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Original Research Article

Grain free diets for utility dogs during training work: Evaluation of the nutrient digestibility and faecal characteristics

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ABSTRACT

Two different diets characterized by the absence of cereals or by the presence of conventional cereals were evaluated on the nutrient digestibility and faecal characteristics and faecal fermentative end-product concentrations of 8 neutered adult Labrador retrievers housed at the Regional Centre Helen Keller (Messina, Italy) during the training work for the service guide for the blind. Dogs (age = 17 ± 1 months, initial body weight [BW] = 26.3 ± 1 kg, and body condition score [BCS] = 4.5 ± 0.11) were divided into 2 homogeneous groups for sex (half males and half females). Dogs in the grain free (GF) group were fed a commercial diet characterized by the absence of grain cereals, and dogs in the control (CTR) group were fed a super-premium pet food characterized by conventional grains as the carbohydrate source. The trial lasted 84 d, preceded by a 7-d of adaption period. Physical examination, digestibility, and faecal characteristics were studied. The statistical model included the effects of diet (GF vs. CTR), time (from d 0 to 84, end of the trial) and the interaction (diet × time). The high-protein, low-carbohydrate dry diet (GF) offered higher apparent nutrient digestibility of protein (+10%; $P = 0.002$) and fat (+7%; $P < 0.001$) and more stable large intestinal fermentation of carbohydrate compared to the commercial high-carbohydrate dry diet, enabling dogs to use nutrients from the diet more efficiently and thus requiring less food (−13%) to satisfy their nutrient requirements, producing less excrement (−33%; $P = 0.033$), and reaching a higher final BW (+8%; $P < 0.0001$) and a higher final BCS (+15%; $P = 0.003$). Therefore, the GF diet appears the nutritional plan most suitable for these animals taking due account not only of the training work done by animals with their increased nutrient and energy needs, but also of the gastrointestinal disorders consequent to stress coming from work and life in kennels, which cause in the Labrador retrievers an unusual weight loss.

1. Introduction

Athletic performance of a working dog is the result of a complex interaction between genetic, workout and nutrition, so no good result can be obtained without one of these 3 elements. Although genetic selection and training are usually done by the breeder and trainer work, nutrition is often underestimated and not properly formulated.

Our research was addressed to identify a nutritional management for guide dogs of blind people, because of the increasing demand in the training centres of dogs that can make the blind people more independent in daily activities. The centre mostly provides Labrador Retriever dogs, characterized by a markedly peaceful temperament with an innate sense of direction, and also commonly provides Golden Retriever and German Shepherd.

In Sicily, the regional government established (Regional Government Law, 2001) the Centre Helen Keller of the Italian Blind and Guide Dog School Union (www.centrohelenkeller.it). The centre is unique in the south of Italy, and it is a member of the International Guide Dog Federation (IGDF), and as such accredited

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to the highest international standards. Each animal of the centre, at about 1 year of age, is assigned to an operator of the centre to begin training, which lasts 5 to 6 months. During the life at the centre, dogs are individually housed, adjacent to a large outdoor space where they can be accessed during rest. Daily, animals perform 2 working sessions, 1 to 3 h per session, both in the school and in the city centre. When dogs end the training period for the guide service for the blind, they must pass an exam in presence of a technical expert. Then, the animal begins the stage of personalized training with the operator of the centre and the blind person to which will be assigned. Annually, the Centre Helen Keller consigns to blinds about 25 dogs, mainly in Italy but also in foreign countries, which signed an agreement with the centre (Malta, Albania, Jamaica, etc.).

A nutritional plan most suitable for the animals trained as guide for blind people must take in account not only of the animal training work, which increases nutrient and energy requirements, but also of the gastrointestinal disorders consequent to stress derived from the changes of life style (work and kennel conditions), which cause to the Labrador retriever, called family dogs, an unusual weight loss such as observed, compared to other breeds in the Livestock Working Dog Animal Welfare Code (2012). In this direction, the potential application of results will be the possibility to obtain diets for working dogs most useful to limit the metabolic, physic and mental stress and to improve animal performances as well as the possibility to set up diagnostic tests with the aim to identify animal welfare. In this way, it will be possible to address the nutritional management of working dogs toward the changed nutritional requirements paying attention more and more aware of the correlation between diet and animal health through the detection of strategies to optimize the nutritional animal status.

Scientific literature regarding the specific nutritional needs of working dogs for the service guide for the blind appears still incomplete and controversial.

As well-known, physical activity causes, first, the increase of energy consumption that changes in relation to the type, intensity, duration of work, and in relation to the environmental conditions in which the animal is working (Case et al., 2000); this complicates the assessment of energy requirements and may influence the digestive tolerance, defined as the overall reaction of the animal to diet in the absence of pathological condition. Previous studies reported poor faecal quality (i.e., faeces of low consistency with high moisture content) in large dogs compared with small dogs (Meyer et al., 1999; Weber et al., 2002), and in certain sensitive breeds such as German Shepherd dogs (Nery et al., 2010; Zentek et al., 2002) fed the same diet.

Knowledge about the undigested protein fractions and their fermentability throughout the different parts of the intestine is still scarce (Pieper et al., 2016). Nonetheless, it is well established that in feedstuffs of lower quality, more undigested dietary proteins will enter the hindgut (Kambashi et al., 2014). Protein quality and quantity may affect the composition of the gastrointestinal microbiota as well as the end products of fermentation should it remained undigested. If a diet contained a protein source of lesser quality, it may affect the amount of protein that remained undigested at the terminal ileum (Yamka et al., 2006), thus affecting the type and amount of protein catabolites generated through fermentation in the colon. Moreover, dietary (i.e. digesta viscosity, dietary particle size, buffering capacity, anti-nutritional factors, diet changes) as well as endogenous factors (i.e. stress or other hormonal signals affecting gastric emptying rate and feed intake) can lead to conditions that may favour bacterial fermentative activity. Understanding the factors influencing intestinal bacterial protein fermentation, the formation of toxic metabolites and subsequent influence on the host is crucial to develop dietary strategies to maintain gastrointestinal tract health.

Some fermentation products, such as short chain fatty acid (SCFA), are important energy sources for the colonic mucosa (Ichikawa and Sakata, 1998). Others like ammonia, amines, phenol, indole, branched chain fatty acids (BCFA) and sulphides can have deleterious effects (Gibson et al., 1989; Hughes et al., 2000; Hussein et al., 1999; Ichikawa and Sakata, 1998; MacFarlane et al., 1988; Mouillé et al., 2004; Musco et al., 2018).

However, to our knowledge, only limited data are available in the literature on the influence of protein quantity on digestive tolerance related characteristics in dogs (Murray et al., 1997; Zentek, 1995a, b; Zuo et al., 1996). If the aim is to decrease fermentation in the hindgut, the choice of protein source used in the dietary formula is of greater importance than protein content. Therefore, nutritional strategies based on manipulation of the protein source and content would be adequate to improve faecal quality in large and sensitive dogs. In particular, using highly digestible protein sources and decreasing the dietary crude protein content could have beneficial effects on fermentation phenomena in the hindgut, and consequently on digestive tolerance (Nery et al., 2012).

In this way, the aim of the study was to evaluate the effects of 2 different diets characterized by the absence of cereals or by the presence of conventional cereals on the nutritional status, diet digestibility and faecal quality of working dogs with a focus on Labrador Retriever, during the training work for the service guide for the blind.

2. Materials and methods

2.1. Animals and diets

The research protocol complies with guidelines of Good Clinical Practices (EMEA, 2000). This study was performed in accordance with the ethical principles that have their origins in the Italian Veterinarians' Ethical Code (Passantino, 2007), and the Italian and European regulations on animal welfare (Anon, 2014; Directive, 2010/63/EU). On the basis of the Italian Legislative Decree, 2014, article 2, the research received the institutional approval by the Ethical Animal Care and Use Committee of the Department of Veterinary Science, of the University of Messina on October 19th 2016, Codex 006/2016 bis.

The study was carried out on 8 neutered adult dogs belonging to the Labrador Retriever breed, housed at the Regional Centre Helen Keller of the Italian Blind and Guide Dog School Union, in Messina (Italy), during the training work to guide service for the blind.

The training consisted of a various phases program in which the dog gradually learned more guide work. This included leading a person in a straight line, stopping at any change in ground elevation as well as overhead obstacles and obstacle avoidance. Feed rewards were used in the guide dogs for the blind training program as a powerful motivation and reinforcement tool for learning and maintaining desired behaviour. During each training session (at d 0, 28, 56, and 84), dogs were introduced to the following specific guide-work behaviours:

- Stopping at streets, regardless of the type of curb or wheelchair ramp;
- Clearing the space around the handler on the right and left sides as well as above the dog's head;
- Crossing streets on a line that efficiently reaches the up curb on the other side;
- Maintaining consistent pace and drive with the verbal cue “forward”;
- Responding to the various uses of the ‘hop-up’ verbal cue – resuming or increasing pace; moving closer to a stopping point; or re-focusing;
- Stopping and standing calmly after the verbal cue “halt”;

- Leading the handler in a 90° turn to the right and picking up the new travel line on “right”;
- Leading the handler in a 90° turn to the left and picking up the new travel line on “left”.

The guide dogs for the blind were trained 3 times a week. Each training session lasted approximately 60 min.

On February, before the beginning of the trial, in order to assess the health status, all dogs were submitted to a physical examination (Ciaramella, 2014), complete blood count, biochemical examination, and urine analysis (data not shown).

Moreover, Indirect Immune Fluorescent Antibody Test (IFAT) for *Leishmania infantum* diagnosis, research of the main vector-borne diseases (Knott and immune-chromatographic test), coprological exams for endoparasite presence were performed.

On February, dogs admitted to the study were divided into 2 homogeneous groups for sex (half males and half females), age (17 ± 1 months), initial BW (BW, 26.3 ± 1 kg) and body condition score (BCS, 4.5 ± 0.11).

The grain free (GF) group received a commercial diet (N&D grain free lamb & blueberry, Farmina Pet Food, Italy), characterized by the absence of grain cereals and by a minimum quantity of carbohydrate from potato; whereas, the control group (CTR) received a super-premium pet food characterized by conventional grains such as carbohydrate source. All the diets were characterised by lamb meal as the main protein source.

Dogs were individually housed in pens of 6 square meters, and the food was administered 2 times a day, in an individual bowl.

The trial was preceded by a 7-d adaptation period (Anonymous was a super-premium pet food usually used in the centre during training work); the quantity of administered diet was the same adopted by the breeder previously.

During the trial, the company sent 3 lots of feed. Each lot was sampled and analysed, separately. Each diet (GF and CTR) was subsampled, composited and lyophilized, and then was ground with dry ice through a 2-mm screen. Diet subsamples (approximately 100 g) were taken twice during each collection period; each subsample consisted of approximately 25 g taken from 4 different sections of the mixer. Diets were evaluated for dry matter (DM), organic matter (OM), and crude protein (CP) (AOAC, 2006); the acid-hydrolysed fat (AACC, 1983; Budde, 1952) and total dietary fibre (TDF) (Prosky et al., 1992) content was determined.

The chemical composition of the diets is reported in the Table 1. At the beginning of the trial (March 1st), the amount of food, daily administered to each dog was calculated on the ratio between the calculated metabolizable energy (ME) requirements, as proposed by Yu et al. (1971), for adult dogs at maintenance and the caloric density of ME of each diet administered, reported in the label by Farmina company:

$$\text{ME requirement} = 145 \times \text{BW}^{0.67} \times k_1 \times k_{2,}$$

where k_1 is the coefficient in relation to the breed, k_2 is the coefficient in relation to the temperament.

During the trial, in relation to the weight and BCS changes, the amount of food had been updated. It was calculated on the ratio between the calculated ME requirements, as proposed by the European Pet Food Industry Federation (FEDIAF) guideline (2018), for adult dogs at maintenance and the caloric density of ME, reported in the label (NRC, 2006), of each diet administered.

From the 6th of April in relation to the weight and BCS loss, the amount of food had been updated. It was calculated on the ratio between the calculated ME requirements, as proposed by Hand et al. (2010), for dogs that perform work, characterized by a moderate duration and frequency:

$$\text{DER} = 2 \times \text{RER}\,(70 \times \text{BW}^{0.75}),$$

where DER is the daily energy requirements, RER is the resting energy requirements, and the caloric density of ME of each food administered. The same formula was updated until the end of the trial in relation to the animal BW.

Table 1

Chemical composition and metabolizable energy (ME) of the diets administered to dogs before and during the trial (g/100 g, as-fed).[1]

Item	Diets[2]		
	Anonymous[3]	GF[4, 5]	CTR[4, 6]
Moisture	9.0	5.42 ± 0.48	6.12 ± 0.57
CP	26	39.24 ± 0.84	24.40 ± 0.32
Fat	15.50	18.69 ± 0.51	11.78 ± 0.29
OM	n.d.	86.83 ± 0.27	86.50 ± 1.20
TDF	2.80	11.59 ± 1.13	13.03 ± 1.46
Ash	4.9	7.91 ± 0.23	7.51 ± 0.55
ME, kcal/kg	3,900	4,330	3,423

CP = crude protein; OM = organic matter; TDF = total dietary fibre.

[1] Values are means ± standard deviation.

[2] Anonymous: super-premium pet food, administered before the trial; GF: grain free diet without grain cereals, administered during the trial; CTR: control diet with conventional grain cereals, administered during the trial.

[3] Values as reported in the label.

[4] Values determined analytically.

[5] Ingredients: fresh grass fed lamb (26%), dehydrated lamb meat (25%), potatoes, dried whole eggs, fresh herrings, dehydrated herring, chicken fat, herring oil, vegetable pea fiber, dried carrots, sun-cured alfalfa meal, inulin, fructooligosaccharide, mannan-oligosaccharides, dehydrated blueberry (0.3%), dehydrated apple, dehydrated pomegranate, dehydrated sweet orange, dehydrated spinach, psyllium seed husk, currant powder, salt, brewers dried yeast, turmeric (0.2%), glucosamine, chondroitin sulfate, tagete flower extract. Analytical compounds (as reported in the label): calcium 1.40%, phosphorus 0.90%, omega-6 fatty acids 3.30%, omega-3 fatty acids 0.90%, docosahexaenoic acid (DHA) 0.50%, eicosapentaenoic acid (EPA) 0.30%, glucosamine 1,200 mg/kg, chondroitin sulfate 900 mg/kg. Nutritional additives (per kilogram, as reported in the label): vitamin A 15,000 IU, vitamin D_3 1,500 IU, vitamin E 600 mg, vitamin C 150 mg, niacin 37.5 mg, pantotenic acid 15 mg, vitamin B_2 7.5 mg, vitamin B_6 6 mg, vitamin B_1 4.5 mg, vitamin H 0.38 mg, folic acid 0.45 mg, vitamin B_{12} 0.1 mg, choline chloride 2,500 mg, beta-carotene 1.5 mg, zinc chelate of the analogous methionine hydroxylase 910 mg, manganese chelate of the analogous methionine hydroxylase 380 mg, ferrous chelate of glycine hydrate 250 mg, copper chelate of the analogous methionine hydroxylase 88 mg, selenomethionine 0.40 mg, DL-methionine 4,000 mg, taurine 1,000 mg, L-carnitine 300 mg. Organoleptic additives: aloe vera extract 1,000 mg, green tea extract 100 mg, rosemary extract. Antioxidants: tocopherol-rich extracts of natural origin.

[6] Ingredients: dehydrated lamb meat (28%), rice (28%), corn (23%), chicken fat (5%), dehydrated fish, dried beetroot pulp (4%), dehydrated whole eggs (4%), fish oil (2%), vegetable oil (1%), sodium chloride, dried brewer's yeast (0.3%). Analytical compounds (as reported in the label): calcium 1.20%, phosphorus 0.90%. Nutritional additives (per kilogram, as reported in the label): vitamin A 10,000 IU, vitamin D_3 1,000 IU, vitamin E 100 mg, vitamin C 100 mg, niacin 25 mg, pantotenic acid 10 mg, vitamin B_2 5 mg, vitamin B_6 4 mg, vitamin B_1 3 mg, vitamin H 0.25 mg, folic acid 0.30 mg, vitamin B_{12} 0.04 mg, choline chloride 1,500 mg, zinc oxide 108 mg, zinc sulphate monohydrate 120 mg, manganous sulfate monohydrate 150 mg, ferrous sulphate monohydrate 44 mg, ferrous carbonate 60 mg, copper sulphate pentahydrate 50 mg, calcium iodate anhydrous 2.4 mg, sodium selenite 0.22 mg, DL-methionine 1,500 mg.

2.2. Physical examination

Weekly, to evaluate the performance of the studied dogs, from the d 0 (start of the administration of the new food) to d 84, all dogs were undergoing physical examinations (Ciaramella, 2014) including:

- Mentation (level of consciousness);
- Posture and gait (watching the animal walk to exam area or to kennel);
- Hydration status - (evaluated from the elasticity of the skin and classified in “adequate”, “marginal” or “inadequate”);

- Rectal temperature (°C);
- Pulse rate (evaluating such pulse of femoral artery);
- Respiratory rate and breath character;
- Perfusion indicators (mucous membrane colour and capillary refill time).

At the same time, on each animal, the following parameters were evaluated: BW, BCS, muscle condition score (MCS), faecal consistency score (FCS) and feed intake.

The determination of BW was measured on fasted animals, in the morning at 09:00, by using a digital scale.

Body condition score was evaluated by assigning a rating scale that ranged from 1 (too thin) to 9 (too heavy) using the table proposed by Nestle Purina (Kealy et al., 2002; Laflamme, 1997; Mawby et al., 2001).

Muscle condition score was assessed using a scale, currently under development and validation, consisting of a score ranging from 3 (no muscle wasting, normal muscle mass) to 0 (marked muscle wasting). The evaluation of muscle mass included the visual examination and the palpation over the temporal bones, scapulae, ribs, lumbar vertebrae, and pelvic bones (Baldwin et al., 2010).

Faecal consistency score was evaluated by assigning a rating scale that ranged from 1 (dry stools) to 7 (liquid stool) using the table proposed by Moxham (2001).

2.3. Digestibility and faecal characteristics

Monthly, for each dog, total faecal output was collected, mixed, dried at 55 °C, and ground through a 2-mm screen. Composited faecal samples were analysed for DM, OM, CP and fat content, as described for diets. Apparent nutrient digestibility values were calculated using the following equation (Kerr et al., 2013):

$$\text{Apparent nutrient digestibility (\%)} = 100 \times [\text{Nutrient intake (g/d)} - \text{Faecal output (g/d)}]/\text{Nutrient intake (g/d)},$$

From d 0, every month, dogs were checked every 15 min in order to obtain a freshly voided faecal sample for protein catabolite component (ammonia, amines, SCFA, BCFA, phenols, and indoles) analysis. One aliquot of faeces (5 g) was acidified with 2 mol/L hydrochloric acid for ammonia, SCFA, and BCFA analysis. For amines, 2 g of sample were weighed in duplicate into 50-mL centrifuge tubes. For phenol and indole components, 2 g of sample were weighed in duplicate into 16-mL centrifuge tubes. All samples were immediately stored at −20 °C until analyses, in order to minimize any loss of volatile components (Propst et al., 2003).

Phenol and indole components were extracted by mixing 2 g of faeces with 5 mL of methanol containing 500 mg/kg 5-methyl indole (internal standard). The faeces-methanol mixture was covered with parafilm, well mixed and incubated for 1 h at 4 °C with frequent mixing. Then, tubes were centrifuged at 29,000 × *g* at 4 °C for 15 min and the supernatant was collected. The remaining pellet was mixed again with 5 mL of methanol and extracted, as detailed above. The 2 supernatant fractions were combined for high resolution gas chromatographic analysis (Flickinger et al., 2003).

Faecal samples for SCFA (acetate, propionate, and butyrate) and BCFA (iso-butyrate) were acidified and diluted 1:5 with 25% metaphosphoric acid. After 30 min, samples were centrifuged at 20,000 × *g* at 4 °C for 20 min. The supernatant was transferred into microfuge tubes and frozen at −20 °C. Following freezing, the supernatant was thawed, centrifuged at 13,000 × *g* at 4 °C for 10 min, and analysed for SCFA and BCFA content, via gas chromatography (Erwin et al., 1961). Concentrations of acetate, propionate, butyrate, and iso-butyrate were determined in the supernatant fluid of acidified faecal aliquots. In the standard solutions, the limit of quantification (LOQ; signal to noise ratio >10) was 0.9 μmol/g for iso-butyrate.

Biogenic amines (phenylethylamine, putrescine, and cadaverine) were analysed as follows: wet faeces (2 g) were mixed with 15 mL of 0.4 mol/L perchloric acid and centrifuged at 2,000 × *g* at 4 °C for 10 min. After aspirating the supernatant, the remaining faecal pellet was mixed with 7 mL of 0.4 mol/L perchloric acid and centrifuged at 2,000 × *g* at 4 °C for 10 min. The supernatants were combined and centrifuged at 12,500 × *g* at 4 °C for 5 min (Flickinger et al., 2003). An aliquot of 1 mL of the final extract was mixed with 5 mL of water, and the pH was adjusted to 9. After o-phthalaldehyde (OPA) derivatization (Kalkan Yýldýrým et al., 2007), biogenic amines were quantified using high performance liquid chromatography (HPLC) with fluorescence detection, according to the method proposed by Preti et al. (2015). In the standard solutions, the L.O.Q. was 0.002 mg/g for phenylethylamine and cadaverine.

Concentrations of ammonia in faeces were determined colorimetrically by spectrophotometry.

2.4. Parasitological evaluation

Before the inclusion of dogs in the trial (d −7), to exclude the presence of parasitic diseases, parasitological exams were performed. In detail, from each dog, a blood sample was collected and divided in 3 aliquots. The first aliquot used to obtain the serum by centrifugation and immunofluorescence antibody test (IFAT) technique and used for the diagnosis of leishmaniosis (Otranto et al., 2009). The second and third aliquots were transferred into test tubes containing K_3EDTA and were used, respectively, for the diagnosis of filariosis through the modified Knott test (Magnis et al., 2013) and to search for *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* and *Anaplasma platys* through rapid immunechromatographic tests. In addition, all dogs were checked by copromicroscopical investigations before inclusion, and every 15 d until the end of the test (total 7 copromicroscopical controls). These times were dictated by the chance of acquisition during the study of some parasites with direct life-cycle which present a very short prepatent period. Faecal samples were maintained at refrigeration temperature and processed within 24 h. The technique used was the flotation with 2 different saline solutions, $NaNO_3$ solution (density 1,300) and zinc sulphate solution (density 1,180) to research respectively eggs of helminths and protozoan cysts (MAFF, 1986). The dogs resulted positive to the presence of parasites were promptly treated and tested again after 7 d.

2.5. Statistical analyses

Data were submitted to statistical analysis by ANOVA using the General Linear Models procedure of SAS (2000) and SPSS (2008). The statistical model included the effects of diet (GF vs. CTR), time (d 0 to 84) and the interaction (diet × time). Data were in least squares means (LSM); the comparison between the LSM was analysed with the Tukey test. The differences between the means were considered significant for $P < 0.05$.

3. Results

3.1. Nutrient intake and growth performances

The amount of food administered to each animal per group during the trial (GF vs. CON) was at d 0: 253 vs. 335 g, at d 21: 287 vs. 335 g, at d 35: 360 vs. 480 g, and at d 63: 370 vs. 425 g. Every day, the food was weighed in an accurate way.

The results of the BW during the trial (Table 2) were significant ($P < 0.0001$) affected by the diet, showing a higher mean value in the GF group than in the CTR group and by the time showing a significant ($P < 0.05$) decrease in the dogs of the CTR group from d 0 to 84 but no significant difference in the GF group (Table 3). The interaction diet × time showed no significant differences ($P = 0.270$). This could be due to the high variability of the BW in each group during the trial.

In contrast to these observations, the trend of the BCS over the whole period of the trial (Table 2) showed significant ($P = 0.003$) differences in relation to the diet with a higher value in the GF group than that in the CTR group, but no significant differences were observed in relation to the time ($P = 0.997$) and to the interaction diet × time ($P = 0.991$) for the whole trial period. All the animals showed a BCS within the ideal range (score 4 and 5).

As regard the MCS (score 3 to 0), no significant differences in relation to the diet ($P = 0.055$), time ($P = 0.938$), and the interaction diet × time ($P = 0.997$) was observed (Table 2). Body condition score and MCS are not directly related. Nevertheless, the BCS does not take in account muscle wasting in animals scoring >2 (Michel et al., 2004), and consequently the MCS was considered to evaluate muscle mass. An animal can be overweight but still have significant muscle loss. Assessing muscle condition is important as muscle loss is greater in patients with most acute and chronic diseases (i.e., stressed starvation) compared to healthy animals deprived of food when primarily fat is lost (i.e., simple starvation) (Baldwin et al., 2010). Therefore, with regard to assessing the impact of the catabolic state on the nutritional status of a patient, assessment of muscle wasting may have the prognostic significance (Michel et al., 2011). In our trial, no animals showed acute and chronic diseases; for this reason, the results of MCS did not cause any concern.

The effect of environmental temperature is unlikely to play a significant role in this population's energy requirement. It is known that temperatures outside of the thermoneutral zone of 20 to 30 °C will increase energy requirements by 1 to 5 kcal × $BW^{0.75}$ per °C per day when above or below this zone (NRC, 2006). During the 3 months of the study period (March 1st to May 24th), the dogs spent their time in thermoneutral zone; considering the kennel's geographic location, it was unlikely that the temperatures at night dropped below the thermoneutral zone.

Table 2
Effect of the diet on BW, BCS and MCS in the trial.[1]

Items	Groups[2]		SEM	P-value
	CTR	GF		
BW, kg	23.44^{b}	25.40^{a}	0.21	<0.001
BCS, score 1 to 9	4.01^{b}	4.64^{a}	0.12	0.003
MCS, score 0 to 3	2.62	2.33	0.09	0.055

BW = body weight; BCS = body condition score; MCS = muscle condition score.
a, b Within a row, means with different superscript letters were significantly different ($P < 0.05$).
[1] Values are least square means (LSM) ± standard error of the mean (SEM).
[2] GF: grain free diet without grain cereals; CTR: control diet with conventional grain cereals.

3.2. Digestibility and faecal characteristics

As regard the FCS (Table 4) during the trial, the diet showed no significant differences ($P = 0.27$) whereas, the variable time ($P < 0.0001$) and the interaction diet × time ($P = 0.018$) showed significant differences with a higher mean value at d 14 in the CTR group than that in the GF group (Table 4). These results show no relation with the parasitological evaluation. In fact, only at d 28 two dogs which, moreover, belonged to the GF group, resulted positive to the coccidia presence whereas, the other dogs were negative to the coprological control.

Except for the values recorded at d 14 in the faeces of the CTR group, all the animals over the whole period of the trial (Table 5) showed a FCS within the ideal range (score 2 and 3).

The quantity of faeces was influenced only by the diet over the whole period of the trial showing significant ($P = 0.033$) lower quantities in the GF group (180 g/d) than those of the CTR group (268 g/d). No significant differences were observed for the variable time ($P = 0.525$) and for the interaction diet × time ($P = 0.967$).

As regards the chemical composition of the faeces during the trial (Table 6), diet affected significantly CP ($P = 0.002$), total fat ($P < 0.0001$), and ash ($P = 0.001$). The GF group showed higher excretion than the CTR group of crude protein and ash and the lower excretion of total fat. The chemical composition of faeces showed no significant differences for time and the interaction for the diet × time.

Over the whole period of the trial (Table 4), the diet influenced the protein and fat digestibility, showing a significant higher value in the GF group than the CTR group for protein ($P = 0.002$) and fat digestibility ($P < 0.001$). Time and the interaction diet × time of digestibility showed no significant differences for protein, fat, DM, and OM digestibility.

On the whole, the protein digestibility in the GF group similar to he 80% value reported in the literature by Atwater (1916) and the fat

Table 4
Effect of the diet on FCS and apparent nutrient digestibility in the trial (%).[1]

Item	Groups [2]		SEM	P-value
	CTR	GF		
FCS, score 1 to 7	2.58	2.72	0.07	0.266
Apparent digestibility, %[3]				
Protein	77.2^{b}	85.3^{a}	1.44	0.002
Fat	90.67^{b}	96.62^{a}	0.49	<0.0001
DM	79.83	79.57	1.70	0.921
OM	84.94	87.29	1.21	0.233

FCS = faecal consistency score; DM = dry matter; OM = organic matter.
a, b Within a row, means with different superscript letters were significantly different ($P < 0.05$).
[1] Values are least square means (LSM) ± standard error of the mean (SEM).
[2] GF: grain free diet without grain cereals; CTR: control diet with conventional grain cereals.
[3] Apparent nutrient digestibility = 100 × (Nutrient intake [g/d] − Faecal output [g/d])/Nutrient intake (g/d).

Table 3
Effect of the diet and time on body weight in the trial (kg).[1]

Groups[2]	Days													
	−7	0	7	14	21	28	35	42	49	56	63	70	77	84
CTR	26.5	26.8^{a}	25.4^{ab}	25.0^{ab}	24.3^{ab}	23.2^{ab}	22.9^{ab}	22.8^{ab}	22.3^{ab}	22.1^{b}	22.7^{ab}	22.8^{ab}	22.8^{ab}	22.0^{b}
GF	24.9	26.8	25.4	25.4	24.7	25.2	24.9	25.1	25.1	25.0	25.7	25.7	25.8	25.8

a, b Within a row, means with different superscript letters were significantly different ($P < 0.05$).
[1] Values are least square means (LSM).
[2] GF: grain free diet without grain cereals; CTR: control diet with conventional grain cereals.

Table 5
Effect of the diet and time on faecal consistency score (FCS) in the trial.[1]

Groups[2]	Days													
	−7	0	7	14	21	28	35	42	49	56	63	70	77	84
GF	2.42	2.83	3.08	3.00^b	3.00	2.50	2.38	2.42	2.25	2.67	2.42	2.33	2.33	2.33
CTR	3.38	2.75^b	2.50^b	4.63^a	2.38^b	2.25^b	2.75^b	2.63^b	3.00^b	2.50^b	2.75^b	2.13^b	2.25^b	2.63^b

[a, b] Within a row and within a column, means with different superscript letters were significantly different ($P < 0.05$).

[1] Values are least square means (LSM; score 1 to 7).

[2] GF: grain free diet without grain cereals; CTR: control diet with conventional grain cereals.

digestibility was higher in the experimental GF group (97%) and lower in the CTR group (91%) than the 92% value reported in the literature by Atwater (1916).

3.3. *Organic acid content in faeces*

During the trial (Table 7), the variables diet influenced significantly the propionate ($P < 0.0001$) and the butyrate ($P = 0.026$); the mean values of the propionate and butyrate content were significant lower in the GF group than the CTR group.

Time influenced significantly the propionate ($P = 0.008$) and butyrate ($P = 0.004$) content, showing a decrease from d 0 to 84, and the interaction diet × time affected significantly the acetate ($P = 0.039$), propionate ($P = 0.016$) and the sum of the organic acids ($P = 0.032$).

The content of iso-butyrate at d 84 was below the L.O.Q. Isobutyrate showed a wide variability even if, this organic acid did not reach the significance in relation to the diet ($P = 0.459$), to the time ($P = 0.279$) and to the interaction diet × time ($P = 0.097$). Branched chain fatty acids are formed by deamination reaction from branched chain amino acids such as valine (iso-butyrate), and arethus indicators of microbial amino acid metabolism (Ríos-Covián et al., 2016).

As regards the sum of SCFA and BCFA, despite of the higher protein content (Yamka et al., 2003) of the GF diet (39.24%, as-fed) than the CTR diet (24.40%, as-fed), dogs of the GF and CTR groups showed similar ($P = 0.913$) content of the sum of organic acids in the faeces (Table 7).

Table 6
Effect of the diet on chemical composition of faeces in the trial (g/100 g, as-fed).[1]

Item	Groups[2]		SEM	*P*-value
	CTR	GF		
DM	31.68	32.50	0.94	0.591
CP	8.15^b	10.37^a	0.39	0.002
Fat	1.61^a	1.12^b	0.06	<0.0001
OM	21.67	18.88	0.88	0.057
Ash	10.01^b	12.39^a	0.39	0.001

DM = dry matter; CP = crude protein; OM = organic matter.

[a, b] Within a row, means with different superscript letters were significantly different ($P < 0.05$).

[1] Values are means (LSM) ± standard error of the mean (SEM).

[2] GF: grain free diet without grain cereals; CTR: control diet with conventional grain cereals.

Table 7
Effect of the diet on organic acids content of faeces in the trial (μmol/g).[1]

Item	Groups[2]		SEM	*P*-value
	CTR	GF		
Acetate	201.54	219.74	14.60	0.431
Propionate	49.79^a	35.62^b	2.78	<0.0001
Butyrate	28.82^a	21.24^b	2.09	0.026
Isobutyrate	12.62	14.32	1.41	0.459
SOA	287.47	284.56	16.84	0.913

SOA = sum of organic acids.

[a, b] Within a row, means with different superscript letters were significantly different ($P < 0.05$).

[1] Values are means (LSM) ± standard error of the mean (SEM).

[2] GF: grain free diet without grain cereals; CTR: control diet with conventional grain cereals.

3.4. *Ammonia, indole and biogenic amines in faeces*

Concerning biogenic amines, in each sample, the phenol resulted under the L.O.D (0.001 mg/g) and/or under the L.O.Q (0.016 mg/g), therefore, it was not identified and/or quantified. Also, phenylethylamine resulted under the L.O.Q (0.002 mg/g) and was not quantified. The biogenic amines identified and quantified were putrescine and cadaverine.

For the whole trial period (Table 8), the variable diet influenced the biogenic amine content showing significant higher values in the GF group than the CTR group for putrescine ($P = 0.009$) and for the sum of biogenic amine ($P = 0.017$), but also for ammonia ($P = 0.050$) and indole ($P = 0.005$).

The variable time affected ammonia ($P = 0.015$) and indole ($P = 0.035$), whereas, the interaction diet × time did not influence ammonia, indole, and the biogenic amine content.

3.5. *Parasitological evaluation*

During pre-screening (d −7), all tested dogs were negative to *L. infantum*, microfilariae presence and vector-borne diseases, i.e. ehrlichiosis, anaplasmosis, borreliosis. At the coprological test, only one dog was positive to *Trichuris vulpis* eggs. The dog was immediately treated with pyrantel and praziquantel (Drontal plus flavour, Bayer SpA) using the recommended dosage of one tablet every 10 kg of BW. The dog was negative at the beginning of the trial (d 0).

During the trial, every 15 d, the dogs were subjected to copromicroscopical evaluation. At d 28, 2 dogs belonging to the GF group resulted positive to the coccidia presence were properly treated with sulfametopyrazine (Vetkelfizina oral solution, Ceva Vetem SpA), with the recommended dosage of 1 mL/kg of BW. One week later, at d 35, the 2 tested dogs were negative to the coprological control.

From d 35, all dogs were negative to the controls until the end of the study (d 84). None positive dogs had symptoms related to the presence of parasites such as diarrhoea and or weight loss.

4. Discussion

4.1. *Growth performances*

All the animals during the trial lost weight; this could be due to the training work for the service guide for the blind; but while the animals of the CTR group showed the highest BW loss (−4.8 kg), the GF group showed the lowest BW loss (−1.0 kg). This could be

Table 8
Effect of the diet on ammonia, indole and biogenic amine content of faeces in the trial (mg/g).[1]

Item	Groups[2]		SEM	P-value
	CTR	GF		
Ammonia	1.225^{b}	1.451^{a}	0.07	0.050
Indole	0.084^{b}	0.125^{a}	0.01	0.005
Putrescine	0.141^{b}	0.364^{a}	0.05	0.009
Cadaverine	0.050	0.152	0.04	0.128
SBA	0.191^{b}	0.516^{a}	0.07	0.017

SBA = sum of biogenic amines.

[a], [b] Within a row, means with different superscript letters were significantly different ($P < 0.05$).

[1] Values are means (LSM) ± standard error of the mean (SEM).

[2] GF: grain free diet without grain cereals; CTR: control diet with conventional grain cereals.

related to amount of all nutrient in relation to the energy intake, which seems more proportionated in the GF group than the CTR one. Nevertheless, weight loss is normal in guide dogs during the training; this is related to the exercise and the life in kennel. However, the GF group showed better BCS and MCS than the CTR group; this could testify the satisfaction of the nutritional needs of the dogs of the GF group.

To date, very few publications include a BCS in combination with a MCS. Taking into account that not only severe acute illness but also more chronic diseases can cause muscle wasting (Safranek et al., 2003), and that muscle wasting can be prevented even in patients with an optimal BCS (75% cats and 25% dogs with abnormal MCS can be ideal or obese) (Michel et al., 2004; Willems et al., 2015), stresses the necessity of performing a MCS in combination with the BCS in every patient.

Consumption of the carbohydrate free diets did not alter the lean body mass or body fat content of the dogs. An increase in the dietary protein intake did not influence body composition of the rats and dogs fed either high carbohydrate or carbohydrate free diets (Kawauchi et al., 2017).

4.2. Nutrient digestibility

The diet influenced the protein and fat digestibility, showing higher values in the GF group than the CTR group. Schauf et al. (2018) demonstrated that feeding a high protein, low carbohydrate diet compared with a commercial high carbohydrate dry diet offered higher apparent nutrient digestibility. The higher apparent digestibility of high protein diet for all nutrients except carbohydrate suggested that it conferred advantages over high carbohydrate diet. Diet, enabling dogs to use nutrients from the diet more efficiently and thus requiring less diet to satisfy their nutrient requirements (Schauf et al., 2018), and producing less excrement (Grandjean and Paragon, 1993). Hill et al. (2009) reported that the low carbohydrate and high protein developed for this study has the potential to be beneficial to working dogs because high protein diet being high in protein and low in carbohydrate is not only closer to the predatory diet the dog evolved on, and that was eaten by its wild ancestors (Case, 2005), but also appeared to confer advantages to the dogs, including higher digestibility of protein and fat and less large intestinal fermentation of carbohydrate.

4.3. Nutrient catabolites in faeces

The lower content of propionate and butyrate recorded in the faeces of the GF group than those in the CTR group could be due to the diets characterized by high protein content in the GF pet food and high carbohydrate content in the CTR pet food. Generally, SCFA (derived from carbohydrate or protein fermentation) are rapidly absorbed from the gut lumen and serve as energy substrates. Acetate acts mainly as precursor for fatty acids synthesis, whereas propionate is mainly used for gluconeogenesis in the liver. Butyrate, in turn, is mainly metabolized by epithelial cells and has been proposed as the main energy source for colonocytes (Hamer et al., 2008). The promotion of lactic acid bacteria and SCFA production (e.g. specifically butyrate) is considered beneficial for the host and may help to maintain a balanced intestinal ecosystem. On the other hand, bacterial protein fermentation taking place mainly in the hindgut, represents a potential risk factor for a compromised intestinal barrier function and increased enteric disease caused by pathogens (Pieper et al., 2016). In addition to SCFA contributions to overall good health, individual SCFA exert specific health benefits. Propionate and butyrate can act independently to stimulate fluid absorption of calcium, magnesium, and other cations in the colon; this action also may be enhanced with the help of acetate (Scharrer and Lutz, 1990). Propionate may enhance the absorptive capacity of the colon by stimulating proliferation of the colonic epithelium, and acetate and propionate were implicated in having regulatory effects on lipid and cholesterol metabolism. Subjects given rectal infusions of acetate and propionate showed a dose-dependent increase in serum total cholesterol and triglyceride levels, providing indirect evidence that SCFA is utilized for lipid synthesis (Wolever et al., 1989). Butyrate, although contributing only 15% to 20% of total SCFA, seems to make the greatest contribution to the integrity of the colon. It is the preferred energy source for colonocytes, contributing directly to energy production and making a trophic effect on the colonic epithelium by increasing crypt height and cecum size (Propst et al., 2003; Topping, 1996).

The similar content of the sum of organic acids in the faeces despite the higher protein content of the GF diet (39.24%, as-fed) than the CTR diet (24.40%, as-fed) could be due to the higher digestibility of the dietary protein (Wiernusz et al., 1995) as discussed above, considering that proteins undigested in the small intestine are subjected to putrefaction in the large intestine, leading to the accumulation of fermentation products such as ammonia, amines, phenol, indole and sulphides, BCFA, SCFA, and gases (hydrogen, carbon dioxide, and methane), as well as intermediate products, such as lactate and succinate (Hughes et al., 2000; MacFarlane and Cummings, 1991).

As reported in the literature, protein catabolites derived from carbohydrate or protein fermentation in the intestine and may be affected by the intensity of fermentation occurring there. High fermentative activity results in an increase in bacterial biomass and considerable production of metabolites due to fermentation, essentially SCFA and lactic acid. As bacterial microflora can retain large amounts of water, its proliferation results in lowering water absorption. At the same time, high SCFA and lactic acid production in colon might disturb water absorption. The great osmotic power of these compounds could increase intra-luminal osmotic pressure resulting in water secretion and/or retention in the colon (MacFarlane and Cummings, 1991). Thus, electrolyte absorption on the one hand, and intensity of fermentation on the other, are 2 key determinants that may affect hydric phenomena. As water content in faeces is closely dependent on its absorption along the entire digestive tract, it would seem reasonable that these 2 parameters play an important role in the predisposition of large dogs to moist stool of poor consistency (Meyer et al., 1999; Zentek and Meyer, 1995).

Various studies have shown that diets can impact upon intestinal health and lead to a high faecal ammonia content. Our results could be related to the higher protein content of the GF diet than the CTR diet, which reflects the higher protein content in the faeces.

According to Zentek (1995a; b), the amount of protein fed may affect protein catabolite generation in the colon because the digestibility of CP would decrease with larger quantities consumed. Nevertheless, in this study, the protein digestibility in the GF group was higher than that in the CTR group. Another explanation could be related to the amount of microbial protein available to the microbiota for fermentation. This microbial protein then can be hydrolysed and fermented to phenols, indoles, and biogenic amines (MacFarlane and Cummings, 1991). These effects would explain the increased content of ammonia and indole in the GF group than those of the CTR group (Table 8), because AA are deaminated to produce these compounds.

Few studies have focused on the association between faecal components and protein source (Yamka et al., 2006). It is well documented that ammonia generated within the colon *in situ* is not only derived exclusively from urea, but also from bacterial deamination of amino acids, peptides, and proteins (Vince et al., 1976). Ammonia have been primarily associated with toxigenic and damaging effects on the intestinal epithelium in human beings (Blaut and Clavel, 2007; Davila et al., 2013). Ammonia can interfere with the oxidative metabolism of SCFA in colonocytes, likely inducing energy deficiency in the cell (Blachier et al., 2007). Whether this is due to direct toxigenic effect on the cells or mediated through specific receptors is not yet clear.

Moreover, the protein source (Nery et al., 2012; Zentek, 1995a; b) and protein content (Dong et al., 1996; Jha and Leterme, 2012; Nery et al., 2012), followed by the carbohydrate content of the diet (Hang et al., 2013; Muir et al., 2004; Nery et al., 2012; Wong et al., 2006), can influence the fermentation of amino acids by microorganisms into SCFA and ammonia to obtain energy (Nery et al., 2012). Therefore, the high content of protein and the lack of carbohydrates in the GF could explain the higher ammonia and indole content as well as the higher SCFA and BCFA content observed in the GF group and consequently to the deamination of amino acids and formation of ammonia; the remaining carbon skeleton can be further metabolized to yield SCFA and BCFA from branched chain amino acids. Thus, bacterial amino acid utilization also contributes to considerable amounts of acetate (e.g. from alanine, aspartate, glycine, threonine, lysine), propionate (e.g. from alanine, threonine) and butyrate (from lysine, glutamate). The faecal BCFA result from the degradation of proteins and their content is a marker of protein fermentation in the hindgut (Blachier et al., 2007; Cherbut et al., 1997).

The observed increases in SCFA and in beneficial biogenic amines, specifically putrescine in the GF group may serve to lessen the effects of the protein catabolites created, but this concept requires further investigation (Barry et al., 2009).

Biogenic amines (putrescine, spermine and spermidine), produced by decarboxylation reactions by bacteria in the colon, are present in all living cells and are required for the metabolic activity and growth process of tissues and organs of the body (Bardocz et al., 1993).

Biogenic amines have several effects on the host even if, little is yet known about their role. The role of polyamines such as putrescine has been studied in detail due to their enhancing effect on cell proliferation (Seiler and Raul, 2007). They stimulate DNA, RNA, and protein synthesis, making them important in the maturation of the intestinal and colonic mucosa (Delzenne et al., 2000; Tabor and Tabor, 1984). Cadaverine and putrescine may reduce the metabolism of histamine in the epithelium as both are also converted by diamine oxidase, thus possibly enhancing negative effect of histamine (Pietrzak et al., 2002).

However, putrescine, spermidine, and spermine are considered beneficial in reduced content as they are associated with normal cell turnover and involved in apoptosis (Delzenne et al., 2000).

5. Conclusions

It is worth noting that guide dogs for the blind are expensive to train, as well as being expensive in personal terms for all concerned if the post-qualification period is unsuccessful, thus this research is intrinsically important for the guide dog trade in several ways.

The starting point of this study was to evaluate the nutrient requirements for working dogs with a focus on Labrador Retriever during the training work in relation to the activity as aid for the blind through the evaluation of the type, intensity and frequency of extended work. In this trial, a low activity of guide dogs was estimated; the exercise was a bodily and mentally work, continuous and demanding, but physical exertion was not particularly intense.

The diets influenced significantly the animal performances in relation to their different protein and carbohydrate levels as well as to their different digestibility related to the ingredients of the formulas. The high-protein, low-carbohydrate dry diet (GF diet) offered higher apparent nutrient digestibility compared with the commercial high-carbohydrate dry diet (CTR), enabling dogs to use nutrients from the diet more efficiently and thus requiring less food (−13%, data not shown) to satisfy their nutrient requirements, producing less excrement (−33%), and reaching a higher final BW (+8%) and a higher final BCS (+15%). The high-protein, low-carbohydrate dry diet used in this study had the potential to be beneficial to working dogs because is not only closer to the predatory diet but also appeared to confer advantages to the dogs, including higher digestibility of protein (+10%) and fat (+7%) and more stable large intestinal fermentation of carbohydrate. Therefore, the GF diet, characterised by high CPand fat content, due to different energy distribution among nutrients, appeared the nutritional plan most suitable for these animals taking due account not only of the training work done by animals, with their increased nutrient and energy needs, but also of the gastrointestinal disorders consequent to stress coming from work and life in kennels which cause in the Labrador Retriever an unusual weight loss.

In relation to the expected results of the study, the most relevant was the increase of the knowledge in the field of dogs involved in animal-assisted intervention programs, through an increased knowledge of the breeding conditions and nutritional characteristics of the diet that may influence the metabolic processes involved in the working activity of the dogs.

Results evidenced that, from a practical standpoint, the nutritional approach is an important tool that may contribute to improve the physical and welfare status in dogs that will have to act throughout their lives as assistants to the blind.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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