImageJ program (Schneider, Rasband & Eliceiri, 2012), and adjustments for scale were made.

2.4. Microbial populations and organic acids content in ceca

Cecal homogenates were prepared for enumeration of the principal bacterial groups present in the Control and M-O group animals' cecum. The ceca obtained from 3 hens of each group, were placed in an anaerobic glove box with a 100% N_2 atmosphere (Anaerobic System model 1024, Forma Scientific, Marietta, USA). The cecal contents, obtained after opening the ceca longitudinally, were weighed and homogenized in pre-reduced sterile saline solution (SS) to obtain uniform slurries. Next, they were diluted in an adequate amount of SS to obtain cecal content suspensions of 10% (w/v) (Gultemirian et al., 2014).

For the bacterial populations counting, successive peptone water dilutions of each cecal suspension were performed and seeded on the following agar culture media: Mac Conkey (Oxoid Ltd., Basingstoke, England) for total enterobacteria; KF (Merck, Darmstadt, Germany) for enterococci; Rogosa (Oxoid, Argentina) for lactobacilli; RCA (Oxoid Ltd., Basingstoke, England) modified with the addition of lithium chloride and sodium propionate for bifidobacteria (Locascio, Pesce de Ruiz Holgado, Perdigón & Oliver, 2001); TSC agar (Merck, Darmstadt, Germany) for clostridia; Brucella agar (Oxoid Ltd., Basingstoke,

England) added vancomycin (750 μg / mL) and blood (10 mL / L) for *Bacteroides* sp. The seeded media for enterobacteria and enterococci were incubated at 37 °C in aerobic conditions while those for lactobacilli, bifidobacteria, clostridia and *Bacteroides* sp. were incubated at 37 °C under anaerobic conditions provided by Anaerocult A (Merck KGaA, Germany) in an anaerobic jar (AnaeroGen system, Oxoid, UK). The results were expressed as cfu / g of cecal content.

The organic acids content in cecal slurries was determined in filtered aliquots of the 10% (w/v) suspensions by high performance liquid chromatography (HPLC) with a HPLC system (Knauer, Germany) equipped with a BIO-RAD Aminex HPX-87H (300 × 7.8 mm) column. Elution was performed at a flow rate of 0.6 mL / min with H_2SO_4 5 mM solution. Sample quantification was carried out by using calibration curves of each SCFA and lactic acid, and results were finally expressed as μmol / g of cecal content.

2.5. Eggs' weight parameters and chemical analysis

Eggs of each group (Control and M-O) were collected daily between 1:00 and 1:30 pm and weighted. Weight of whole egg, edible portion (white and yolk) and eggshell were determined with a balance with precision of 0.01 g (Denver Instruments, USA). Mean values of eggs weight corresponding to the adaptation period were recorded in the first day of the trial, and mean values corresponding to three 14-days periods were recorded at days 14, 28 and 42. Iron, calcium and cholesterol (mg / 100 g egg) of the whole edible portion and shell calcium content (g / 100 g egg) of each period were analyzed on days 1, 14, 28 and 42 of the trial. In order to determine egg's minerals content, edible portion was homogenized, frozen 24 h at −20 °C and lyophilized (Lyovac GT2; Leybold, Köln, Germany) 16 h at 0.3 mbar to get less than 1% residual humidity. Samples of lyophilized edible portion and dried shell of eggs were disaggregated with HCl solution prior to the quantitative determinations. Minerals quantification was carried out by FAAS as was described previously in the second subsection. Cholesterol content was determined in whole edible portion of eggs by an enzymatic method (Enzymatic Colestat Kit, Wiener Lab, Argentina) after lipidic extraction (Boselli, Velazco, Caboni & Lercker, 2001).

2.6. Liver, muscle and bone minerals content

Leg muscles were extracted from 3 animals of each group (second subsection), and after discarding the connective and adipose external tissue, cut in pieces and dried in a heater until reaching constant weight. Livers were immediately weighed as a whole piece, and later dried until constant weight was reached. Dry samples were disaggregated with concentrated HCl solution for calcium and iron determination by FAAS as was described in the second subsection.

Femurs were dried in a heater and later in a muffle oven until reaching a constant weight to get the ashes (Association of Official Analytical Chemists International, 1995). These were treated with concentrated HCl solution for calcium quantification using the same procedure described above.

2.7. Statistical analysis

The results were expressed as mean ± standard deviation (SD) and statistically evaluated by Tukey's test after analysis of variance (ANOVA) with OriginPro 8 SR0v8.0724 (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Hens' weight and blood parameters

The weight of the birds that received the Control diet and the conventional diet modified by inclusion of molasses and oligofructose did not shown significant differences through time. Mean body weight of hens in Control and M-O groups was 1368 ± 83.67 and 1392 ± 92.15 gs, respectively at the 1st day, and 1327 ± 120.5 and 1378 ± 117.2, respectively at the day 42 of the trial. There were no significant differences in feed intake between Control and M-O groups throughout the experimental period (data not shown).

The animals did not show alteration caused by the change of diet in the WCB concentration or cells type percentage, which were similar throughout the trial in both feeding groups (Table 2). Furthermore, there were no significant differences between the Control and M-O groups in total values of RCB, HCT and Hb content.

The plasma calcium and iron values found were within normal parameters for laying hens and there were not significant differences when compared Control and M-O groups.

3.2. Epithelium intestinal structure by tem

No damage to different segments of the intestine was observed with the naked eye in hens of the Control and M-O groups when the organs were removed. After tissue processing, ultrathin sections were examined by transmission electron microscopy. Photomicrographs of the small bowel epithelial cells apical surface of hens are shown in Fig. 1. Images correspond to Control group (A and B) and M-O Group (C and D). After the feeding period with molasses and oligofructose, unions between the cells did not show any ultra-structural alteration. The cellular organelles were preserved and no shedding of microvilli of the epithelial cells surface was observed in any group of feeding. Regarding the apical morphology of enterocytes, significantly longer microvilli were detected

Table 2
Hematological parameters and minerals plasma content at days 1 and 28 of the trial.

Days	1		28	
Group	Control	M-O	Control	M-O
Parameters				
RBC (× 10^6 / μL)	2.40 ± 0.08	2.40 ± 0.09	2.35 ± 0.14	2.37 ± 0.17
Hb (g / dL)	11.8 ± 0.20	11.4 ± 0.60	10.8 ± 0.70	11.1 ± 0.60
HCT (%)	30.3 ± 0.60	30.0 ± 1.40	30.3 ± 2.50	32.0 ± 3.00
WBC (× 10^3 / μL)	13.70 ± 2.30	13.20 ± 0.99	14.00 ± 2.80	13.40 ± 2.70
Differential WBC count				
Heterophils (%)	25.0 ± 4.00	29.0 ± 1.50	33.0 ± 2.50	31.0 ± 2.50
Eosinophils (%)	0	0	0	0
Basophils (%)	0	0	0	0
Lymphocytes (%)	71.0 ± 3.00	67.0 ± 3.00	70.0 ± 2.00	69.0 ± 4.00
Monocytes (%)	6.50 ± 2.00	6.00 ± 2.00	6.00 ± 1.00	7.00 ± 3.00
Plasma minerals				
Calcium (mg / 100 mL)	28.0 ± 0.85	28.5 ± 0.76	28.3 ± 0.90	29.70 ± 0.87
Iron (μg / 100 mL)	810 ± 50.0	808 ± 78.0	823 ± 66.0	900 ± 59.0

Results are expressed as means ± SD ($n = 6$ per group). Means without superscripts are not significantly different at significance level of 0.05.

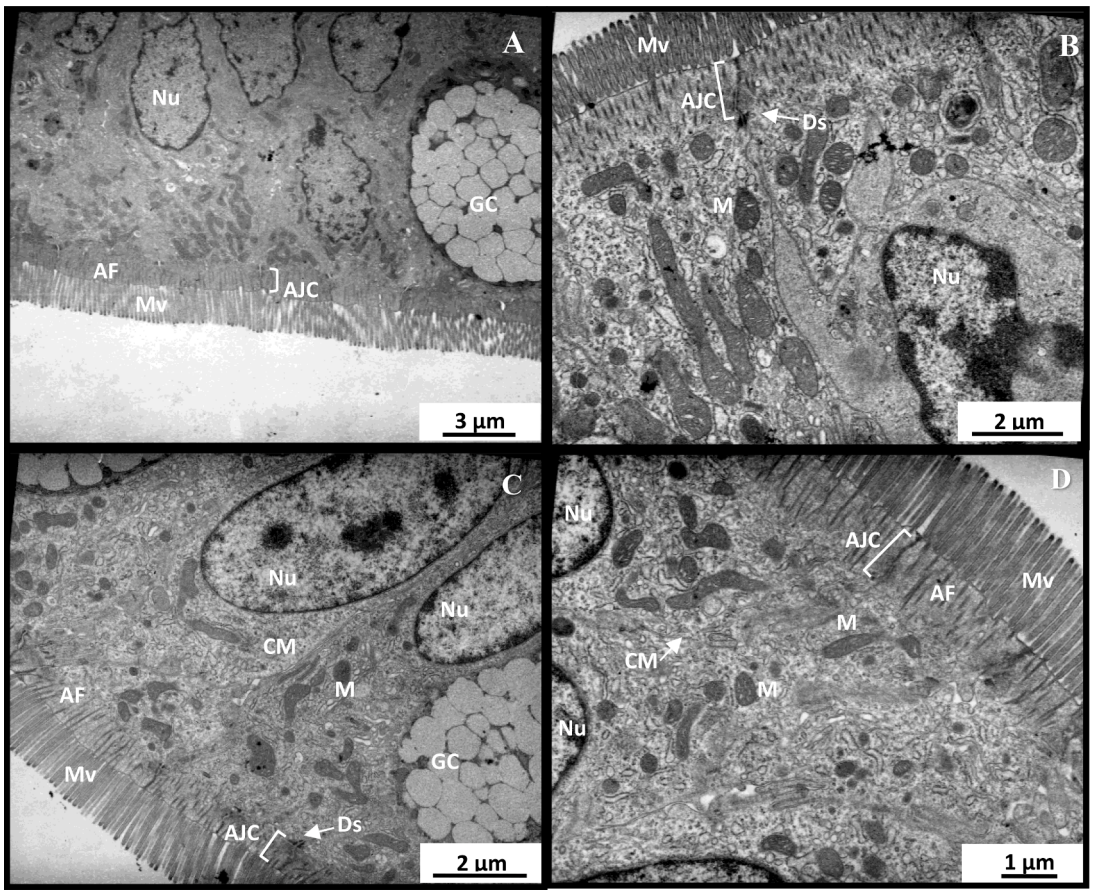

Fig. 1. Transmission electron microscopy (TEM) photomicrographs illustrating the organization of the brush border of epithelial cells of the jejunum of layer hens. Images correspond to Control group (A, B) and M-O group (C, D) at the end of the trial. Enterocytes and goblet cells (GC) are observed in A and C images. The cellular structures and organelles highlighted are: enterocyte nucleus (Nu), mitochondria (M), cell membrane (CM), apical junctional complex (AJC), desmosome (Ds), actin filaments (AF), microvilli (Mv). Scale bars are: A, 3 µm; B and C, 2 µm; D, 1 µm.

Table 3
Eggs weight, cholesterol and mineral content.

Days	1[1]		14		28		42	
Group	*Control*	*M-O*	*Control*	*M-O*	*Control*	*M-O*	*Control*	*M-O*
Eggs' weight parameters								
Whole egg weight (g)	59.0 ± 3.10	57.6 ± 5.10	60.6 ± 3.40	58.2 ± 5.30	59.1 ± 3.90	60.6 ± 5.00	61.5 ± 2.50	59.6 ± 6.00
Edible portion weight (g)	54.1 ± 2.30	52.0 ± 4.60	56.1 ± 3.50	54.5 ± 5.10	55.2 ± 3.80	56.5 ± 4.40	56.6 ± 2.70	56.2 ± 5.20
Shell weight (g)	4.73 ± 0.50	4.41 ± 0.19	4.59 ± 0.22	4.68 ± 0.39	4.34 ± 0.30	4.65 ± 0.52	4.66 ± 0.42	4.73 ± 0.60
Cholesterol content								
Cholesterol (mg / 100 g)	475 ± 6.50	508 ± 48.8	495 ± 4.79	486 ± 10.8	493 ± 9.30	497 ± 2.33	530 ± 54.8	500 ± 43.3
Minerals in edible portion								
Ca (mg / 100 g)	59.5 ± 1.70 [a]	58.7 ± 2.22 [a]	56.7 ± 3.50 [a]	59.5 ± 0.90 [a]	59.7 ± 0.30 [a]	59.0 ± 1.19 [a]	60.9 ± 0.44 [a]	67.3 ± 2.04 [c]
Fe (mg / 100 g)	2.00 ± 0.12 [a]	2.00 ± 0.07 [a]	2.00 ± 0.16 [a]	2.10 ± 0.03 [a]	2.10 ± 0.06 [a]	2.40 ± 0.04 [b]	2.00 ± 0.05 [a]	2.40 ± 0.06 [b]
Shell calcium								
Ca (g / 100 g shell)	37.2 ± 0.29 [a]	37.0 ± 0.33 [a]	37.7 ± 0.48 [a]	36.7 ± 0.37 [a]	37.7 ± 1.12 [a]	37.5 ± 0.72 [a]	36.9 ± 0.16 [a]	40.1 ± 0.60 [d]

[a – d]Values within a row with no common superscript letters differ significantly at $P < 0.01$ (b), $P < 0.05$ (c) or $P < 0.005$ (d).
[1] Eggs processed at day 1 were collected during the two weeks adaptation period before the M-O diet administration. Results are expressed as means ± SD ($n = 10$).

in hens that consumed the M-O diet. Mean length of microvilli in cells of jejunum was 1.376 ± 0.043 ($n = 3$) in hens of Control group and 1.664 ± 0.079 ($n = 3$) in birds of the M-O one ($P < 0.001$).

3.3. Egg's weight and content

The weight of whole eggs, edible portions, and eggshells on days 14, 28 and 42 of the trial did not show significant differences between the Control and the group treated with the mix of molasses and oligo-fructose, and were similar to those of the adaptation period as shown in Table 3.

The cholesterol content in eggs was not modified when the M-O combination was included in the diet. There was no change in the egg calcium content until day 28. However, a significant increase of this mineral was noticed in the edible portion of eggs from animals that received the M-O diet at day 42 ($P < 0.05$). Shell calcium increase was also observed in eggs of birds fed the supplemented diet at the last day of the trial ($P < 0.005$). By contrast, iron amount in the eggs edible portion was significantly higher at days 28 and 42 in the group fed with the experimental diet than in the Control one ($P < 0.01$).

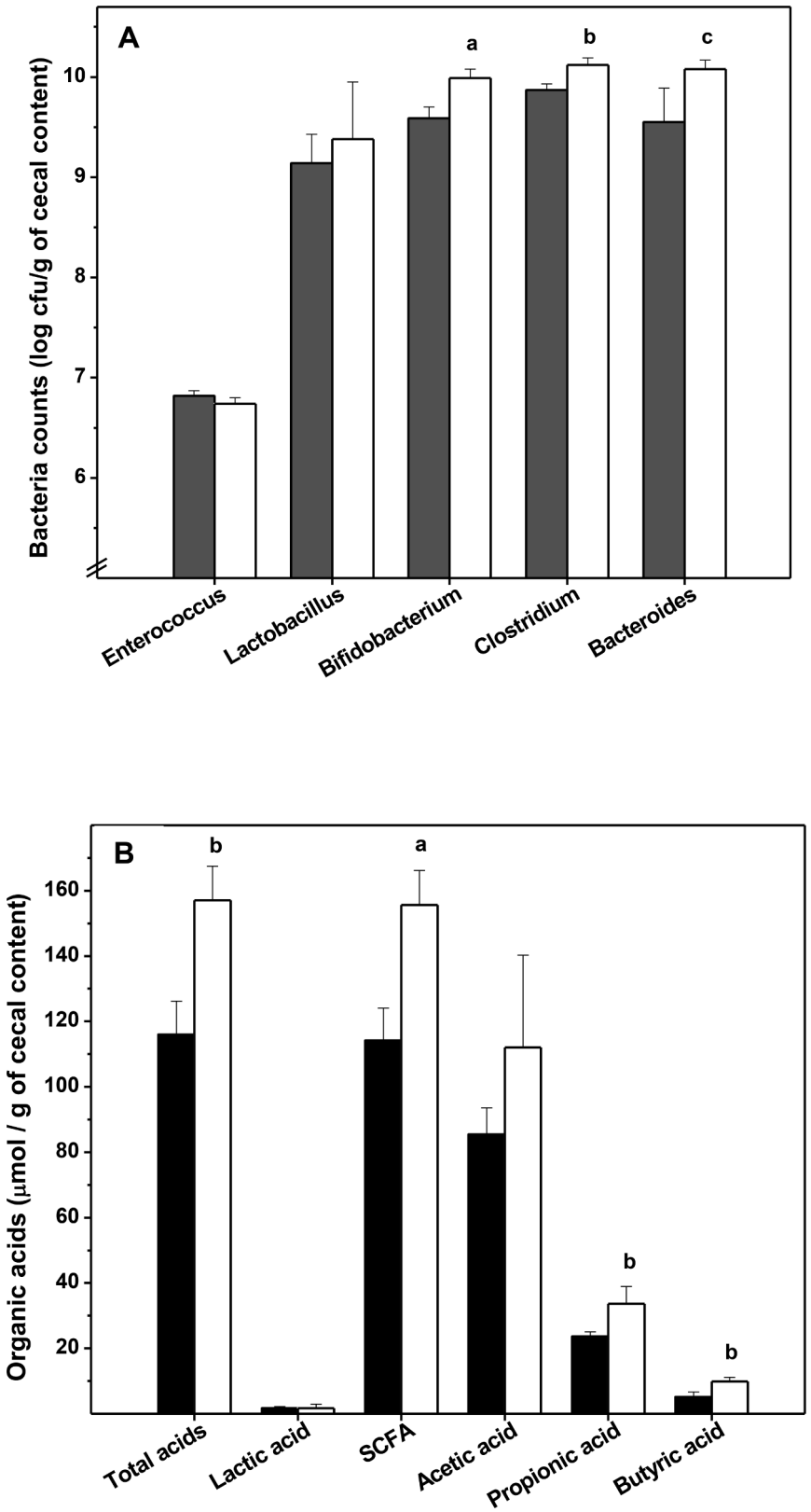

Fig. 2. **A -** Counts of relevant bacteria genera in the cecal content of birds fed with the control diet (□) and M-O diet (■) at day 42 of treatment. **B -** Organic acids in the cecal content of hens at the day 42 of the trial. Results are expressed as means ± SD ($n = 3$). Symbols on columns of the M-O group of feeding indicate values significantly different compared to the Control group (a, $P < 0.01$; b, $P < 0.05$; c, $P < 0.1$).

3.4. Microbiota and SCFA content in the cecum

Main bacterial genera in the cecal content of hens at the end of the trial are shown in Fig. 2-A. No differences in the number of enterococci were observed in birds fed with the diet supplemented with molasses and oligofructose, related to Control feeding group. Enterobacteria were not a relevant population and reached values lower than 10^5 cfu / g of cecal content in both feeding groups. Counts of *Lactobacillus* in the M-O group evidenced no significant increase with respect to the Control group. Significantly higher counts of the genera *Bifidobacterium* ($P < 0.01$), *Clostridium* ($P < 0.05$) and *Bacteroides* ($P < 0.1$) were observed in the M-O group with respect to Control group.

Concentrations of the main organic acids were also evaluated in cecal content of hens at the end of trial (Fig. 2-B). There were significant increments in total acids ($P < 0.05$) and SCFA concentrations ($P < 0.01$) in the hens fed the supplemented diet. Propionic and butyric acids contents were higher in the ceca of M-O group than in the Control animals ($P < 0.05$). By contrast, acetic and lactic acids contents were not significantly modified by the addition of molasses and oligofructose to the diet. In accordance with the increased concentration of total acids, pH of the cecal slurry of M-O group of hens was 5.37 ± 0.12, in relation to Control group in which pH was 6.77 ± 0.15 (data not shown).

3.5. Minerals in liver, muscle and bone

There were no differences between Control and treated hens' groups in liver Ca and Fe and in bone Ca concentrations, but a significant increment ($P < 0.05$) was evidenced in Fe concentration in the muscle of the treated hens as compared with the Control group (Table 4).

4. Discussion

During the last decades, the poultry industry has made great efforts to increase production and profitability through different strategies These include genetic selection of animals, feed quality improvement, and using of multi-enzyme additives that favor the proper digestion of dietary ingredients and conversion into body mass and eggs (Khan, Atif, Mukhtar, Rehman & Fareed, 2011). However, consumer demand for foods with higher healthy properties, such as low cholesterol, higher content of polyunsaturated fatty acids and minerals, as well as high-quality protein, poses new challenges for the poultry industry.

In recent years, valuable insights have been gained on the impact of new feed additives on the digestion and nutrient absorption that improve the nutritional value of poultry products (Alagawany et al., 2015; Sharma, Dinh & Adhikari, 2020). In addition, new ingredients that partially replace existing ones have been used to modify the composition of eggs for human consumption. Eggs of lower cholesterol content, higher amount of n-3 polyunsaturated fatty acid (PUFA) and reduced n-6:n-3 ratio were obtained without affecting performance (Aguillón-Páez, Romero & Diaz, 2020; Saleh et al., 2021).

Cane molasses, is a residual product of sugar cane juice processing that usually retains sugar in mean concentration higher than 43% (range of 35.65 – 52.27%) depending on cane variety and agronomic management, according to internal reports of EEAOC-Estación Experimental Agroindustrial Obispo Colombres (Tucumán, Argentina). Molasses may be used for industrial ethanol production by yeasts fermentation, or in animal husbandry to reduce dust in feeds. In some countries, it can be useful for partial substitution of corn in animal diets, when the grain access is limited (Mordenti et al., 2021).

Table 4
Minerals in liver, muscle and bone at the day 42 of trial.

Organ	Mineral (μg/g)			
	Calcium		Iron	
	Control group	M-O group	Control group	M-O group
Liver	126 ± 21.2	137 ± 22.6	234 ± 30.9	226 ± 14.1
Muscle	124 ± 14.8	153 ± 11.3	53.7 ± 7.23 [a]	77.3 ± 7.02 [b]
Bone	17.3 ± 0.85	18.5 ± 0.28	-	–

Results are expressed as means ± SD ($n = 3$). Means with different superscript in the same row and mineral assayed are significantly different ($P < 0.05$).

Hussein et al. (2018) reported that inclusion of sugar syrup in avian diets, a high-quality molasses with 76% sugar, should not be higher than 10% for growing pullets or 20% for laying hens, to avoid laxative effects and increased drinking water ingestion. According to this recommendation, in the present study molasses was used in combination with the prebiotic additive oligofructose, in concentrations that did not exceed 10 g / kg of diet each. At this inclusion level, no significant differences were observed in hematological parameters in blood, suggesting that M-O diet did not produce detrimental effects on the health status of the animals. RBC, WBC and different WBC types were in normal values for laying hens, indicating that there was no concentration of blood cells caused by dehydration in hens of M-O group Kang, Park, Kim and Kim (2016). reported changes on the number of heterophiles (H), linfocytes (L) and the H/L ratio, a hematological stress indicator, due to the increase the stock density. However, in the present experiment, the animals were housed to be comfortable and there were no hematological signs of stress caused by the change of the diet in M-O group, or by the stock density during the trial. By contrast, the results were in agreement with Mugnai, Dal Bosco, Moscati, Battistacci and Castellini (2011), who studied the effect of organic production *vs* standard cage system on welfare and performance of two genotype of laying hens, without detecting hematological changes. Related to cholesterol and minerals content in blood, concentrations were not significantly modified during the trial. Plasma iron and calcium were similar to other research reports in laying hens (Hussein et al., 2018; Lopez-Berjes & Recio, 1981).

Molasses also contains vitamins, amino acids, fatty acids and minerals (Mordenti et al., 2021), but these have negligible impact in feeding poultry when diets contain the recommended value of 10 g molasses per kg feed. Although some components of molasses do not impact directly on poultry nutrition, they positively influence the development of the intestinal microbial populations. Fructans have received considerable attention in human and animal diets, as only few species of gut bacteria can easily break them down, enhancing their growth. *Bifidobacteria* sp. and *Lactobacillus* sp. are specifically stimulated when inulin, oligofructose or fructooligosaccharides (FOS) are included in the diets (Gibson, 1999). In addition, other saccharolytic genera benefit from the availability of fructans, including *Bacteroides* sp., which use them and release oligosaccharides of different chain lengths (Falony, Calmeyn, Leroy & De Vuyst, 2009; Macfarlane & Macfarlane, 2003). In the present study, the consequence of bacterial fermentation of the complex mixture of these oligosaccharides and residual sucrose, resulted mainly in significant increase of *Bifidobacterium* and *Bacteroides* genera, and higher SCFA production. The limited concentration of lactic acid detected in the hens' intestinal content of both groups, could be attributed to the development of intestinal species of *Clostridium* that use lactate as energy source and produce acetic acid and propionic or butyric acids, or to the presence of other butyrogenic bacteria, which prevent excessive lactate accumulation (Duncan, Petra & Flint, 2004).

Fructans fermentation, as well as other fibers collectively named prebiotics (Campbell, Fahey & Wolf, 1997; Gibson & Roberfroid, 1995), can promote the proliferation of epithelial cells and lead to increased growth of the intestinal mucosa, which in turn participates in the hydrolysis of complex nutrients and absorption of smaller molecules (Scheppach, 1994). In the present study, no damages were observed in the intestinal epithelium of hens of both groups at the end of the trial, and microvilli of cells' apical surface preserved integrity and morphology. However, feeding with M-O combination induced a significant increase ($P < 0.001$) on microvilli length, which could positively influence ions transport across the epithelium.

Absorption of SCFA, produced in higher amount by the intestinal microbiota in hens from M-O group, could drive the passive mineral

transport predominantly in the large intestine. Lactic acid and SCFA are produced mainly at the distal region of small intestine and in cecum due to the higher concentration of fermentable carbohydrates and fibers that reach these intestinal regions and the presence of bacterial genera able to metabolize them (Macfarlane & Macfarlane, 2003). Dietary calcium may have higher bioavailability from the jejunum to large intestine due to fibers fermentation and subsequent pH descent (Kruger, Brown, Collett, Layton & Schollum, 2003; van Der Wielen et al., 2000; Younes, Demigne & Remesy, 1996), as was verified in the cecal content in the present investigation. This is in agreement with the concept that calcium absorption takes place mainly in the jejunum and large intestines, driven by transepithelial active and paracellular passive transport, respectively (Gloux et al., 2019) Chen and Chen (2004). reported increased plasma calcium content when supplementing the diet of Leghorn layers with oligofructose or inulin. Tese findings differ to those of the present study where M-O diet did not induce significant increment of plasma calcium compared to Control diet. This result was probably due to the fact that blood samples were taken in the morning after 12-hour fasting.

Calcium content in the edible portion and eggshell did not vary throughout the trial in the Control group of hens, and was similar to results of previous reports (Grobas & Mateos, 1996; Nys & Sauveur, 2004). Six weeks after the beginning of the trial, feeding with the experimental diet supplemented with M-O led to higher level of calcium in the edible portion ($P < 0.05$) and eggshell ($P < 0.005$), without affecting calcium bone content. These results confirmed that the M-O supplementation improved the intestinal absorption of dietary calcium and favored eggs development.

Iron absorption and the impact on egg's composition, was previously studied using iron-soy proteinate and iron-methionine chelate in the hens' diet (Pal & Molnár, 2021), resulting in improved egg quality parameters and increased iron content in yolk using both types of organic supplement Sarlak et al. (2021). also demonstrated that ferrous sulfate can be replaced by ferrous glycine, with more favorable impacts on egg quality and iron enrichment. Although non digestible carbohydrates are known to increase *in vitro* (Gultemirian et al., 2014) and *in vivo* (Scholz-Ahrens, Schaafsma, van den Heuvel & Schrezenmeir, 2001) mineral absorption in animals, results depend on the carbohydrate used, animal species, sex, age, dose, animal husbandry, among other factors. In the present work, the effects of combining molasses and oligofructose on iron enrichment in eggs and tissues in laying hens were evaluated during 6 weeks, to ensure the metabolic impact of the dietary modification and to determine the time need to detect significant changes Ali and Ramsay (1974). and later Lopez-Berjes and Recio (1981), reported that plasma iron in layer hens reach higher amounts than in non-layer or in male birds, and that the difference is associated to the phosphoprotein phosvitin presence only in layer hen's plasma. Part of the plasma iron is transported by phosvitin for egg formation while other part is transported by the blood transferrin to tissues and bone marrow, to take part in red blood cells formation. In the present work, the higher absorption of iron from intestinal sources increased significantly the egg iron content in hens of M-O group compared to Control group, from the day 28 to 42 ($P < 0.01$), while muscle reached higher amount of iron in hens of M-O group only at the end of the trial ($P < 0.05$). These results are in agreement with other reports about the function of specific iron carriers in hens that mainly drive the mineral to ova to guaranty the embryo development.

5. Conclusions

Molasses and oligofructose included in the diet of laying hens in equal amounts did not affect the animals' health and favored the development of bacterial genera that produce SCFA in the intestines. Feeding with the M-O combination did not induce damages at the intestinal tissue, which exhibited normal appearance, but increased the length of microvilli at the apical surface of epithelial cells and increased dietary calcium and iron absorption. This subsequently led to improved calcium content in the edible portion of eggs and their shells, and iron in eggs and muscles. This diet modification could be useful to improve the mineral content of poultry products and thus human nutrition. In addition, diversifying the use of molasses in aviculture may be a strategy to reduce the environmental impact of discarding this valuable by-product.

Author contributions

María L. Gultemirian conceived and designed the experiments, performed the experiments, analyzed the data, and approved the final draft. Bernardo F. Iglesias designed the experiments, performed the experiments, analyzed the data, and approved the final draft. Adriana Perez Chaia analyzed the data, prepared figures and/or tables, authored and reviewed drafts of the paper, and approved the final draft. María C. Apella conceived and designed the experiments, analyzed the data, authored and reviewed drafts of the paper, and approved the final draft.

Declaration of Competing Interest

The authors declare there are no competing interests in this study.

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