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PhD THESIS

DIGESTIBILITY AND NUTRITIONAL ADEQUACY OF INNOVATIVE MATERIALS IN PET FOOD

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"The world is too much with us; late and soon,

Getting and spending, we lay waste our powers;

Little we see in Nature that is ours;

We have given our hearts away, a sordid boon!

This Sea that bares her bosom to the moon;

The winds that will be howling at all hours,

And are up-gathered now like sleeping flowers;

For this, for everything, we are out of tune;

It moves us not. Great God! I'd rather be

A Pagan suckled in a creed outworn;

So might I, standing on this pleasant lea,

Have glimpses that would make me less forlorn;

Have sight of Proteus rising from the sea;

Or hear old Triton blow his wreathèd horn."

The World is too much with us by WILLIAM WORDSWORTH

SUMMARY

A continuous and increasing effort has been promoted in the last few decades regarding environmental sustainability. Even tough pet food has always been considered as a valuable asset in reducing food waste there is still a wide margin of improvement. As such, new ingredients are constantly tested in the pet food market for both their nutritional value as well as their particular properties. One of the most promising ingredients can be considered insects, due to their potential sustainability features and nutritional aspects. Their high levels of both protein and fat content alongside their supposed low environmental footprint has gain the attention of the pet food market. Another set of ingredients which are increasing steadily in the pet food manufacturing are legumes. In particular, legumes by-product can obtain new value while providing interesting nutritional characteristics in pet food diets. Their higher level of protein and fibre compared to the more commonly used cereals can affect the gastrointestinal tract of dogs and cats.

Aim of the PhD project was to assess the nutritional quality, digestibility, safety and dietary characteristics of diets based on new materials for pet food production. Considering the novelty of insects, a diet based on black soldier fly larvae was tested for its digestibility as well as for its safety, following the nutritional adequacy trial according to AAFCO guidelines. Regarding legumes it was chosen to test lentils pasta by-product as this ingredient is discarded from the human-graded food production just for aesthetic reasons (e.g. discoloured or broken), retaining all its nutritional value for a possible use in pet food industry.

The first trial was conducted at the University of Turin (Italy) following FEDIAF Guidelines for *in vivo* digestibility trials using both total faecal collection and marker methods. Six West Highland White Terrier (3 males and 3 females, median of 3 years old) were allocated into 2 groups, one receiving an experimental diet based on black soldier fly larvae meal (BSF) while the other receiving a control diet based on venison meat (CTRL), in a cross-over design, so that each dog was the control of itself. The two diets were formulated to be isonitrogenous and isoenergetic, and they were also tested for *in vitro* digestion afterwards. Digestibility of nutrients for both TFC and marker methods were tested using two-way ANOVA for the comparison of both diets (CTRL vs BFL) and methods (TFC vs marker). The two methods used showed similar results while the diets differed regarding crude fibre digestibility (lower in the BSF diet compared to the CTRL diet), crude protein and calcium digestibility (higher in the BSF diet compared to the CTRL diet). *In vitro* results slightly overestimated OM and CP digestibility compared to *in vivo* results, even though they were substantially similar from a nutritional perspective. This overestimation was probably due to the fact that prediction equations

were derived from feedstuff based on vertebrates; as a consequence, diets formulated with insects may need further studies to derive more reliable prediction equations. The nutritional adequacy trial involved 16 mixed-breed dogs (8 males and 8 females, median of 5 years old) for a total of 26 weeks, according to AAFCO guidelines. The dogs were fed the same diets used for the digestibility trial and their clinical parameters as well as their weight were checked at the beginning (T0) and at the end of trial (T1). No variation of weight, haemoglobin, PCV, serum albumin and ALP was recorded, and all parameters were within normal ranges (according to AAFCO) during all the duration of the trial. Consequently, the diet based on black soldier fly larvae meal was deemed suitable for adult dogs maintenance diets.

The second trial was conducted at the UNESP (Brazil) and involved diet formulation and processing as well as the digestibility trial itself. Since legume-based diets for pets are already on the market the main focus of this research involved both the extrusion technique, as it is one of the major concerns for the pet food manufacturers, and the nutritional outcomes of such diet (e.g. faecal biogenic amines and volatile fatty acid production or glycaemic and insulinemic curve). In fact, legumes have specific nutritional properties, such as higher protein and fibre content and lower starch content compared to cereals, which make them a challenging ingredient to extrude while, at the same time, giving the diet particular intestinal features. Five experimental diets were formulated: a basal diet (CO) was based on rice and poultry by-product meal; while other 3 experimental diets were made only with the replacement of rice with different inclusion levels (33%, 66% and 100%) of lentil pasta by-product (LP33, LP66 and LP100). A fifth experimental diet (LPS) was formulated with 70% of the basal diet (CO) and 30% of the lentil pasta by-product in order to evaluated the ingredient digestibility alone. The diets were produced in an experimental extruder and the processing parameters were regularly checked and recorded while samples were collected at periodic intervals. The results showed a linear increase in extruder pressure, hardness and bulk density of kibbles when increasing the level of LP inclusion ($P < 0.05$), without affecting starch gelatinization. The ATTDC of dry matter, organic matter, and gross energy presented a quadratic reduction, while the dietary fibre and crude protein ATTDC increased quadratically with LP inclusion ($P<0.05$). According to the polynomial contrasts analysis, up to 66% of LP replacement did not reduce ATTDC, with similar or increased values compared to the BR diet. Nitrogen balance did not change ($P>0.05$), but a linear increase in faeces production and moisture, and a linear decrease in faeces pH was observed with higher LP ($P<0.05$), without changing faecal score. Faecal acetate, propionate, total short-chain fatty acids (SCFA), branched-chain fatty acids, and lactate all increased linearly with LP inclusion ($P<0.05$), without altering ammonia concentration in faeces. The increase in LP inclusion

promoted a linear increment in cadaverine, tyramine, histamine and spermidine ($P<0.05$); while spermine exhibited higher concentrations in faeces for the LP 33% diet ($P<0.05$). The effects on both extrusion parameters and kibble formation, as well as in ATTDC and fermentation of by-products in faeces can be explained by the higher dietary fibre and lower starch content of LP in comparison with BR. The increase in fibre with lower starch may increase the mass resistance to flow inside the extruder, increasing shear, temperature and pressure, but the reduced starch content ended reducing expansion due to the lower plasticised mass necessary for cell structure formation. The higher fibre intake by dogs due to LP inclusion can also explain the reduction in digestibility. The dietary fibre of LP showed to be fermentable by gut microbiota, increasing the concentration of desirable fermentation products including SCFA, and spermidine. The glycaemic and insulinemic curve was performed on 2 groups of 8 Beagle dogs fed the BR diet and the LP100 diet respectively. The results showed a reduction in post-prandial glucose and insulin response for the LP100 diet in comparison to BR diet, suggesting that this ingredient can be used in diet designed to have low glycaemic response. Finally, a palatability study was performed on 38 dogs of different breeds and body weight in a completed randomized design using the two-bowl method, comparing the BR diet with the LP100 diet. The results showed a preference for the LP100 ration in both the "first choice" test and the "consumption rate" evaluation ($P<0.05$). The trial revealed how a by-product discarded for just aesthetic reason from human-graded food chain can still retain its highly palatable organoleptic properties.

Overall, both trials performed showed that the ingredients tested can have a future application in the pet food market, both from a nutritional as well as a sustainable perspective, according to the pillars of the circular economy.

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1.INTRODUCTION

It is undeniable nowadays that the world is changing from a climatic and demographic point of view (Fróna et al., 2019). The rapid increase in food demand will rise ever so greatly during the next years and a challenge for the welfare quality of people (FAO, 2009) and their pets life is looming (Swanson et al., 2013). Protein sources are the most expensive and ecologically demanding ingredients, accounting also the fact that they have a substantial impact on environment, land and water usage (Okin, 2017). Nevertheless, protein (as a macronutrient) is a key point for pet owners while choosing pet food products (Laflamme et al., 2008; Nijdam et al., 2012; Berardy et al., 2019). On that extent, alternative raw materials (such as by-products from human food industry) in pet food can increase the provision and/or digestibility of different nutrients combining, at the same time, an enhanced sustainability for what concerns the world economy, society and environment (Meeker et al., 2015). Indeed, a nutritional approach is considered sustainable if it is in accordance with the three-P dimensions (Planet, People and Profit) (Makkar et al., 2014 a). These changes, alongside the increased food-quality awareness of pet owners (Swanson et al., 2013), are leading manufacturers to introduce products with new sources of non-structural carbohydrates (NSC) and proteins. As this new trend is rising, concerns about food safety and quality are going hand to hand and need to be addressed (van der Spiegel et al., 2013). As a consequence, numerous factors need to be studied and conveyed before the introduction of these novel materials in the pet food production chain.

2. SUSTAINABILITY IN PET FOOD

Pet industry is one of the most prolific and steadily growing industry around the world. In 2022, Italian pet owners spent 11.4% more than the previous year on their pet food and treats ('Rapporto Assalco-Zoomark', 2023), despite the COVID-19 pandemic lowered the population spending power. If seen globally this increase is also similar for most of the western countries (Alexander et al., 2020), with 11% global growth between 2017 and 2020 (Alltech, 2020). With the livestock industry at its limit in terms of sustainable production capacity, and the pet food business in constant growth, new sources of protein are being sought in order to meet the market's demand and the expectations of pet owners (Bosch et al., 2016). The potentially increasing burden associated with feeding dogs and cats need to be accounted for in order to face constructively the future food system challenges. Recent estimations reported an average annual impact for 1 pet ranging from 27 to 1444 kg of CO_2-equivalents per year for dogs and from 43 to 228 kg CO_2-equivalents for cats (Acuff et al., 2021). Similarly, another assessment evaluated that the annual global production of dry pet food could be responsible for up to 2.9% of CO_2-equivalents emission and up to 1.2% of agricultural land use (Alexander et al., 2020). For this reason, the revalorization of food by-product, otherwise destined to compost or landfill, should be prioritize in such a flourishing industry. In particular, the concept of upcycling, i.e. using still nutritionally valuable but discarded by-product, even if already quite established in the pet food industry, has stirred research interests in various fields, which include also marketing, in the light of the magnitude of the recent food waste crisis (McCarthy et al., 2017, 2020; Bolton, 2022). Opposite to this idea there is the so-called "premiumization" trend of pet food, i.e. the use of higher cost and expensive ingredients which, in certain cases, are directly taken from what would be considered perfectly suitable for human consumption (Alexander et al., 2020). Similarly, raw meat diets, which have inherently a greater environmental burden, have increased in quantity despite the poor evidence that this kind of diet can give health benefit to dogs and cats (van Bree et al., 2018). As such, the growing belief among owners that these "natural" pet foods (i.e. containing natural preservatives, meats, fruits and vegetable, and designed according to ancestral or instinctive nutritional philosophies (Buff et al., 2014)) induced to more than double their sales between 2008 and 2012 (Carter et al., 2014). As a consequence, the use of higher-grade raw meat or commercially available food with the so-called "natural" ingredients can increase the associated environmental impact (Swanson et al., 2013). The level and the scale of the environmental paw print appears therefore disproportionately small if we look at the few research and data on the topic (Alexander et al., 2020). The current role of research as well as pet food manufacturers lays on mitigation options in order to increase sustainability while enhancing

economic growth with an appropriate marketing. A current trend to achieve an improved sustainable use of agriculture while increasing the planetary health is to facilitate the adoption of human plant-based diets (Clark et al., 2017; Willett et al., 2019). Similarly, in order to reduce the carbon footprint of pet foods, it has been proposed to switch from a meat-based diet to a vegetable-based diet (Knight et al., 2016). Although this statement may be perceived more as a provocation than a real possibility, a plant-based diet has good potential, both for its digestibility, nutritional value and environmental sustainability. Mainly, if combined with the proper meat-based ingredients, plant proteins can overcome their deficiencies and form part of the diet for dogs (Li et al., 2020), while for cats this can be a greater challenge due to their carnivorous nature (Legrand-Defretin, 1994). From an environmental standpoint, plant-based diets require lower energy, land, and water usage compared to meat-based diet (Swanson et al., 2013; Okin, 2017). The main opportunity to increase the sustainability of our pets diets is to increase the knowledge about new ingredients and correctly target consumer choices through scientific evidence in order to ensure animal food safety (Acuff et al., 2021).

3. NOVEL AND SUSTAINABLE INGREDIENTS FOR PET FOOD

3.1 Insects

Insects may provide a possible solution as an alternative ingredient, since they can partially replace traditional feed sources, whilst they also provide a means to bio-converting organic waste (Food and Agriculture Organization of the United Nations, 2013). In addition, insect-based pet food offers a potentially more sustainable alternative through reduced water consumption, land use and emissions compared to commonly used animal protein (Alexander et al., 2017).

There are mainly 3 species of insects currently reared for pet food, since they can be grown in large quantities in "mini-farms", and these are: black soldier fly larvae (*Hermetia illucens*), mealworm larvae (*Tenebrio molitor*) and adult house crickets (*Acheta domesticus*) (van Huis, 2020). The level of crude protein among them is similar or higher than soybean meal (40-45%) and meat meal (40-50%), with a range that varies from around 41% up to 69% on dry matter (DM) basis (Makkar et al., 2014 b). Furthermore, insect are rich in essential amino acids (Bosch et al., 2014), while the limiting amino acids are considered to be methionine and threonine (Bosch et al., 2020). Additionally, insects appear to be rich in several microminerals such as iron, manganese, copper, selenium and zinc, and contain high concentration of calcium, phosphorus and magnesium as well (Makkar et al., 2014 b; Kouřimská et al., 2016). The lipid content of insects, especially during the larval stages, provide a large amount of energy, but varies in composition depending on the insect diet and species (Bukkens, 2005; Valdés et al., 2022). Insects contain significant amount of crude fibre, however most of the fibre content can be assimilated to chitin, a structural polysaccharide which compose the exoskeleton of insects (Tabata et al., 2018). Chitin itself, alongside the presence of catalase, antibacterial peptides and superoxide dismutase confer to insects a high antioxidant capacity (He et al., 2019), while combining an enhancement for the local immunity (Gasco et al., 2018; Lopez-Santamarina et al., 2020). Future studies need to address the role of insects in gastrointestinal health but they appear to have the protentional to be a functional ingredient for promoting gut health in pet food diets.

3.1.1 Black soldier fly

Of the various insects being considered, the black soldier fly (*Hermetia illucens*) is showing particular promise due to its immediate potential for large-scale production (Veldkamp et al., 2012). The black soldier fly (BSF) has a balanced protein composition and one of the highest amino acid scores

compared with other currently reared insects or traditional protein sources (such as fish meal) (Bosch et al., 2014). Compared with crickets and mealworms, BSF boasts a more stable nitrogen and phosphorus composition, and has a more advantageous feed conversion ratio (Oonincx et al., 2015). It can also be considered a possible sustainable solution due to the possibility of rearing the insects on materials deemed unsuitable for human nutrition, such as alimentary by-products and organic substrates (Spranghers et al., 2017). Moreover, it has been estimated that commonly used meat-based sources have 2 to 5 times higher environmental impact compared to diets based on black soldier fly (Smetana et al., 2016, 2019).

As pointed out by Böhm et al. (2018) (Böhm et al., 2018), insects may constitute an appropriate novel protein source for dogs presenting cutaneous adverse food reactions. Nevertheless, societal negative opinions about the use of insect meal in pet nutrition have arisen, especially due to insect phobia and concerns about safety. Security aspects about insects consumption were also discussed critically in (Scientific Committee EFSA, 2015), where uncertainty regarding the risk of non-processed items, due to the lack of data, has been acknowledged. However, EFSA concluded that microbiological risks are expected to be comparable with other food raw materials, provided that insects are fed with allowed feedstuff. Consumers from Western countries still continue to have prejudices regarding the introduction of insects in their diet (Moruzzo et al., 2021) and, due to the current "humanization trend" (Okin, 2017), this fact could be also translated to their pets. Notwithstanding that, public opinion seems to be less concerned about the use of veterinary-prescribed diets based on insects (Leriche et al., 2017). Indeed, veterinarians have expressed interest in hypoallergenic food alternatives prepared using insects (Pagani et al., 2016). According to the Commission Regulation (EU) 2020/354 (4 March 2020) (European Commission, 2020) a product can be claimed to reduce ingredient and nutrient intolerances if it is composed by hydrolysed proteins or selected and limited protein sources or selected carbohydrate sources. Therefore, according to the current European Regulations, a product composed only of insects as the main source of protein could be considered with the particular purpose of reduction of food intolerance. Concurrently, and reflecting the growing interest in this field of research (Gasco et al., 2019), various recent studies have investigated the possibility of feeding BSF larvae to poultry (Marono et al., 2017; Schiavone et al., 2017; Gariglio et al., 2019; Biasato et al., 2020), fish (Shakil Rana et al., 2015; Renna et al., 2017; Belghit et al., 2019), and swine (Biasato et al., 2019; Heugten et al., 2019). Recently, a thorough review from Bosch & Swanson (2020) (Bosch et al., 2020) explored in depth palatability, digestibility and nutritional aspects of the inclusion of insects in dogs and cats diet, showing the potential of insects as future pet food products (Penazzi et al., 2021).

3.2 Legumes

In an approach similar to novel ingredients also legumes have been rediscovered by the pet food industry as new or alternative sources of both carbohydrates and proteins. The marketing focus on these ingredients has been promoted by manufacturers to create a new appealing option to more traditional components (such as cereals) of the pet food diet. In fact, grain-free diets (based on legumes and tubers as the main carbohydrate source) are largely present nowadays on the pet food market (Corsato Alvarenga et al., 2020 a). Even though starch is not considered an essential nutrient for dogs, it is a major source of energy for dog food formulation as well as an essential component for the extrusion process and kibble formation (Allen et al., 2007; Tran et al., 2008; Carciofi et al., 2012; Monti et al., 2016; Baller et al., 2018; Pacheco et al., 2018; Corsato Alvarenga et al., 2020 b). Different factors may affect starch digestibility and animal metabolism, such as the carbohydrate source (Murray et al., 1999; Carciofi et al., 2008; Domingues et al., 2019; Pezzali et al., 2019; Ribeiro et al., 2019), starch type (Rooney et al., 1986; Svihus et al., 2005), processing conditions (Moore et al., 1980; Ribeiro et al., 2019), starch-protein interactions (Dhital et al., 2017) and physical granule form (Svihus et al., 2005; Dhital et al., 2017). These factors have been thoroughly addressed in dog foods based on traditional cereals, although little has been studied on alternative carbohydrate sources, especially pulses (Tran et al., 2008; Corsato Alvarenga et al., 2021). Non-structural carbohydrates are also the main nutrients affecting glucose and insulin responses in dogs (Nguyen et al., 1998; Carciofi et al., 2008; Teshima et al., 2021). In both humans and dogs a faster digestion and absorption of starch appear to lead to greater post-prandial insulin peak (Wolever et al., 1996; Murray et al., 1999; Carciofi et al., 2008). Taking into account this fact there are situations were minimize the insulin response may be advantageous, especially in condition of impaired carbohydrate metabolism such as diabetes mellitus (Teixeira et al., 2018), old age (Ribeiro et al., 2019) or obesity (Brunetto et al., 2011). Since legumes contain a higher fibre content compared to cereals (Carciofi et al., 2008; Corsato Alvarenga et al., 2020 a; Pacheco, 2020), this may affect the production of short-chain fatty acids (Bach Knudsen, 2015), reduce nutrient and energy digestibility (Kawauchi et al., 2011) as well as alter the glucose and insulin postprandial responses (Alminger et al., 2008; Gemen et al., 2011). Therefore, the type and amount of starch is not the only factor affecting the metabolism of the animal, but the interaction itself with other nutritional components of the ingredient (such as fibre) appear to have a role in the fermentation profile and postprandial response.

3.2.1 Lentils

The production of lentil pasta has been increasing steadily due to the raise of celiac people (Laleg et al., 2016); as a result, the by-product of this human food (discarded due to discoloration or because it is not perfectly shaped) has grown in quantity and has received the attention of the pet food industry as a possible alternative ingredient for dogs. Lentils are richer in total dietary fibre compared to other commonly used legumes (such as peas and chickpeas) (Brummer et al., 2015). Similarly to other pulses, lentils are a good source of protein, having between 20.6% and 31.4% on dry matter basis, but generally a low percentage of sulphur-containing amino acids (Jarpa-Parra, 2018). In particular, pulses seem to have high concentration of lysine but low level of methionine, a precursor for taurine synthesis (Marinangeli et al., 2017). On this matter the Food and Drug Administration announced on July 2018 that diets containing pulses and potatoes as main ingredient may be associated with the development of dilated cardiomyopathy (DCM) in dogs (FDA, 2018). However, a thorough review on the matter (McCauley et al., 2020) outlined the fact that studies performed so far showed no definitive evidence on the matter as multiple variables and confounding factors may have affected this correlation. In particular, diets not correctly formulated to provide an optimal intake of sulphur-containing amino acids may have been the cause of the related DCM in dogs rather than pulse ingredients themselves. Nevertheless, knowing these factors a careful evaluation on the inclusion level of lentils (and pulses in general) should be taken into account during the formulation for dogs food.

Lentils appear to be a possible useful ingredient for specific dietary conditions such as diabetes mellitus, obesity, pregnancy, stress, infection, cancer and senescence, as it minimizes (while prolonging) postprandial glucose and insulin response, possibly improving glycaemic control (Carciofi et al., 2008). In human studies lentils have been associated with cholesterol- and lipid- lowering effects, reducing also the incidence of colon cancer and type-2 diabetes as well (Roy et al., 2010). The efficacy of other legumes (such as peas) has been studied with beneficial effects for dogs with diabetes, minimizing the concentration of plasma triglycerides and cholesterol (Adolphe et al., 2015; Teixeira et al., 2020). In a recent study using in vitro analysis, data suggested that legumes (green lentils, yellow peas, black bean beans, sea bean powder and chickpeas) appear to be a source of slow fermentable fibre, which may have beneficial implications in the proportions of saccharolytic to proteolytic fermentation towards the distal colon (Traughber et al., 2020). Besides, lentils showed higher antioxidant capacities in the gastrointestinal tract than other legumes such as soybeans, chickpeas and peas (García-Mora et al., 2017).

4. PET FOOD PRODUCTION

Complete diets for dogs appeared on the market since 1930 in both forms of canned wet food as well as dry meat meals, but only during the 1960s the extruded diets for pets started to grew constantly in quantity and income. Nowadays, the most common types of pet food are dry food (kibbles) followed by wet food (canned or packed), treats and a small portion of semi-moist food (Krestel-Rickert, 2005; Zicker, 2008). Since the major segment of the pet food production is dry food (Spears et al., 2004) it is necessary to understand how the ingredients can affect the extrusion procedure as well as the processing parameters in order to make the desired product (Allen et al., 2007; Tran et al., 2008; Carciofi et al., 2012).

4.1 Extrusion process

Produce extruded dry diets for pets is not an easy task as the ingredients need to pass through a series of steps before the actual extrusion. Raw materials need to be grinded and mixed properly, the level of grinding also will determine the physical characteristics of the final kibble as well as affecting their digestibility (Riaz, 2000 a; Riaz et al., 2012; Bazolli et al., 2015). Since extruded pet food formulations have a complex matrix (animal derived proteins, non-structural carbohydrates, fats, fibre, and mineral sources) compared to human-graded food, such as pasta or breakfast cereals (composed mainly by starch), several aspects of both the hardware and software conditions need to be altered in order to obtain the proper shaping, cooking, and texturization (Camire et al., 1991; Lue et al., 1991; Guy, 2001; Monti et al., 2016; Baller et al., 2018). Preconditioning (using a horizontal blender with injectors) is an essential phase for these kind of products as water and steam can be added into the mass in order to achieve the desired objective of cooking and expansion (Pansawat et al., 2008; Baller et al., 2018, 2021). In addition, moisture is necessary to hydrate the ingredient particles as well as cooking starch and protein (Riaz, 2007). The final shaping and texturization of the extrudates is in fact the result of a good balance with water as a fluidizing agent, mass resistance to flow and viscosity, as the steam flash-off during the cutting of the dough promotes kibble expansion (Guy, 2001; Onwulata et al., 2001; Ding et al., 2005; Baller et al., 2018). Besides, achieving a suitable level of moisture in the mass may also avoid undesirable chemical reactions which can be induced by the relative high temperatures reached during the extrusion process, such as vitamin destruction, Maillard reactions and lipid oxidation (Lankhorst et al., 2007; Riaz, 2007; Rooijen et al., 2013). After pre-cooking the ingredients in the pre-conditioner, the dough

is moved into the extruder, a horizontal jacketed barrel tightly fitted with a single or twin screw (Riaz, 2000 a). In general, single screw extruders are adopted in the majority of the case in the pet food industry, but if the feed material used has a high level of moisture (including in the formulation for example fresh meat) or contains more than 12% of fats, the twin extruder is usually preferred (Riaz et al., 2012). At the end of the extruder the mass passes through a die, with different holes according to the desired shape of the kibble. In fact, the rotating knife cuts the material at the desired size while the sudden change of pressure and water evaporation let the expansion and hardening of the final kibble. The subsequent phase is therefore drying the formed kibbles to the desired moisture content, using a hot air flux. Final step is the coating, in which kibbles are covered with fats and/or palatability enhancers (Riaz, 2000 a; Riaz et al., 2012).

4.2 Effect of extrusion on nutrients

Depending on the length of the thermal treatment, the moisture injected and the intensity of the shear forces applied, the feed materials can undergo different changes (Tran et al., 2008). In order to both achieve desired modifications of the ingredient (such as reduce anti-nutritional factors) and to avoid decrease in the nutritional quality of the products (such as vitamin loss), several processing parameters can be monitored (Riaz, 2000 a). The extrudates can be influenced by the feed mixture particle size, moisture, pressure and temperature in both the pre-conditioner as well as the extruder barrel, material retention time, and knife rotation speed. The effect of the reduction in the particle size corresponds to an increase in starch gelatinization, water absorption and expansion ratio (Chauhan et al., 1985; Lue et al., 1991; Mathew et al., 1999; Carvalho et al., 2010) but seems to have an ingredient dependant effect on digestibility and faecal fermentation products (Bazolli et al., 2015; Abd El-Wahab et al., 2022; Chuppava et al., 2023).

4.2.1 Extrusion effect on proteins

Proteins can be modified during the extrusion process by the pressure and the temperature applied. In particular the loss of their quaternary and tertiary structure can expose the molecule to react with reducing sugars and other components of the feed mixture. In particular, the main chemical reaction involved is the Maillard reaction, responsible for both the flavouring and browning of the feed components, but also accountable for decreasing the nutritional quality (Camire, 1991) and increasing the production of possibly toxic compounds (Singh et al., 2007 b; Rooijen et al., 2013).

Factors influencing the extent of the Maillard reaction are the amount of amino acids and reducing sugars found in the feed material, temperature as well as time of heat exposure, pH and moisture level (van Boekel, 2006; Baller et al., 2018).

4.2.2 Extrusion effect on starch

Starch is composed by glucose residues organized in 2 different polymers: amylose, with linear α 1-4 bonds; and amylopectin, with additional branched α 1-6 linkages aside from the α 1-4 bonds (Haralampu, 2000). The branched arrangement of the amylopectin chains in the starch granules originate a compact radial formation composed of alternated crystalline and amorphous structures. Among the amylopectin clusters amylose molecules are randomly interposed, and the ratio between these two polymers can vary depending on the botanical origin of the starch. When water and heat are added to the starch granule the molecules undergo to the process called gelatinization, in which starch granules acquire water, swell and disrupt the crystalline structure (Zeng et al., 1997). The gelatinization and the expansion ratio of the final extrudates can be used to evaluate the quality of the extrusion process, being moisture and temperature, the main factors involved. Since the feed materials for pet food are a complex matrix for the extrusion process, containing both proteins and fats aside from starch, this can affect the level of gelatinization. In fact, the insulating effect of lipid on water absorption can reduce the degree of starch gelatinization (Lin et al., 1997). In addition, the formation during the extrusion process of V-amylose complexes between lipids and starch can reduce the susceptibility to the enzymatic digestion (Singh et al., 2007 b), even though the fat utilization does not appear to be impacted when these complexes are not present in large amount (Tran et al., 2008). The addition of fibre sources to dog food products appear to reduce starch cooking and kibble expansion, with denser and harder kibbles as a result (Monti et al., 2016). Resistant starch, composed of starch or its degraded products which cannot be digested and absorbed by the small intestine, has been proposed as a candidate prebiotic for dogs rations due to its effect on large intestine fermentation (Nugent, 2005; Fuentes-Zaragoza et al., 2010; Peixoto et al., 2018). Changing the mechanical and thermal energy applied during the extrusion process can affect the resistant starch content of the diet. In fact, implementing a low-shear extrusion process compared to medium-shear conditions (usually performed in pet food products) can have an impact on gelatinization and, therefore, on resistant starch levels. Recent studies showed higher short-chain fatty acids content in faeces of dogs fed foods with lower starch gelatinization (Bazolli et al., 2015) or higher resistant starch levels (Peixoto et al., 2018; Ribeiro et al., 2019); showing, together with an almost complete starch apparent total tract digestibility coefficient, that nondigested starch is

readily fermented in dogs colon (Moreau et al., 2003). In addition, increasing the level of resistant starch in the diet appears to reduce the postprandial glucose response in old dogs (Ribeiro et al., 2019).

4.2.3 Extrusion effect on dietary fibre

The extrusion process appears to increase the total dietary fibre content in the diet especially due to the formation of soluble dietary fibre from enzyme-resistant indigestible glucans, the formation of resistant starch or the transformation from insoluble fibre to soluble dietary fibre (Björck et al., 1983; Lue et al., 1991; Vasanthan et al., 2002; Dust et al., 2004). In general, however, using the medium shear extrusion cooking typical of the pet food industry does not appear to change in a significant manner the level of dietary fibre but rather increase the soluble fibre components (Singh et al., 2007 b). Inclusion of sources rich in fibre, however, influences the processing parameters, potentially altering the final product characteristics (Mendonça et al., 2000). In fact, fibre can affect viscosity, mass flow inside the barrel, and the formation of the cellular structure of kibbles, altering consequently the textural characteristics such as hardness and crispiness (Karkle et al., 2012 a). Kibble characteristics appear to be significantly impacted not only by the level of inclusion of fibre, but also by fibre type and particle size (Monti et al., 2016).

4.2.4 Extrusion effect on lipids

Extrusion of feed materials with high levels of lipids is usually not advisable as it may impair the extruder performances, in particular for expanded products (Riaz, 2000 a). Levels of fat above 7% can reduce product expansion due to increased slipping within the barrel and, consequently, lower pressure (Riaz, 2000 a; Riaz et al., 2012). Besides, the lower torque and shear force applied due to high fat formulations can also decrease product cooking while increasing the bulk density, even when efforts are made to maintain it. At the same time, a small lipid content (below 5%) is advisable to maintain a steady extrusion and improve the texture. Nonetheless, during the extrusion, lipids contained in the feed mixture can be affected by chemical modification such as hydrogenation, oxidation, isomerization and polymerization (Tran et al., 2008). The oxidation of lipids is usually one of the major challenges for pet food preservation as lipid inclusion need to be correctly balanced with the appropriate amount of antioxidants in order to prevent this phenomenon (Lin et al., 1998; Riaz, 2007). Many factors can influence lipid oxidation rate during the extrusion and, in particular, moisture content and the degree of kibble expansion play a crucial role (Singh et al., 2007 b). On

the other hand, lipase and lipoxidase present in foods are inactivated during the extrusion process, resulting in products less susceptible to oxidation (Lin et al., 1998; Tran et al., 2008).

4.2.5 Extrusion effect on vitamins and minerals

Several vitamins are sensitive to both physical and chemical treatments and their stability depends on their specific structure. Exposure to light, oxygen, heat, pH, moisture and processing time during extrusion can increase vitamins degradation (Killeit, 1994); while minimizing shear force and temperature protects most of them (Singh et al., 2007 b; Tran et al., 2008). Similar to lipids, in pet food the extrusion cooking seems to be detrimental to vitamins content especially due to oxidative processes (Cheftel, 1986), with the iron level in the mixture catalysing furthermore the oxidation (Tran et al., 2008). Among vitamins, vitamin D and choline appear to be quite stable; while vitamins from the B group, vitamins A, K and E seem to suffer a higher degradation rate in the presence of oxygen and heat (Singh et al., 2007 b; Riaz et al., 2009).

On the contrary, extrusion cooking appears to enhance the absorption of most minerals as it has a positive effect on the reduction of antinutritional factors (such as phytates or tannins) as well as the chemical alteration of compounds such as fibre (Singh et al., 2007 b). Nevertheless, little research has been carried out on the various essential elements, as minerals are considered heat stable and unlikely to be lost during the food processing (Camire et al., 1990). However, it is important to notice that recently a study by Baller *et al.* (2020) showed how selenium, depending on the source (organic or inorganic), can be partially lost during dog food extrusion processing. In particular, the addition of selenium-yeast to the formulation led to a minimum reduction in the final product while sodium selenite presented recovery rates between 62 and 86%, due to the possible conversion to hydrogen selenide, lost as a gas, during the extrusion process (Baller et al., 2020). Therefore, it may be necessary in the future to assess the recovery rate also of other microelements as some of their sources may not be stable during the extrusion treatment.

4.3 Effect of extrusion on palatability

Diet palatability plays a key role in pet food industry for dogs and cats themselves as well as regarding owners' acceptance (Samant et al., 2021). It can be influenced by several factors, including diet nutritional composition, kibble macrostructure, flavour enhancers and processing

techniques (Hullar et al., 1998; Tran et al., 2008; Viera, 2010). Extrusion itself can affect palatability by controlling the level of specific mechanical energy (SME) and specific thermal energy (STE). In particular, cats seem to prefer foods extruded with higher mechanical energy configuration (Kvamme et al., 2003); on the other hand dogs appear to increase their food acceptance when products are more thermally cooked (Dunsford et al., 2002). The production of a mild browning due to the Maillard reaction (i.e. the chemical reaction involving certain amino acids with reducing sugars when heat is applied) appears to have desirable effects regarding both taste and appearance, even though data are still limited on this regard (Riaz et al., 2012; Rooijen, 2015). However, an excessive browning may result in a burnt appearance and taste along with a series of Maillard reaction products which may have the potential to be toxic for the organism (Rooijen, 2015).

4.4 Effect of extrusion on digestibility

Alongside with the modifications which can affect the chemical structure of the various nutrients, the extrusion process can affect also their digestibility and utilization. In particular, protein digestibility of extrudates appears to be higher than their non-extruded counterparts, probably due to the denaturation of the protein structure and inactivation of antinutritional factors (Singh et al., 2007 b). Especially, vegetable proteins appear to benefit from extrusion cooking due to the inactivation of enzyme inhibitors (Colonna et al., 1989; Arêas, 1992). Among the process variables, the increase of the extrusion temperature and the feed ratio showed the greatest changes in protein digestibility (Bhattacharya et al., 1985, 1988; Fapojuwo et al., 1987; Camire et al., 1990). Regarding starches, the factors which can affect their digestibility are temperature, moisture level, amylose and lipid content, which may all lead to structural alterations of starch granules (Trần, 2008). Nonetheless, gelatinization level may affect digestibility depending on the starch source; specifically, when increasing temperature and, consequently, gelatinization, digestible starch increased in tapioca, barley and corn, but did not change in wheat or oat bran (Wolter et al., 1998; Dust et al., 2004). It is important to remember that extrusion may enhance the formation of lipid-amylose complexes (Lin et al., 1997), thus inhibiting the digestion by amylase. In fact, under specific extrusion conditions (such as high moisture and high temperature), complexes of lipid-protein or lipid-starch may impair the utilization of fats if this phenomenon occurs to a large extent (Trần, 2008). However, under normal conditions, extrusion inactivates lipase and lipoxidase resulting in less oxidation of fatty acids during storage (Lin et al., 1998), while the formation of complexes is

not enough to cause any decrease in fat digestibility, which is usually particularly high in both canine and feline diets (Hullar et al., 1998)

5. AIM OF THE STUDY

The project was developed in order to evaluate the digestibility and the effects of two different ingredients for dog pet food. Both of the ingredients were chosen as they are claimed to be possible alternative and sustainable sources of nutrient, so as to enhance the concept of circular economy, which is a paramount key point nowadays in the pet food industry.

A first trial using black soldier fly larvae (*Hermetia illucens*) was realized in order to assess the digestibility parameters as well as the nutritional adequacy of this innovative ingredient. An in vitro analysis was also conducted to evaluate the possibility to avoid the use of live dogs for the digestibility trials and check if the prediction equations could be appliable also for a complex protein matrix such as insects.

A second trial was performed in order to assess the digestibility, faecal characteristics and fermentation products using a by-product of the pasta industry. In particular, we used red lentils pasta by-product as it is a valuable source of both carbohydrates and protein. As such, a glycaemic and insulinemic curve as well as a palatability trial were developed so as to evaluate also the effect of this ingredient on the metabolic responses of the animal and its acceptance.

6. MATERIALS AND METHODS

6.1 Animals and Experimental Design

6.1.1 Trial 1

The study was performed at the University of Turin (UNITO), Department of Veterinary Sciences, Grugliasco (Turin), Italy. All the experimental procedures were approved by the Bioethics Committee of the University of Turin (Italy) (prot. n. 336595).

Six clinically healthy West Highland White Terrier adult dogs [of which three males and three females, median of 3 years old (IQR 2-5), 7.2 ± 0.8 kg BW, with a median BCS of 5 (IQR 4.5-5.5) on a nine-point scale (Laflamme, 1997)] were fed two isonitrogenous and isoenergetic dry extruded diets (control vs. insect diet) according to a cross-over design. During the digestibility experiment, the dogs were housed individually in 3 × 3-m kennels and had *ad libitum* access to fresh water. The dogs were allowed to walk freely for 1 h per day in a concrete outside the pen and play with toys during the adaptation periods.

For the nutritional adequacy trial a total of 16 mixed-breed adult dogs [of which 8 females and 8 males, all neutered, median of 5 years old (IQR 3-6), 25.7 ± 4.6 kg BW, with a median BCS of 5 (IQR 5-6) on a nine-point scale (Laflamme, 1997)] were enrolled. The dogs were fed the same isonitrogenous and isoenergetic diets mentioned above (control vs. insect diet). During the trial the dogs were housed in 7 x 5-m kennels (two animals fed the same diets for each kennel) and had *ad libitum* access to fresh water. The dogs were able to play with toys and have contacts with the other dogs for all the duration of the trial.

6.1.2 Trial 2

The study was performed at the "Universidade Estadual Paulista" (UNESP), "Faculdade de Ciências Agrárias e Veterinárias", Jaboticabal (São Paulo), Brazil. All the experimental procedures were approved by the Bioethics Committee (CEUA) of the "Universidade Estadual Paulista" (UNESP) – Campus of Jaboticabal (Brazil) (prot. n. 1501/21).

Forty clinically healthy adult Beagle dogs [of which 19 males and 21 females, median of 3 years old (IQR 2-7), 11.9 ± 2.0 kg BW, with a median BCS of 5 (IQR 5-6) on a nine-point scale (Laflamme, 1997)] were fed 5 dry extruded diets. In particular, the diets were formulated as follow: a basal diet

(CO), whose main ingredients were rice and poultry by-product meal, three experimental diets with lentil pasta by-product (LP) at different inclusion levels in place of rice 33% (LP33), 66% (LP66) and 100% (LP100), and a fifth experimental diet (LPS) formulated with 70% of the basal diet (CO) and 30% of the lentil pasta by-product. During the digestibility experiment, dogs were individually housed in 1 × 1 × 1 m stainless steel metabolic cages, purposely made to separate and collect both faeces and urine.

6.2 Diets and digestibility protocols

6.2.1 Trial 1

Two extruded diets were tested during the trial. The diets were formulated to be isoenergetic and isonitrogenous. In the control diet (CTRL diet), the protein source was provided in the form of processed [rendering process, method III, according to the EU Reg. 142/2011 (European Commission, 2011)] deer (Cervus elaphus) protein, whereas the insect diet (BSF diet) provided defatted BSF (H. illucens) larvae meal as its sole protein source. The chemical composition, amino acidic profile, and ingredient composition of both diets are shown in Table 1. Diets were formulated and balanced in order to meet nutrient requirements in accordance with the FEDIAF (FEDIAF, 2020) nutrient guidelines for dogs. Venison was chosen as the primary protein source for this trial since it is one of the protein sources usually incorporated in commercial foods for dogs which show adverse food reactions; similarly, insect meal showed a similar potential (Böhm et al., 2018). Nevertheless, venison meal is more expensive than other common sources of proteins as well as insect meal so far and, for these reasons, was deemed eligible for the comparison of the diets.

Table 1 Ingredients and nutritional composition of the experimental diets

	CTRL[1]		BSF[2]	
Ingredients	*(% as fed)*	*(% of DM)*	*(% as fed)*	*(% of DM)*
Potato meal	51.5		54	
Venison meal	40		-	
Black soldier fly meal	-		36.5	
Vitamin and mineral premix	3		3	
Oils and Fats[3]	2.5		2	
Yeast (hydrolysate)	2		2	
Calcium carbonate	-		1.5	
Other ingredients[4]	1		1	
Nutrient and chemical composition [5]				
Dry matter	93.80	-	96.04	-
Organic matter	86.11	91.80	90.21	93.93
Crude protein	16.97	18.09	20.70	21.55
Ether extract	17.42	18.57	15.61	16.25
Crude fiber	5.77	6.15	4.09	4.26
Ash	7.69	8.20	5.83	6.07
Calcium	1.03	1.10	0.87	0.91
Phosphorus	0.93	0.99	0.53	0.55
Collagen	2.72	2.90	0.88	0.92
Hydroxyproline	0.34	0.36	0.11	0.11
Amino acidic profile[5]				
Aspartic acid		1.88		2.09
Serine		0.68		0.79
Glutamic acid		1.98		2.19
Glycine		1.14		1.01
Histidine		0.31		0.49
Arginine		0.86		1.02
Threonine		0.60		0.68
Alanine		0.87		1.15
Proline		1.12		1.07
Cysteine		0.15		0.16
Tyrosine		0.40		0.78
Valine		0.71		1.01
Methionine		0.23		0.39
Lysine		0.80		0.97
Isoleucine		0.53		0.69
Leucine		1.03		1.23
Phenylalanine		0.64		0.79
ME (MJ/kg)[6]	15.66		16.44	

[1] CTRL: control diet; [2] BSF: Black soldier fly diet; [3] poultry purified fat, sunflower oil; [4] digest (hydrolyzed poultry liver), mineral and vitamin pre-mix; [5] analyzed; [6] estimated according to FEDIAF (FEDIAF, 2020)

The trial was conducted according to the guidelines of Carciofi et al. (Carciofi et al., 2007) regarding the use of a marker method and the total collection method for assessing in vivo total tract apparent digestibility. Chromium oxide (Cr2O3) was used as digestibility marker. It was added to a final concentration of 2.5 g/kg of diet. A 5-day test diet adaptation period preceded 5 days of faeces collection during the experimental trial. Food was weighed each day, divided into two equal portions, and given to the animals at 9 a.m. and 5 p.m. in stainless-steel bowls. Food quantity was administered considering maintenance energy requirements according to the FEDIAF equation (110 kcal × $BW^{0.75}$) (FEDIAF, 2020). Bowls were removed before the next meal, and any uneaten food was weighed and recorded. Faeces were collected twice daily, weighed, and kept frozen at −20°C until analysis.

The nutritional adequacy protocol was performed according to AAFCO guidelines (AAFCO, 2018) as outline in the "AAFCO Methods For Substantiating Nutritional Adequacy of Dogs and Cats Foods". Briefly, the dogs were fed the two respective diets (control vs insect diet) for a period of 26 weeks according to their energy requirements (FEDIAF, 2020). The dogs were assessed clinically at the beginning (T0) and at the end (T1) of the trial and their weight was recorded as well. At the beginning and at the end of the study a blood test was performed to ensure there was no variations of the following parameters: haemoglobin, PCV, serum albumin and ALP.

6.2.2 Trial 2

Five experimental diets were formulated to supply the nutritional requirements of adult dogs according to FEDIAF (FEDIAF, 2020). A basal diet (CO) was formulated based on rice and poultry by-product meal; while other 3 experimental diets were made only with the replacement of rice with different inclusion levels of lentil pasta by-product (LP), in particular substitution of 33% (LP33), of 66% (LP66) and of 100% (LP100). The other ingredients were not altered in the formula. A fifth experimental diet (LPS) was formulated with 70% of the basal diet (CO) and 30% of the lentil pasta by-product so as to evaluate the ingredient digestibility alone. The ingredient composition and particle size of the diets are shown in (Table 2)

Table 2 Ingredients composition and particle size of the experimental diets

	Experimental diets[1]			
Ingredients (g/kg, as-fed basis)	*CO*	*LP33*	*LP66*	*LP100*
Broken rice	571.7	381.2	190.6	-
Red lentil pasta by-product	-	190.6	381.2	571.7
Poultry by-product meal	281.8	281.8	281.8	281.8
Poultry fat	86.4	86.4	86.4	86.4
Palatability enhancer[2]	20.0	20.0	20.0	20.0
Beet pulp	20.0	20.0	20.0	20.0
Potassium chloride	4.7	4.7	4.7	4.7
Sodium chloride	4.5	4.5	4.5	4.5
Vitamin-mineral premix[3]	3.2	3.2	3.2	3.2
Mold inhibitor[4]	1.0	1.0	1.0	1.0
Antioxidant[5]	0.5	0.5	0.5	0.5
DL-Methionine	0.2	0.2	0.2	0.2
Mean geometric diameter (μm)	208	223	211	224
Geometric standard deviation (μm)	1.45	1.28	1.38	1.28

[1]CO, control diet; LP33, substitution 33% rice with red lentil pasta by-product; LP66, substitution 66% rice with red lentil pasta by-product; LP100, substitution 100% rice with red lentil pasta by-product.
[2]D'TECH 10L, Palatabilizante Líquido, SPF do Brasil Indústria e Comércio Ltda., Descalvado, Brazil.
[3]Added per kg of food: Vitamin A, 24,000 IU; Vitamin D3, 1,350 IU; Vitamin E, 150 IU; Vitamin K3, 1.5 mg; Vitamin B1,4 mg; Vitamin B2, 9 mg; Pantothenic Acid, 20 mg; Vitamin B6, 3 mg; Vitamin B12, 54 mcg; Vitamin C, 90 mg; Nicotinic Acid, 25 mg; Folic Acid, 0.45 mg; Biotin, 0.09 mg; Iron, 60 mg; Copper, 8.3 mg; Manganese, 6.7 mg; Zinc, 110 mg; Iodine, 1.3 mg; Selenium, 0.35 mg.
[4]Mold-Zap Citrus, Alltech do Brasil Agroindustrial Ltda., Araucária, Brazil.
[5]Banox, Alltech do Brasil Agroindustrial Ltda., Araucária, Brazil.

In order to assess the implications of the red lentil pasta by-product inclusion on extrusion processing and kibble formation, an extrusion study was conducted following a completely randomized design (all diets were extruded on the same day). For statistical comparisons, the experimental unit (treatment repetition) was used to establish the processing information and material samples that were taken every 15 min during a stable extrusion processing. At least four samplings per treatment were obtained for processing parameter evaluation. Kibble characteristic evaluation was analysed in a completely randomized design, and each kibble was considered an experimental unit with 20 repetitions (kibbles) analysed per treatment.

The *in vivo* study included nutrient digestibility, faecal fermentation end-product, glycemic and insulinemic curve, biogenic amines and nitrogen balance evaluation. These assessments were organized in a completely randomized block design with five diets, two blocks of 20 dogs each, and 4 dogs per diet in each block, totaling eight dogs per diet and 40 dogs in total. The limiting factor for each block was time, due to the impossibility of handling the 40 dogs at the same time in the

laboratory. The experimental unit was an individual dog. The palatability trial was conducted with 38 dogs, 19 males and 19 females, of different breeds and body weight, in a completely randomized design according to the two-bowl method (Griffin, 2003). The CO and the LP100 were compared for dog palatability and the comparisons were performed at Panelis Latin America (Descalvado, Sao Paulo, Brazil) using a qualified panel of dogs. The first choice (first product consumed) and preferred product (product consumed in greater amount) were determined using the two-bowl method (Griffin, 2003). Dogs were housed individually. Due to the differences in body weight, the results were calculated as the relative consumption of each diet, and the mean intake of the two meals was compared.

The ingredients of each diet were weighed individually for each batch, mixed in a horizontal single shaft mixer with a capacity of 450 kg/batch directly coupled into the mill, and ground in a high-speed hammer mill fitted with a 0.8 mm size screen sieve (1000 kg/h capacity, Sistema Tigre de Mistura e Moagem, Tigre, Sao Paulo, Brazil). Extrusion processing was carried out on a single-screw extruder (MEX- 250, Manzoni Industrial Ltda., Campinas, Brazil) with a production capacity of 250 kg/h, equipped with a differential diameter cylinder preconditioner. The preconditioner shaft speed in the small and large cylinders was set at 60 and 30 rpm, respectively, resulting in an average retention time of dough in the preconditioner of 180 seconds. An extruder screw profile typical for pet foods was used: first section – single flight screw and no steam lock; second section – single flight screw and small steam lock; third section – double flight uncut screw and small steam lock; fourth section – double flight uncut screw and medium steam lock; fifth section – double flight cut cone screw. For operation conditions, steam and water application was implemented only into the preconditioner. The extruder screw was fixed at 607 rpm for all treatments. The extruder die was equipped with a probe to measure the mass temperature at the center of the product flow. A circular die with one opening of 8.0 mm in diameter was used, with an open are of 50.2 mm^2. The extruder knife speed was set at 940 rpm for all treatments. After extrusion, the extrudates were dried in a dual pass dryer at 110 °C for 24 min.

Processing parameters were stabilized for the CTRL diet and kept unchanged for the other treatments, in order to describe the effect of the LP inclusion on extrusion traits and kibble macrostructure. After reaching a stable processing condition (approximately after 30 min), the extrusion parameters were recorded, and samples from the preconditioner, extruder and dryer were collected four times every 15 min for each treatment and properly stored for later analysis (experimental unit). The specific mechanical energy (SME, kW-h/ton), specific thermal energy (STE, kW-h/ton) and total specific energy (TSE, kW-h/ton) implementations were calculated with recorded

parameters for each treatment repetition using the equation proposed by Riaz (Riaz, 2000 b) as described in more detail by Pacheco (Pacheco et al., 2018). As a complementary analysis of processing, the starch gelatinization (Sá et al., 2013) was determined for each treatment repetition through enzymatic methods previously described.

The mean geometric diameter (MGD) and geometric standard deviation (GSD) of the raw material mixture after grinding were calculated using 100 g of samples in a sieve agitator with a coupled hammer (ABRT 820, Bronzinox, Sao Paulo, Brazil). An agitation time of 10 min and 12 screen sieve sizes were used: 1.000 mm, 0.841 mm, 0.710 mm, 0.595 mm, 0.500 mm, 0.354 mm, 0.297 mm, 0.250 mm, 0.210 mm, 0.149 mm, 0.125 mm, and 0.062 mm plus plates (Zanotto et al., 1996). The results were integrated using Granucalc software (Granucalc, Embrapa Suínos e Aves, Concordia, Brazil).

Kibble characteristics and macrostructure were analysed using kibble samples collected from the dryer. The radial expansion ratio, specific length and piece density were determined by measuring the length, diameter, and mass of 20 representative extrudates, as described by (Karkle et al., 2012 b). Hardness was analysed in 20 kibble pieces (first stabilized at the same moisture in an oven at 35 °C for 24 h) using a texture analyser (TAX/T2I, Stable Micro Systems, Godalming, UK) equipped with a load cell of 50 kgf (kilogram force) and a cone probe.

The total tract apparent digestibility of nutrient evaluation was carried out through the quantitative collection of faeces, according to the recommendations of FEDIAF (FEDIAF, 2020). Forty clinically healthy adult Beagle dogs were fed 5 dry extruded diets. The health of the animals was previously evaluated by physical examinations. Each 17-d digestibility trial included a 12-d food adaptation phase and a 5-d total collection of faeces. During the adaptation period, dogs were housed in 1.5 × 4.0 m kennels with a solarium and were released daily for 6 hours into a collective playground for exercise and socialization. During the collection period, the dogs were individually housed in 1 × 1 × 1 m stainless steel metabolic cages. The amount of food for individual dogs was calculated to satisfy the metabolizable energy (ME) requirement for adult dogs in maintenance (kcal/d = 110 x $BW^{0.75}$) according to FEDIAF (FEDIAF, 2020) and was offered twice daily (10 h and 16 h). Offered and refused amounts were weighed, and the intake was recorded. Faeces were quantitatively collected and weighed at each feeding time and immediately frozen at -20 °C. At the end of the digestibility period, faeces were thawed, homogenized, pooled by dog, pre-dried in a forced-air oven (MA035, Marconi, Piracicaba, Brazil) at 55 °C for 72 h, and stored for chemical analysis. The faecal quality was scored on a 0 – 5 scale (Carciofi et al., 2008): 0 = watery liquid faeces that can be

poured; 1 = soft faeces, formless; 2 = soft, unformed stool which assumes the shape of the container; 3 = soft, formed and moist stool that retains shape but leave traces on the floor when picked up; 4 = well-formed and consistent stool; the faeces do not leave traces on the floor when picked up; 5 = hard, dry pellets in the form of small and hard mass. During the faecal collection phase, samples of fresh faeces were collected within 15 min after excretion (cages were continuously observed) on three consecutive days to measure faecal pH, biogenic amines and the fermentative end-products short-chain fatty acids (SCFA), lactate and ammonia.

The glucose and insulin postprandial responses of dogs were determined only in the control diet (CO) and the formulations with 100% replacement of LP (LP100). A total of 16 healthy adult Beagle dogs, [of which 9 females and 7 males, all neutered, median of 3 years old (IQR 2-5.5), 11.9 ± 1.9 kg BW, with a median BCS of 5 (IQR 5-6) on a nine-point scale (Laflamme, 1997)] were enrolled. Postprandial response profiles were evaluated after the digestibility trials following the procedure described by (Carciofi et al., 2008), with modifications to blood collection times: 0 (zero before the meal), 15, 30, 60, 120, 180, 240, 300, 420, 540 and 720 min after the end of the meal. Dogs were conditioned to ingest all food within 10 min, in one single meal per day. Thereby the animals were tested 24 h after the last meal. The time for blood sample collection was started immediately after the end of the meal. Dogs that did not eat all food within 10 min were not tested on that day and was tested again on the next day. On the day of blood collection, each dog was aseptically catheterized using a peripheral intravenous catheter inserted into the cephalic vein (Angiocath 20 GA x 1.16 in., Becton-Dickinson, Franklin Lakes, NJ, USA). In each collected time, 3.0 ml of blood were collected and divided in two containers. Immediately after blood collection, each catheter was flushed with saline solution to maintain patency. For glucose analysis, 1.0 ml of blood was deposited into sodium fluoride and EDTA tube (NaF/Na_2EDTA, BD Vacutainer), centrifuged at 2000 g for 10 min at 4°C, and the plasma separated into a polypropylene tube. For insulin test, 2.0 ml were deposited in the container with anticoagulant (Tube serum, BD Vacutainer), centrifuged at 2000 g for 10 min at 4°C and the serum separated into a polypropylene tube. Glucose and insulin samples were stored at −80°C until analysis. Before collecting the samples, 0.3 ml of blood was withdrawn and discarded to avoid any dilution of the analytes of interest by saline in the catheter.

6.3 Chemical analyses

6.3.1 Trial 1

At the end of the collection period, pooled individual faeces were thawed, homogenized, and freeze-dried. Faeces samples were freeze-dried using a laboratory freeze dryer (5Pascal, Trezzano sul Naviglio, Italy). The process of lyophilization consisted of dry sublimation with water evaporation under low pressure (0.200 mbar) until the samples reached room temperature (25◦C). Both the foods and freeze-dried faeces were ground to pass through a 1-mm sieve and stored in airtight plastic containers for laboratory tests. The dry matter (DM) of the foods was determined by drying the samples at 103°C to constant weight. The foods and faeces were analysed according to the AOAC (Association of Official Analysis Chemists International (AOAC), 2000) standard procedures; thus, ash was determined by muffle furnace incineration (section 942.05), crude protein (CP) was ascertained using the Kjeldahl method (section 954.01), and ether extract (EE) was analysed following acid hydrolysis (section 954.02). In addition, diet crude fibre (CF) was determined using the method described in section 962.09 (29), and amino acid content by HPLC (Waters Alliance System with a Waters 1525 Binary HPLC Pump, Waters 2707 Autosampler, and Waters 2475 Multi l Fluorescence Detector, Milford, USA) after pre-column derivatization (Madrid et al., 2013) in samples ground to pass a 0.5-mm sieve. The detection limit ranged from 2.9 to 20.1 pmol/µl depending on the amino acid. Tryptophan was not analysed. Samples of foods and faeces were burnt to ashes and acid digested in the microwave (García-Rico et al., 1999), prior to the determination of chromium concentrate by inductively coupled plasma optical emission spectrometry (ICP-OES). Calcium and phosphorus were also determined by ICP-OES in the absence of the previous incineration. Hydroxyproline and the related collagen content were assessed according to the colorimetric method adapted by Kolar (Kolar, 1990) and described in the AOAC (Association of Official Analysis Chemists International (AOAC), 2000) section 990.26. The acid hydrolysis of the sample was performed under heat; an oxidizing agent was added to the sample, and oxidized hydroxyproline was measured photometrically.

6.3.2 Trial 2

Samples of ingredients, diets and pre-dried faeces were ground in a cutting mill using a 1 mm screen sieve (MA680, Marconi, Piracicaba, Brazil). The chemical composition was determined according to official methods of the AOAC (Association of Official Analysis Chemists International (AOAC), 2000) for dry matter (method 934.01); crude fat was determined by acid hydrolysis (method 954.02); ash

content, by muffle furnace incineration (method 942.05); crude fibre (method 962.09) and crude protein (method 990.03) using a LECO nitrogen/protein analyser (FP-528, LECO Corporation, Saint Joseph, USA); and total and insoluble dietary fibre by the enzymatic-gravimetric method (method 991.43). Soluble dietary fibre was calculated as total fibre minus insoluble fibre. Organic matter (OM) was calculated as DM minus ash. Gross energy (GE) was determined in a bomb calorimeter (IKA C2000 Basic, IKA-Werke GmbH & Co., KG, Staufen, Germany). The total starch content was determined using an enzymatic method (Hendrix, 1993). Amylose and amylopectin were analysed according to the method of (Knutson, 1986) and expressed as a percentage of the total starch. To analyse the starch cooking on extrusion, the starch gelatinization degree was determined using the amyloglucosidase method (Sá et al., 2013). All the analysis was conducted in duplicate and repeated when the variation between duplicates was greater than 5%.

Faecal pH was measured using a digital pH meter (DM20, Digimed Analítica Ltda., São Paulo, Brazil) immediately after collection by mixing 2 g of fresh faeces with 6 ml of ultrapure water. The concentrations of faecal SCFA were analysed by gas chromatography (GC-2014, Shimadzu Corporation, Kyoto, Japan) according to (Erwin et al., 1961). Briefly, 10 g of faeces was mixed in 30 ml of formic acid solution at 4.2 N (1:3 w/v), precipitated at 4 °C for 72 h, and centrifuged (5,000 G at 15 °C for 15 min). Lactic acid was measured according to (Pryce, 1969) by mixing 3 g of faeces with 9 ml of milli-Q water and subsequent evaluation with a colorimetric method (Spectrophotometer Quick-Lab, Drake, Sao José do Rio Preto, Brazil). Ammonia was assessed in the extracts prepared for SCFA according to (Vieira, 1980) in a nitrogen distillation system (Tecnal TE-036/1, Tecnal, Piracicaba, Brazil).

The faecal concentrations of biogenic amines were evaluated using five grams of fresh faeces which were homogenized and added to 7 mL of a 5% trichloroacetic acid solution and then mixed for 3 min by vortex and centrifuged at 10000x*g* for 20 min at 4°C (5810R; Eppendorf, Hamburg, Germany), according to (Vale et al., 1997). The supernatant was filtered through qualitative filter paper, and the residue was extracted twice using 7 and 6 mL of a 5% trichloroacetic acid solution, separately. Then, the supernatants were filtered and pooled. The final volume obtained was recorded and frozen. Biogenic amine concentrations in the supernatant were determined by HPLC (HPLC model LC-10AD; Shimadzu Corporation, Kyoto, Japan).

The plasma glucose concentrations were determined with the glucose oxidase test (GOD-ANA, Labtest Diagnóstica S.A., Lagoa Santa, Brazil) using a semiautomated glucose analyzer (Labquest model BIO-2000, Labtest Diagnóstica S.A., Lagoa Santa, Brazil). All the analysis was conducted in

duplicate and repeated when the variation was greater than 5%. The serum insulin concentration was performed using a kit Quantikine ELISA (human/canine/porcine insulin immunoassay, R&D Systems, Minneapolis, USA), following the recommendations of the manufacturer. The reading was performed in microplate reader (Biochrom Asys Expert Plus, Biochrom, Cambourne, Cambridge, UK), using a 450 nm filter. The insulin analysis inter-assay coefficient of variation was 5.7% and the intra-assay coefficient of variation was 3.6%. The responses profiles were compared computing the basal, minimum, mean, and maximum concentrations, and the time to peak of the absolute and incremental values (absolute minus basal metabolite value of the animal). The integrated areas under the postprandial glucose and insulin response curves were calculated by numerical integration by the trapezoidal method using R (R Core Team, 2019).

6.4 Calculations

6.4.1 Trial 1

6.4.1.1 *In vivo* digestibility

Apparent total tract digestibility coefficients (ATTDC) of the individual dietary elements of the two diets were calculated as follows:

a) Total fecal collection method (TFC):

$$ATTDC\ X_{diet}\ (\%) = [(total\ X_{diet} - total\ X_{feces}) / total\ X_{diet}] \times 100$$

where X is the total content of: DM, organic matter (OM), CP, EE, ash, calcium, or phosphorus in the consumed food or feces produced (X_{diet} and X_{feces}, respectively);

b) Marker method (Cr_2O_3):

$$ATTDC\ X_{diet}\ (\%) = \{[(X/Cr_2O_3)_{diet} - (X/Cr_2O_3)_{feces}]/ (X/Cr_2O_3)_{diet}\} \times 100$$

where X represents the concentration of DM, OM, CP, EE, ash, calcium, or phosphorus in the diet or feces;

Cr_2O_3 represents the chromium oxide concentration in the diet or feces;

$(X/Cr_2O_3)_{diet}$ = ratio between nutrient (X) and Cr_2O_3 concentration in the diet;

$(X/Cr_2O_3)_{feces}$ = ratio between nutrient (X) and Cr_2O_3 concentration in the feces.

6.4.1.2 *In vitro* digestibility

The *in vitro* digestibility (or degradability) of DM, CP and OM of the food was determined (in triplets) employing the methods described by Hervera et al. (2007) (Hervera et al., 2007) and Hervera et al. (2009) (Hervera et al., 2009). The methods involve two phases: the first entails incubation for 2 h under conditions simulating gastric digestion (pH 2, 39ºC, and inclusion of pepsin); whereas the second phase simulates 4 h of post-gastric digestion (pH 6.8, 39ºC, and inclusion of a pancreatin preparation for enzymatic digestion). The resulting residue was filtered, dried, and weighed to determine the remaining DM content, and incinerated to determine the residual OM content. Residual CP was determined by ascertaining the nitrogen content of the residue (using the Kjeldahl method) and considering a N:P conversion factor of 6.25. The *in vitro* digestibility of DM, OM, and CP were calculated as the difference between the amount of each initial nutrient in the sample vs. the undigested residue, divided by the initial nutrient content of the sample.

6.4.1.3 Estimated digestibility

Data from the *in vitro* digestibility analyses were also used to estimate *in vivo* OM and CP digestibility according to the regression equations reported by Hervera et al. (Hervera et al., 2007, 2009):

Estimated digestibility of OM (%) = – 9.15 + 1.06 x *in vitro* OM digestibility (%) (Hervera et al., 2007);

Estimated digestibility of CP (%) = 37.91 + 0.52 x *in vitro* CP digestibility (%) (Hervera et al., 2009).

6.4.2 Trial 2

6.4.2.1 *In vivo* digestibility of the diets

Apparent total tract digestibility coefficients (ATTDC) of the individual dietary elements of the two diets were calculated as follows:

Total fecal collection method (TFC):

$$ATTDC\ X_{diet}\ (\%) = [(\text{total } X_{diet} - \text{total } X_{feces}) / \text{total } X_{diet}] \times 100$$

where X is the total content of: DM, organic matter (OM), CP, EE, ash, calcium, or phosphorus in the consumed food or feces produced (X_{diet} and X_{feces}, respectively)

6.4.2.2 *In vivo* digestibility of the ingredient

The calculation of the apparent digestibility and metabolizable energy of the red lentil pasta by-product ingredient was performed using the substitution method, or the difference method, whose equation was proposed by Matterson et al. (Matterson et al., 1965) and Sakomura & Rostagno (Sakomura et al., 2007) and it is described below. For the calculation, the inclusion percentage of the ingredient was corrected for the dry matter

$$\mathrm{ATTDCing} = \mathrm{ATTDCrd} + \frac{\mathrm{ATTDCtd} - \mathrm{ATTDCrd}}{\mathrm{Incl}(\frac{\mathrm{g}}{\mathrm{kg}})/1000}$$

Where:

ATTDCing = Apparent Total Tract Digestiblity Coefficient of the ingredient;

ATTDCrd = Apparent Total Tract Digestiblity Coefficient of the reference diet (CTRL);

ATTDCtd = Apparent Total Tract Digestiblity Coefficient of the test diet (LPS);

Incl(g/kg) = Inclusion level of the ingredient (LP) in the reference diet (CTRL)

6.4.2.3 Extrusion processing

The SME (kW-h/ton) was calculated for each treatment repetition (experimental unit) using the following equation (Riaz, 2000 b):

$$\mathrm{SME}\ (\frac{\mathrm{kwh}}{\mathrm{t}}) = \frac{\left((\sqrt{3}\ \mathrm{x}\ \mathrm{Voltage}\ \mathrm{x}\ (\mathrm{At} - \mathrm{Av})\ \mathrm{x}\ (\cos \mathrm{Fi}\ )\right)}{\mathrm{M}}$$

Where: Voltage = 220 V; At = torque load working amperage (A); Av = no torque load working amperage (A); cosFi = power factor (0.80); M = mass flow rate from extruder (kg/h).

The STE (kW-h/ton) in the preconditioner and extruder was calculated by mass and energy balance equations according to Riaz (2000). The feed, water and steam total input and output mass amounts were determined. These mass values and the corresponding specific heat values from each component of the system were used to calculate the amount of heat produced, as described below.

A. Mass Balance:

1) For Preconditioner

$M_r + M_{sp} + M_{wp} = M_p + M_{slp}$

Where:

M_r = raw material feed rate (kg/hr);

M_{sp} = steam injection into preconditioner (kg/hr);

M_{wp} = water injection into preconditioner (kg/hr);

M_p = preconditioner product flow rate (kg/hr);

M_{slp} = steam loss from preconditioner (kg/hr).

2) For Extruder

$M_p + M_{we} = M_{sle} + M_e$

Where:

M_p = preconditioner product flow rate (kg/hr);

M_{we} = water injection into extruder (kg/hr);

M_{sle} = steam loss from extruder (kg/hr);

M_e = product flow rate (kg/hr)

B. Energy Balance:

1) For Preconditioner

$Q_r + Q_{sp} + Q_{wp} = Q_p + Q_{slp} + Q_{\Sigma\Delta hP} + Q_{LP}$

Where:

Q_r = energy flow with raw material (kJ/hr);

Q_{sp} = energy flow with steam injection (kJ/hr);

Q_{wp} = energy flow with water injection (kJ/hr);

Q_p = energy flow with flow rate (kJ/hr);

Q_{slp} = energy flow with steam loss (kJ/hr);

$Q_{\Sigma\Delta hP}$ = energy flow to cook starch and protein (kJ/hr) in the preconditioner;

Q_{LP} = preconditioner energy loss (kJ/hr).

2) For Extruder

$$Q_p + Q_{we} + Q_{SME} = Q_{sle} + Q_{\Sigma\Delta hE} + Q_{LE} + Q_e$$

Where:

Q_p = energy flow with raw flow rate (kJ/hr);

Q_{we} = energy flow with water injection (kJ/hr);

Q_{SME} = energy flow with specific mechanical energy (kJ/hr);

Q_{sle} = energy flow with product flow rate (kJ/hr);

$Q_{\Sigma\Delta hE}$ = energy flow to cook starch and protein (kJ/hr) in the extruder;

Q_{LE} = extruder energy loss (kJ/hr);

Q_e = energy flow with flow rate (kJ/hr).

The amount of heat (Q) was obtained from the formula:

$$Q = m \times c \times T$$

Where: m = mass; c = specific heat capacity; T = temperature

The STE was calculated as follow:

$$\mathrm{STE}\left(\frac{\mathrm{kWh}}{\mathrm{t}}\right) = \left(\frac{Q\Sigma\Delta hE + Qwe + Qr + Qwp + Qsp + Q\Sigma\Delta hP}{Me}\right) \div 3.6$$

The TSE (kW-h/ton) was obtained by the summation of SME and STE.

6.4.2.4 Kibble macrostructure

For each treatment, the length (l_e), diameter (d_e) and mass (m_e) of 20 extrudate kibbles were measured by using a vernier calliper and a precision scale. Data were used to obtain the radial expansion ratio (ER), specific length (l_{sp}) and piece density (ρ), as described below (Karkle et al., 2012 b). The die diameter (dd) used was 8 mm:

$$\mathrm{ER} = \frac{d_e^2}{d_d^2} \qquad l_{sp}\left(\frac{m}{kg}\right) = \frac{l_e}{m_e} \qquad \rho\left(\frac{kg}{m^3}\right) = \frac{m_e}{\pi \times (\frac{d_e}{2})^2 \times l_e}$$

6.4.2.5 Nitrogen balance

Nitrogen levels in the urine samples were determined using a LECO nitrogen/protein analyser (FP-528, LECO Corporation, Saint Joseph, USA). The nitrogen (N) balance was therefore calculated as the difference between the ingested N (Ni) and the N excreted with faeces (Nf) and urine (Nu), as described in the following equation:

Nitrogen balance (mg/kg$^{0.75}$/day) = Ni (mg/kg$^{0.75}$/day) – [Nf (mg/kg$^{0.75}$/day) + Nu (mg/kg$^{0.75}$/day)]

6.5 Statistical analyses

6.5.1 Trial 1

The statistical unit was the individual dog (n=6) for *in vivo* digestibility trials, and the diet (n=2) for *in vitro* digestibility trials. The comparisons between diets (CTRL vs. BSF) and methods (*in vivo* TFC vs. Cr_2O_3) were analyzed using two-way ANOVA, considering the diet (D) and the method (M) of *in vivo* digestibility calculation as the source of variation, respectively. Before testing for group and method differences, the non-normality of the data distribution and the homogeneity of variance were assessed by the means of the Shapiro-Wilk test and Levene's test, respectively. For the nutritional adequacy the two diets were assessed used a Student t-test. The significance level was set at $p = 0.05$. A statistical trend was considered for $p \leq 0.10$. All statistical analyses were performed using R Software (version 3.6.1) (R Core Team, 2019).

6.5.2 Trial 2

The results of the extrusion parameters were analysed as a complete randomized design with four replications (sampling time) per treatment. The kibble characteristics were evaluated in a complete randomized design, with 20 experimental units (individual kibble) per treatment. Data on apparent digestibility and faecal parameters were analysed in a completely randomized block design, with two blocks of 20 dogs and 8 experimental units (dogs) per treatment. All data were tested for normality (using Lilliefors test) and homogeneity of variances (using Levene's test) and then analysed by one-way ANOVA test. When significant differences were found by the ANOVA, polynomial contrasts were used to compare means according to the red lentil pasta by-product inclusion (33%, 66% and 100%), and orthogonal contrasts (based on Tukey HSD test) were used to compare the diets. The faecal score was analysed by the Kruskal-Wallis test. For the glycaemic and insulinemic curve analysis, the area under the curve (AUC) of the postprandial responses was calculated by numerical integration using the trapezoidal method. Data were submitted to repeated measures ANOVA ($P<0.05$). The time to peak in the glycaemic and insulinemic curve analyses were submitted to Wilcoxon test. Data were evaluated using R Software (version 3.6.1) (R Core Team, 2019). In the palatability study, the first preference was evaluated using the χ^2 test, and the food intake ratio was evaluated by Students t-test, after checking for the normality (using Lilliefors test) and homoscedasticity (using F-test). Statistical significance was set at the level of 5% ($p < 0.05$), while a statistical trend was considered at the level of 10% ($p < 0.1$).

7. RESULTS

7.1 Trial 1

7.1.1 Digestibility trial

The foods were well accepted during all the trial length and no episode of nausea or vomiting has been reported. The *in vivo* ATTDC digestibility results are summarized in Table 3. The two methods used to estimate *in vivo* digestibility (TFC and Cr_2O_3) showed similar results between the CTRL and BSF groups in relation to DM, OM, EE, ash, and phosphorus. However, the ATTDC of CF was significantly lower ($p < 0.001$) in the BSF diet compared with the CTRL diet. On the contrary, the ATTDC of calcium was significantly higher ($p < 0.05$) in the BSF compared with the CTRL diet. A statistical trend ($p = 0.066$) was observed for the ATTDC of CP, being higher in the animals fed the BSF compared with the CTRL diet.

Table 3 Comparison of the *in vivo* digestibility using the total fecal collection method (TFC) and *in vivo* digestibility with marker (Cr_2O_3) in 6 dogs (mean values are presented).

In vivo digestibility (%)

	TFC[1]		Cr_2O_3			p-value		
	CTRL[2]	BSF[3]	CTRL[2]	BSF[3]	SEM	D[4]	M[5]	DxM[6]
Dry matter	82.11	82.17	83.05	83.83	0.52	0.698	0.241	0.740
Organic matter	86.23	85.04	86.98	86.46	0.45	0.358	0.247	0.719
Crude protein	72.41	75.80	74.04	78.22	1.01	0.066	0.311	0.842
Ether extract	96.58	96.40	96.72	96.75	0.14	0.800	0.411	0.717
Crude fiber	43.13	18.83	45.78	23.60	3.18	<0.001	0.393	0.798
Ash	32.73	35.76	35.88	41.39	1.95	0.292	0.280	0.757
Calcium	12.16	24.88	19.19	31.62	2.61	0.018	0.162	0.976
Phosphorus	20.77	21.46	26.17	25.83	2.00	0.946	0.280	0.908

[1]TFC: total fecal collection;
[2]CTRL: control diet;
[3] BSF: Black soldier fly diet;
[4] D: Diet;
[5] M: Method;
[6] DxM: diets and methods interaction.

No statistical differences were observed between the two ATTDC methods (TFC vs. Cr_2O_3). Furthermore, no statistical interaction between diets and methods was found.

The *in vitro* digestibility data and estimated *in vivo* digestibility results, obtained utilizing the regression equations described in Hervera et al. (2007) (Hervera et al., 2007) and Hervera et al. (2009) (Hervera et al., 2009), are reported in Table 4. The digestibility values for DM, OM, and CP obtained using the *in vitro* method were higher for both the CTL and the BSF diet (by an average of: +8.43%, +5.25%, and +6.08%, respectively) compared with those obtained using *in vivo* methods. The estimations of *in vivo* digestibility of OM and CP (based on *in vitro* data) were consistently higher than the data obtained using *in vivo* ATTDC methods: *in vitro* estimation of *in vivo* digestibility overestimated OM and CP digestibility by up to 4.0% and 9.8%, respectively, compared with the *in vivo* methods.

Table 4 Comparison of the *in vitro* digestibility of the two diets (CTRL vs. BSF) and estimated *in vivo* digestibility based on the *in vitro* results.

In vitro digestibility (%)

	CTRL[1]	BSF[2]
Dry matter	90.65	91.79
Organic matter	90.82	92.04
Crude protein	80.06	82.33

Estimated in vivo digestibility (%) based on the vitro results

	CTRL[1]	BSF[2]
Organic matter[3]	87.12	88.41
Crude protein[4]	79.54	80.72

[1]CTRL: control diet;
[2] BSF: Black soldier fly diet;
[3] according to Hervera et al. (2007) (Hervera et al., 2007) for OM estimation;
[4] according to Hervera et al. (2009) (Hervera et al., 2009) for CP estimation.

7.1.2 Nutritional adequacy trial

During the nutritional adequacy trial, the dogs did not show any signs of inappetence or food refusal. Two dogs of the control group and 1 dog from the BSF group were removed from the trial for reasons unrelated to the study (adoption). There were no statistical differences between groups at T0 and T1 for all the parameters considered (weight, haemoglobin, PCV, albumin and ALP). All the blood parameters remained within normal ranges according to (AAFCO, 2018) with no individual

with levels of haemoglobin < 12.0 g/dL, PCV < 36%, albumin < 2.4 g/dL or ALP > 300 IU/L. No individual dog lost > 15% of the initial BW and the average change in the BW was < 10%. Data are shown in Table 5.

Table 5 Weight and blood parameters of the dogs at T0 and T1 in the two different diets considered for the nutritional adequacy trial

Parameters	T0		T1	
	CTRL	BSF	CTRL	BSF
Weight (kg)	24.0±5.0	27.1±4.0	24.2±5.2	27.6±4.1
Haemoglobin (g/dL)	16.7±1.8	16.3±2.1	17.4±2.0	16.2±2.0
PCV (%)	47.8±4.9	47.6±4.7	47.1±4.7	44.6±4.5
Albumin (g/dL)	3.0±0.3	3.0±0.3	3.4±0.3	3.2±0.3
ALP (IU/L)	64.0±26.0	43.8±18.2	78.5±13.7	60.2±17.9

7.2 Trial 2

7.2.1 Experimental diets composition and extrusion variables

The diets were formulated to have, as the only difference, a different level of substitution of the main carbohydrate source. As expected, the levels of protein, starch and dietary fibre differed (as shown in Table 6) as the broken rice showed a lower content of protein and dietary fibre and a higher level of starch compared to the red lentil pasta by-product. Level of ash, acid-hydrolysed fat, crude fibre, gross energy, Ca, P, and moisture content were similar among CO, LP33, LP66 and LP100.

Table 6 Analysed chemical composition (g/kg, DM basis) of the dog foods and main carbohydrate sources used in the experimental trial

Item	Experimental diets and ingredients[1]						
	CO	*LP33*	*LP66*	*LP100*	*LPS*	*BR*	*LP*
Moisture	88.7	106.8	101.5	101.9	96.9	104.3	89.4
Ash[2]	51.4	53.7	57.2	58.4	44.7	8.4	23.3
Acid-hydrolyzed fat[2]	133.3	131.7	135.5	138.3	120.6	18.8	19.6
Crude protein[2]	296.0	318.7	355.9	388.7	295.7	113.3	299.0
Starch[2]	411.6	376.6	315.1	269.0	418.9	744.1	557.9
Resistant (%)	0.17	0.14	0.20	0.55	0.30	14.07	18.74
Amylose (%)	9.31	9.20	8.50	8.55	10.92	14.89	12.29
Amylopectin (%)	90.69	90.80	91.50	91.45	89.08	85.11	87.71
Amylose:Amylopectin	0.10	0.10	0.09	0.09	0.12	0.17	0.14
Crude fiber	22.8	24.4	25.2	28.1	24.9	12.3	15.6
Total dietary fiber[2]	105.9	110.6	122.4	127.9	102.0	51.1	95.1
Insoluble dietary fiber	98.4	102.4	113.5	117.7	94.7	44.8	87.9
Soluble dietary fiber	7.5	8.1	8.8	10.2	7.3	6.3	7.2
Ca	7.34	7.60	7.58	7.59	6.23	0.06	0.33
P	6.31	6.83	7.16	7.52	5.87	2.00	4.43
Gross energy (kcal/g)	4.89	5.00	5.05	5.05	4.88	4.24	4.42
Gross energy (MJ/kg)	20.4	20.9	21.1	21.1	20.4	17.7	18.5

[1]CO, control diet; LP33, substitution 33% rice with red lentil pasta by-product; LP66, substitution 66% rice with red lentil pasta by-product; LP100, substitution 100% rice with red lentil pasta by-product; LPS, with 70% of the basal diet (CO) and 30% of the lentil pasta by-product; BR, broken rice; LP, red lentil pasta by-product.

During the extrusion procedures, the preconditioner discharge mass temperature was kept at approximately 88.3 ± 1.5 °C for all diets ($p>0.05$) (Table 7). As planned, the throughput as well as the in-barrel moisture remained similar for all diets ($p>0.05$), so these parameters did not interfere on results interpretation. The extruder motor amperage, pressure and mass temperature increased quadratically with LP inclusion ($p<0.05$), indicating higher resistance to mass flow. This can also be observed by the quadratic increase in SME application with higher LP inclusion ($p<0.05$). However, STE, TSE and STE/SME ratio were similar for all the formulations considered ($p>0.05$).

It should be noted that the bulk density increased linearly ($p<0.001$) while increasing the level of LP inclusion in the diets. Increasing the LP inclusion promoted a linear increment ($p<0.001$) of the kibble hardness while, at the same time, fostered a linear decrease ($p<0.001$) of the specific length. The expansion rate and the piece density reduced quadratically ($p<0.001$) with the increase in LP.

Table 7 Processing variables, kibble characteristics and macrostructure of the diets

Item	Experimental diets[1]				SEM[2]	p-value	Polynomial contrasts	
	CO	*LP33*	*LP66*	*LP100*			*Linear*	*Quadratic*
Preconditioner								
Temperature (°C)	88.0	88.3	88.5	88.5	0.373	0.96	0.64	0.88
Extruder								
Motor amperage (A)	38.5^{a}	38.8^{a}	40.0^{b}	38.9^{ab}	0.182	<0.01	<0.05	<0.05
Pressure (MPa)	24.8^{a}	30.0^{b}	30.9^{b}	29.4^{b}	0.653	<0.001	<0.001	<0.001
Mass temperature (°C)	118.5^{a}	122.8^{ab}	129.8^{c}	126.8^{bc}	1.265	<0.001	<0.001	<0.05
Throughput (kg/h)	192.9	193.8	198.9	193.8	1.424	0.47	0.45	0.27
Bulk density (g/L)	383.8^{a}	402.5^{b}	433.3^{c}	451.0^{d}	6.842	<0.001	<0.001	0.95
In-barrel moisture (%)	26.0	25.3	25.4	24.8	0.283	0.55	0.25	0.97
Energy balance (kW-h/ton)								
SME[3]	11.1^{a}	11.1^{a}	13.0^{b}	11.7^{ab}	0.252	<0.01	<0.05	<0.05
STE[4]	63.5	65.5	71.6	63.9	2.337	0.64	0.67	0.29
TSE[5]	74.6	76.7	84.6	75.6	2.401	0.48	0.54	0.22
STE/SME ratio	58	5.9	5.5	5.5	0.218	0.90	0.49	0.92
Kibble macrostructure								
Hardness (N)	84.7^{a}	95.9^{a}	96.9^{ab}	115.6^{b}	2.842	<0.001	<0.001	0.34
Expansion rate	2.69^{b}	2.68^{b}	2.51^{a}	2.74^{b}	0.020	<0.001	0.63	<0.001
Piece density (g/cm^3)	0.49^{a}	0.48^{a}	0.54^{b}	0.50^{a}	0.004	<0.001	<0.001	<0.01
Specific length (cm/g)	15.24^{b}	15.48^{b}	14.81^{a}	14.60^{a}	0.063	<0.001	<0.001	0.08
Starch gelatinization (%)	89.7^{a}	90.7^{a}	95.9^{b}	97.2^{b}	0.871	<0.001	<0.001	0.80

[1]CO, control diet; LP33, substitution 33% rice with red lentil pasta by-product; LP66, substitution 66% rice with red lentil pasta by-product; LP100, substitution 100% rice with red lentil pasta by-product; LPS, with 70% of the basal diet (CO) and 30% of the lentil pasta by-product.
[2] SEM = standard error of the mean
[3] SME = specific mechanical energy
[4] STE = specific thermal energy
[5] TSE = total specific energy
[a, b, c, d] Mean values in the same row not sharing a common superscript letter differ (P < 0.05).

7.2.2 Nutrient intake, ATTDC, faecal characteristics and fermentation end products

Nutrient intake and ATTDC of nutrients are reported in Table 8. All diets were well accepted by the dogs, with no episodes of refusals, vomiting or diarrhoea.

The inclusion of LP did not affect the intake levels of dry matter, organic matter or acid-hydrolysed fat. However, as expected, the intake level of the crude protein and total dietary fibre increased linearly ($p<0.001$) with the increment of the LP inclusion level; on the contrary the intake of starch decreased linearly ($p<0.001$) with the addition of LP in the diets.

Dry matter, organic matter and gross energy digestibility appeared to be lower ($p<0.01$) in the LP100 diet compared to the LP33 and LP66 diets. Crude protein and total dietary fibre digestibility resulted to be higher ($p<0.05$) in the LP66 diet compared to the CO diet. Starch ATTDC was higher ($p<0.01$) in the CO diet compared to the LP100 diet, even though the difference appears to not have relative importance from a nutritional standpoint (0.999 vs 0.997).

There was no difference ($p>0.05$) in the nitrogen balance results and all the diets had a positive balance. The results of the ATTDC of the ingredient alone (LP) calculated using Matterson et al. (1965) are presented in Table 9. It is possible to observe that the ingredient has high apparent nutrient digestibility by dogs.

Table 8 Nutrient intake and apparent total tract digestibility coefficient of the different nutrients according to the diets tested

Item	Experimental diets[1]				SEM[2]	p-value	Polynomial	
	CO	*LP33*	*LP66*	*LP100*			*Linear*	*Quadratic*
Nutrient intake (g/kg $BW^{0.75}$/day)								
Dry matter	24.46	24.07	24.26	24.07	0.102	0.49	0.37	0.79
Organic matter	23.23	22.78	22.72	22.54	0.107	0.14	0.09	0.68
Acid-hydrolysed fat	3.29	3.21	3.26	3.30	0.016	0.24	0.47	0.14
Crude protein	7.43^{a}	7.64^{a}	8.58^{b}	9.31^{c}	0.140	<0.001	<0.001	<0.01
Starch	10.33^{d}	9.03^{c}	7.60^{b}	6.44^{a}	0.267	<0.001	<0.001	0.85
Total dietary fibre	2.66^{a}	2.65^{a}	2.95^{b}	3.06^{c}	0.035	<0.001	<0.001	0.09
ATTDC								
Dry matter	0.847^{ab}	0.858^{b}	0.855^{b}	0.825^{a}	0.004	<0.01	<0.05	<0.01
Organic matter	0.872^{ab}	0.884^{b}	0.876^{b}	0.847^{a}	0.004	<0.01	<0.01	<0.01
Acid-hydrolysed fat	0.920	0.920	0.922	0.917	0.002	0.89	0.81	0.53
Crude protein	0.764^{a}	0.802^{ab}	0.808^{b}	0.777^{ab}	0.005	<0.05	0.18	<0.01
Starch	0.999^{b}	0.999^{b}	0.998^{ab}	0.997^{a}	0.0002	<0.01	<0.001	0.26
Total dietary fibre	0.512^{a}	0.580^{ab}	0.604^{b}	0.546^{ab}	0.011	<0.05	0.12	<0.01
Gross energy	0.866^{ab}	0.880^{b}	0.876^{b}	0.846^{a}	0.004	<0.01	<0.05	<0.01
Nitrogen balance								
mg N/kg $BW^{0.75}$/day	67.0	76.7	164.8	102.2	24.7	0.52	0.34	0.39

[1]CO, control diet; LP33, substitution 33% rice with red lentil pasta by-product; LP66, substitution 66% rice with red lentil pasta by-product; LP100, substitution 100% rice with red lentil pasta by-product

[2] SEM = standard error of the mean

[a, b, c, d] Mean values in the same row not sharing a common superscript letter differ ($P < 0.05$).

Table 9 Apparent total tract digestibility coefficient of the different nutrients for the ingredient tested (LP) calculated according to Matterson et al. (1965) using the results from LPS

	ATTDC						
	Dry matter	Organic matter	Acid-hydrolysed fat	Crude protein	Starch	Total dietary fibre	Gross energy
LP[1]	0.939	0.938	0.811	0.877	0.998	0.765	0.938

[1]LP, red lentil pasta by-product

The incrementing inclusion of LP linearly increased ($p<0.05$) the faecal production and linearly decreased ($p<0.05$) both the faecal DM and faecal pH (Table 10). However, despite the increase in the moisture content, the faecal score remained similar with a median of 4 (well-formed and consistent stools) in all the diets during the digestibility trial. It is important to notice that during the period of adaptation 2 dogs of the LP100 and 1 dog of the LP66 diets had loose faeces (FS of 2 or 3 out of 5) for 5 to 7 days. Nonetheless, thereafter there were no more signs of diarrheic stools, suggesting a possible need, for the gastroenteric tract of certain dogs, of a period of adjustment with these levels of LP inclusion.

Rising the level of LP inclusion showed a linear increase of acetic acid ($p<0.01$), propionic acid ($p<0.001$), valeric acid and total VFA ($p<0.05$), as well as lactate ($p<0.01$). On the contrary, there was a linear decrease in isovaleric acid and total bVFA ($p<0.05$) when increasing the LP inclusion in the formulations. Levels of butyric acid, iso-butyric acid and ammonia were similar among dogs fed the diets tested ($p>0.05$)

Table 10 Faecal characteristics and fermentation products of dogs fed diets with different inclusion of red lentil pasta by-product

Item	Experimental diets[1]				SEM[2]	p-value	Polynomial contrasts	
	CO	*LP33*	*LP66*	*LP100*			*Linear*	*Quadratic*
Faeces characteristics								
g/kg $BW^{0.75}$/day (as fresh matter)	10.9^{a}	10.2^{a}	11.3^{a}	14.6^{b}	0.43	<0.001	<0.05	0.07
Faecal DM (g/kg)	353.6^{c}	334.1^{bc}	311.3^{ab}	288.9^{a}	5.20	<0.001	<0.05	0.18
Faecal score[3]	4.0	4.0	4.0	4.0	-	-	-	-
pH	6.24^{b}	6.30^{b}	6.19^{ab}	5.96^{a}	0.04	<0.01	<0.05	0.27
Fermentation products (mMol/g of DM)								
Acetic acid	275.2^{a}	284.1^{a}	307.5^{ab}	376.7^{b}	12.1	<0.05	<0.01	0.13
Propionic acid	159.8^{a}	181.2^{ab}	214.8^{b}	290.8^{c}	10.5	<0.001	<0.001	<0.05
Butyric acid	66.9	64.8	57.6	64.4	1.97	0.40	0.33	0.23
Total acetic, propionic, butyric acids	503.3^{a}	530.2^{a}	578.1^{a}	718.4^{b}	21.5	<0.001	<0.001	0.07
Valeric acid	6.64^{ab}	4.99^{a}	7.45^{ab}	14.5^{b}	1.26	<0.05	<0.05	0.06
Isobutyric acid	15.4	15.12	12.5	13.1	0.49	0.07	0.17	0.10
Isovaleric acid	27.6^{ab}	28.4^{b}	22.2^{ab}	21.5^{a}	0.97	<0.05	<0.05	0.26
Total bVFA	43.0^{ab}	44.0^{b}	34.7^{ab}	33.9^{a}	1.49	<0.05	<0.05	0.25
Total VFA	553.0^{a}	579.2^{a}	620.3^{a}	766.8^{b}	21.8	<0.001	<0.05	0.35
Ammonia (mMol/kg of DM)	350.6	361.1	316.1	318.2	10.9	0.37	0.20	0.50
Lactate (mMol/kg of DM)	4.98^{a}	5.22^{ab}	6.34^{bc}	7.24^{c}	0.22	<0.001	<0.01	0.54

[1]CO, control diet; LP33, substitution 33% rice with red lentil pasta by-product; LP66, substitution 66% rice with red lentil pasta by-product; LP100, substitution 100% rice with red lentil pasta by-product

[2] SEM = standard error of the mean

[3] Faecal score on a 0–5 scale (Carciofi et al., 2008): 0 = watery liquid faeces that can be poured; 1 = soft faeces, formless; 2 = soft, unformed stool which assumes the shape of the container; 3 = soft, formed and moist stool that retains shape but leave traces on the floor when picked up; 4 = well-formed and consistent stool; the faeces do not leave traces on the floor when picked up; 5 = hard, dry pellets in the form of small and hard mass. Calculated as the median of all of the observations

[a, b, c, d] Mean values in the same row not sharing a common superscript letter differ ($P < 0.05$).

Serotonin and agmatine biogenic amines were below the limit of detection (0.4 mg/kg of DM) for all the faecal samples analysed. Putrescine, phenethylamine and tryptamine did not differ ($p>0.05$) among the different diets (Table 11). Increasing LP inclusion led to a linear increment of cadaverine, histamine and spermidine ($p<0.001$) as well as tyramine ($p<0.01$). Spermine concentration, on the other hand presented a quadratic increase and it values reduced for LP100 and CO in comparison to LP33 ($p<0.01$).

Table 11 Faecal biogenic amines concentration (mg/kg of dry matter) in dogs fed diets with different inclusion of red lentil pasta by-product

Item	Experimental diets[1]				SEM[2]	p-value	Polynomial contrasts	
	CO	*LP33*	*LP66*	*LP100*			*Linear*	*Quadratic*
Putrescine	478.3	757.6	688.5	832.5	61.2	0.20	0.51	0.82
Cadaverine	192.8^{a}	512.8^{ab}	777.1^{ab}	1163.3^{b}	103.7	<0.01	<0.001	0.74
Tyramine	19.2^{a}	37.5^{a}	85.6^{ab}	188.2^{c}	20.1	<0.05	<0.01	0.19
Histamine	8.1^{a}	9.4^{a}	35.0^{a}	118.6^{b}	11.5	<0.001	<0.001	<0.05
Spermidine	65.7^{a}	79.7^{ab}	105.6^{bc}	117.0^{c}	5.7	<0.01	<0.001	0.88
Phenethylamine	5.3	7.8	6.6	8.2	1.2	0.86	0.61	0.96
Spermine	14.8^{a}	90.9^{b}	64.6^{ab}	25.4^{a}	9.2	<0.01	0.97	<0.01
Tryptamine	23.0	28.7	13.1	26.6	3.2	0.33	0.68	0.37

[1]CO, control diet; LP33, substitution 33% rice with red lentil pasta by-product; LP66, substitution 66% rice with red lentil pasta by-product; LP100, substitution 100% rice with red lentil pasta by-product; LP, red lentil pasta by-product (as ingredient calculated using (Matterson et al., 1965) from the results of the LPS diet).
[2] SEM = standard error of the mean
[a, b, c, d] Mean values in the same row not sharing a common superscript letter differ ($P < 0.05$).

7.2.3 Palatability study

Two animals were excluded for the test analysis due to underconsumption. The dogs ate normally without any signs of vomit and the quality of the faeces produced was adequate. From the analysis of the "first choice" (first feed consumed by the animal) it was verified that, regardless of breed, there was a preference for the LP100 diet ($P<0.05$) in comparison to the CO diet (Figure 1). By assessing the "intake rate" (amount of feed consumed by the animal, as a percentage of the total consumed), it was found that, regardless of the breed, there was a preference for the LP feed ($P<0.001$), as shown in Figure 2.

Figure 1 Analysis of the percentage of animals which consumed first one diet proposed compared to another (LP100 vs CO)

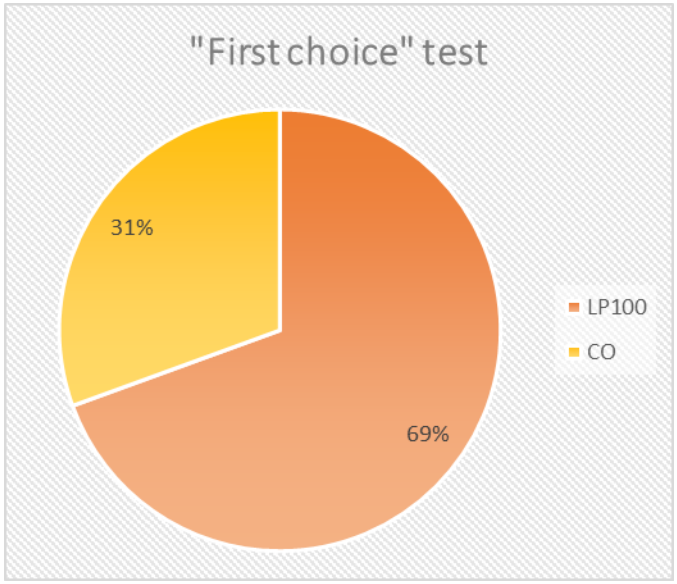
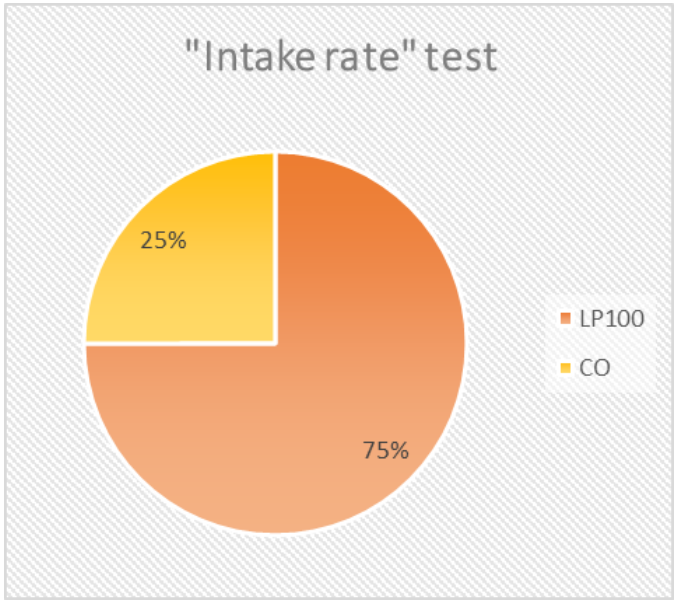


Figure 2 Analysis of the food consumed by the animals as a percentage of the total consumed (LP100 vs CO)



7.2.4 Glycaemic and insulinemic postprandial curve

Basal, minimum and mean glucose concentrations were higher ($p<0.05$) in the CO diet compared to the LP100, as well as the maximum glucose concentration which showed a statistical trend ($p=0.07$), as outlined in Table 12.
The time to glucose peak was slower in the LP100 diet (median of 180 minutes) compared to the CO diet (median of 150 minutes). The AUC 0-60' and 60-120' of glucose, as well as the total AUC of glucose, were lower ($p<0.05$) for dogs fed the LP100 diet compared to the CO diet. There was a statistical difference in the glucose concentration at 60, 420 and 720 minutes, with LP100 group showing a lower concentration compared to dogs fed CO diet (Fig. 3). Similarly, the glucose incremental curve showed a delayed glucose peak as well as a decreased total AUC ($p<0.05$) in the LP100 diet compared to the CO diet; while the maximum glucose incremental concentration was higher ($p<0.05$) in the CO diet (18.4 mg/dL) compared to the LP100 diet (9.3 mg/dL; data not shown).

The serum insulin curve of dogs fed LP100 diet showed a lower and delayed peak (300 minutes, IQR= 240-300) compared to the CO group (180 min; IQR= 180-180; $p<0.05$). The AUC 60-240' was also lower ($p<0.05$) compared to the CO group, showing a constant increase during the first 4 hours after the meal (Fig. 4). The basal insulin concentration was different between treatments ($p<0.05$) with dogs fed the LP100 diet showing a higher level (40.9±10.7 mg/dL) compared to the CO group (27.3±7.6 mg/dL), as shown in Table 13. It should be also observed that the two groups returned to their respective basal level (LP100 = 42.7±20.9 mg/dL; CO = 32.4 ±6.7 mg/dL) at time 720', possibly meaning a less sharp decrease of the curve for the LP100 diet compared to the CO diet. The serum insulin incremental curve showed even more conspicuous results when comparing the insulin incremental concentrations in the 2 groups. In fact, LP100 group had lower insulin incremental levels at 30, 60, 120 minutes ($p<0.05$), and 180 minutes ($p<0.01$) compared to the CO group. Likewise, the serum incremental insulin curve of dogs fed LP100 diet showed a lower and delayed peak (median of 300 minutes) compared to the CO group (median of 180 min; $p<0.05$). In addition, the AUC 0-60' and AUC 60-240' were lower ($p<0.01$) in the LP100 group in contrast to the sharp increase in the CO group (data not shown).

Figure 3 Postprandial responses of serum glucose (mg/dL) according to the time of adult dogs fed CO vs LP100 diets

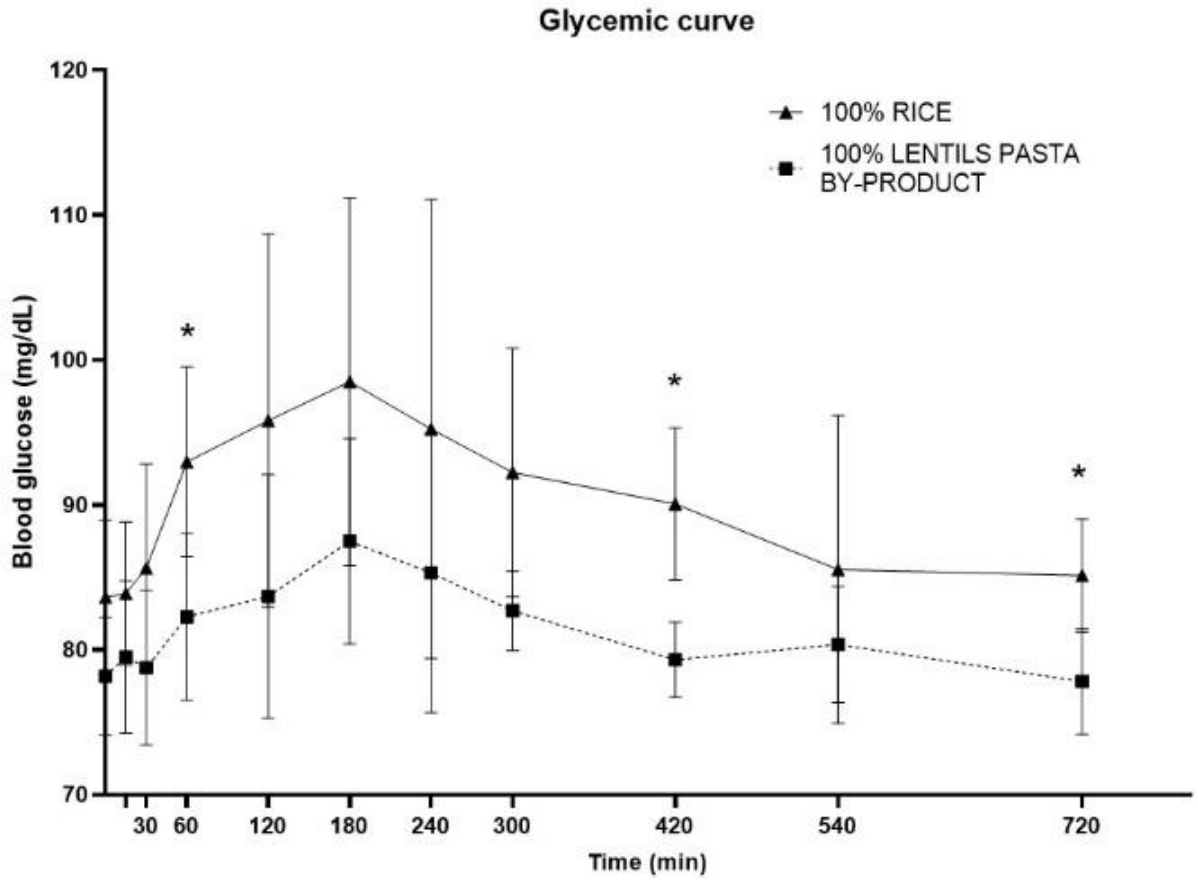


Figure 4 Postprandial responses of serum insulin (mg/dL) according to the time of adult dogs fed CO vs LP100 diets

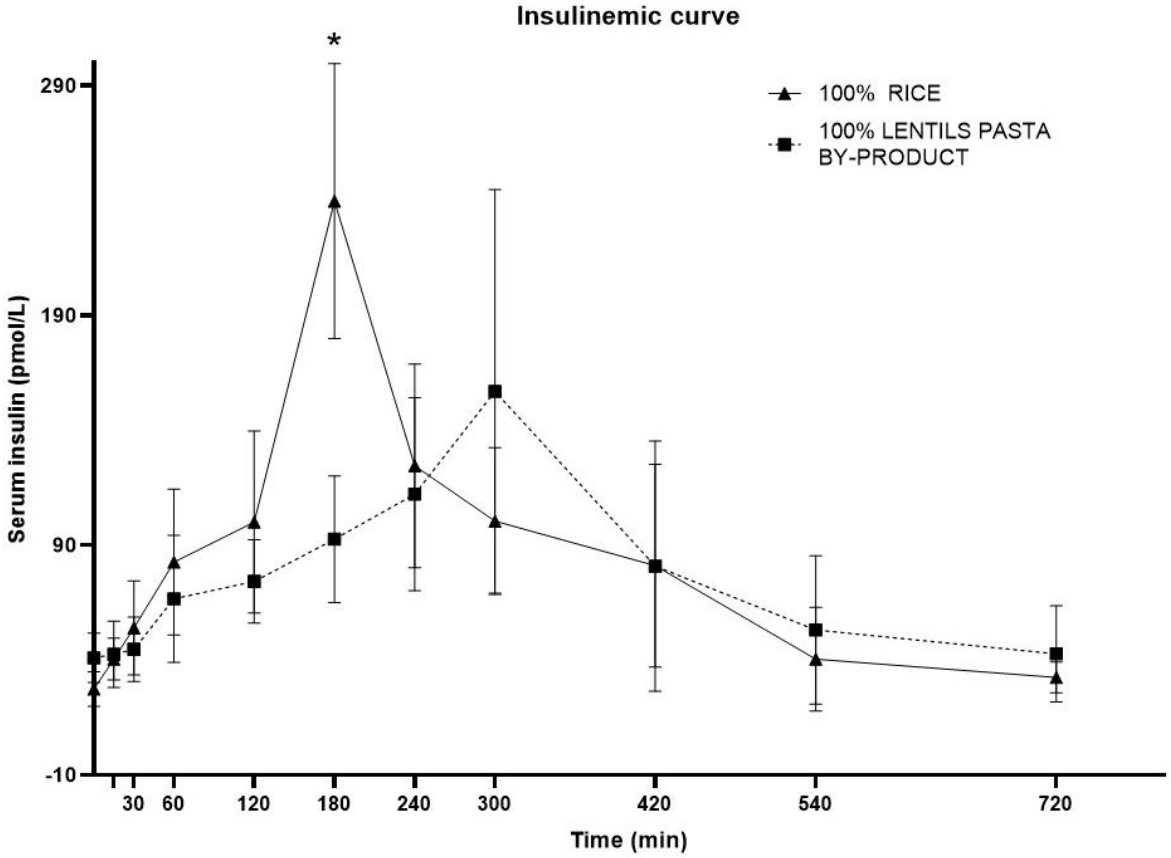


Table 12 Basal, minimum, mean, maximum, time to peak, and area under the curve (AUC) of glucose of dogs fed diets formulated with alternative carbohydrate sources

Item	Experimental diets and ingredient[1]		p-value
	CO	*LP100*	
Basal glucose (mg/dL)	83.6±5.3	78.2±4.0	<0.05
Minimum glucose (mg/dL)	79.8±5.6	73.9±3.9	<0.05
Average glucose (mg/dL)	89.6±7.7	81.6±2.6	<0.05
Maximum glucose (mg/dL)	101.4±12.1	91.7±5.5	0.07
Time to peak (min)	150 (60-180)	180 (165-240)	<0.05
AUC (mg/dL/h)			
0-60’	86.6±5.3	79.6±3.8	<0.05
60-120’	94.4±9.5	83.0±4.7	<0.05
120-300’	276.8±57.1	256.5±15.8	0.36
300-720’	593.2±92.6	562.3±17.1	0.38
0-720’	1080.9±104.0	981.5±30.9	<0.05

Values expressed as average±standard deviation or median (interquartile range)

Table 13 Basal, minimum, mean, maximum, time to peak, and area under the curve (AUC) of insulin of dogs fed diets formulated with alternative carbohydrate sources

Item	Experimental diets and ingredient[1]		p-value
	CO	*LP100*	
Basal insulin (mg/dL)	27.3±7.6	40.9±10.7	<0.05
Minimum insulin (mg/dL)	23.6±2.9	28.7±8.9	0.15
Average insulin (mg/dL)	78.0±24.3	73.7±21.6	0.71
Maximum insulin (mg/dL)	189.5±83.0	166.8±79.5	0.58
Time to peak (min)	180 (180-180)	300 (240-300)	<0.05
AUC (mg/dL/h)			
0-60’	54.4±13.1	45.8±14.0	0.23
60-240’	393.0±13.1	263.6±90.9	<0.05
240-420’	268.9±110.8	372.2±181.4	0.19
420-720’	217.4±91.4	277.6±147.5	0.35
0-720’	972.2±281.0	969.6±351.7	0.99

Values expressed as average±standard deviation or median (interquartile range)

8. DISCUSSION

8.1 Trial 1

8.1.1 Digestibility trial

This study evaluated the nutritional quality of defatted BSF larvae meal as a potential sustainable novel raw material for pet food, to be integrated into extruded diets as a protein source. In addition, it explored the suitability of the *in vivo* marker method and the *in vitro* digestibility method with the traditional *in vivo* total collection method.

Although the control (containing venison meal) and insect-based diets were formulated to be isonitrogenous, our analysis showed CP content to be almost 4% lower in the former (16.97% vs. 20.70%, respectively); the discrepancy between the diets was nevertheless within the limits stipulated in the EU regulation 2017/2279 regarding "Tolerances for analytical constituents" (Commission, 2017). It is also important to remember that since chitin is a nitrogen-containing polysaccharides, this could also have led to a mild overestimation of the protein content in the BSF diet (Diener et al., 2009; Spranghers et al., 2017).

We must also acknowledge that the higher crude protein content of the BSF diet compared with the CTRL diet could be an overestimation due to our use of a nitrogen to protein (N:P) conversion factor of 6.25. In fact, several authors recently pointed out that this conventionally used conversion factor may lead to the overestimation of protein content in a variety of feedstuffs (Mariotti et al., 2008; Sriperm et al., 2011), including insect meals (Janssen et al., 2017; Nery et al., 2018). Furthermore, although Finke *et al.* (Finke, 2007) estimated that the amount of nitrogen in insect chitin would not significantly affect the total amount of nitrogen, other authors support the hypothesis that the presence of non-protein nitrogen (NPN) in insect CP could cause the overestimation of CP (Janssen et al., 2017; Nery et al., 2018).

In our trial, the ATTDC of DM, OM, and EE were similar in both BSF and CTRL groups, whereas the ATTDC of CP was higher in the BSF vs. CTRL group. A similar result was obtained by Lei *et al.* (Lei et al., 2019), where increasing levels of BSF meal inclusion (at 0%, 1% and 2%) in Beagle dog rations raised nitrogen digestibility, whereas EE digestibility remained similar to that of the control diet. A digestibility trial using the precision-fed cecectomized rooster assay demonstrated that the AA digestibility of BSFL of various ages was high and BSFL was considered a high-quality protein and amino acid source (Do et al., 2020). However, Gariglio *et al.* (Gariglio et al., 2019) observed that up to 9% BSF meal inclusion in the diet of growing Muscovy ducks did not change diet

digestibility, with the exception of the ATTDC of EE, which was improved in BSF groups. In line with these data, Biasato *et al.* (Biasato et al., 2019) observed no change in the ATTDC of BSF diets (up to 10% inclusion) in growing piglets. Similarly, Freel *et al.* (Freel et al., 2021) did not notice any difference in ATTDC of DM, CP and EE in a trial involving 56 Beagle dogs fed with diets containing graded levels of BSFL meal (5.0, 10.0, and 20.0%) and BSFL oil (1.0, 2.5, 5.0%). Furthermore, in a study where BSF meal completely replaced soybean meal in the diet of laying hens, Cutrignelli *et al.* (Cutrignelli et al., 2018) found BSF to correlate with lower crude protein digestibility, whereas lipid digestibility remained unaffected. Likewise, Kröger *et al.* (Kröger et al., 2020), in a study involving 12 Beagles, observed a decrease in ATTDC of CP in the BSF group compared to the control group, while the ATTDC of DM was increased when dogs were fed the diet containing the BSF meal (at 20.0% of inclusion). This result could be explained by differing levels of chitin, which can negatively affect protein digestibility (Longvah et al., 2011). Indeed, the reported difference in fiber digestibility between the diets supports this result and explanation, since chitin gets recognized as part of the crude fiber fraction during the analysis (Nafisah et al., 2019). As well, the mean values of crude protein ATTDC (for HI based diets) observed in our study were in line with those found in Kröger *et al.* (Kröger et al., 2020) and Jian *et al.* (Jian et al., 2022) but below those recovered in Freel *et al.* (Freel et al., 2021).

Hydroxyproline can be used as an index of protein quality (Messia et al., 2012), due to it being a marker of collagen content (Colgrave et al., 2012). The level of collagen and of hydroxyproline were higher in the control diet compared with the BSF diet, probably due to the fact that collagen is limited in insects meal compared to vertebrates protein meal. This could also explain the higher level of digestibility of the BSF diet compared with the control diet, at least with regard to crude protein digestibility, since the net protein utilization of collagen is zero (Hand et al., 2010). Collagen content also influences the N:P ratio of protein sources, and consequently the real CP content of the diets, in particular that of the control diet (Mariotti et al., 2008). It may also be speculated that the control diet had a decreased crude protein digestibility due to the higher ash content; however, high levels of crude ash did not appear to decrease protein digestibility, as previously reported by Bockskopf and Kamphues (Bockskopf et al., 2015).

The difference in calcium digestibility could be due to the use of different ingredients to adjust the calcium level of the diets. Indeed, calcium carbonate was added to the BSF diet to obtain the minimum requirements for dogs, whereas in the CTRL diet the calcium requirements were satisfied by the presence of ground bone in the venison meal (thus avoiding the need for any calcium salt addition), and this could have led to the discrepancy. Interestingly, Lei *et al.* (Lei et al., 2019) noticed significant increases in the level of calcium in the blood of Beagles as the BSF larvae meal content

of their food was increased. This result points toward a potential increase in the bioavailability of this macroelement that depends on the inclusion of BSF larvae meal in the diet; however, further investigations are required to confirm and understand the basis of any possible relationship.
It is important to note that no statistical differences were observed between the ATTDC values determined using the marker method and the total collection method for both CTRL and BSF diets, confirming the validity of the marker method as an alternative to the total collection method (Carciofi et al., 2007). The values of *in vitro* DM, OM, and CP digestibility were also similar to the results obtained with the two *in vivo* methods; even though, in line with the previous literature (Hervera et al., 2007, 2009), slightly overestimated in the former. We also evaluated whether the equations for the estimation of *in vivo* crude protein and organic matter digestibility, utilizing *in vitro* digestibility data, as described in Hervera et al. (Hervera et al., 2007) and in Hervera et al. (Hervera et al., 2009), fitted with the results obtained in this study (shown in Table 4). Since the predictive equations proposed were only used to assess feedstuff based on vertebrates and, to our knowledge, no other study inspected if they could be applicable to invertebrates, we decided to include these findings. For both the venison and insect diet, the predictive equations gave slightly overestimated values compared with the mean of the *in vivo* digestibility results, even though they were substantially similar from a nutritional perspective. Indeed, the discrepancy between the crude protein digestibility estimated using the equation and the *in vivo* crude protein digestibility results ranged from 3.2-9.8%, whereas the overestimation of the organic matter digestibility ranged from 0.2-4.0%, with lower deviations and a narrower range. According to these results, predictive equations utilizing *in vitro* digestibility values appear to constitute a valid tool for the analysis of feedstuff digestibility, and therefore offer a means to reduce, if not avoid, the use of live animals.

8.1.2 Nutritional adequacy trial

The nutritional adequacy trial assessed if the diet tested (with 36.5% of inclusion of BSF larvae) could lead to any change in body composition and blood parameters when using it in the long term (6 months). No significant differences were found regarding body weight, hemoglobin, PCV and ALP, and all the parameters were within the normal ranges according to AAFCO (AAFCO, 2018). As such, the insect formulation was deemed suitable for dog adult maintenance diets.

8.2 Trial 2

In the present study, an alternative carbohydrate source, i.e. red lentil pasta by-product, was evaluated for both the implications involving the extrusion process as well as digestibility parameters in dogs. In general, the outcome suggested that the novel ingredient used in the formulation can induce alteration during the extrusion processing and kibble formation, which need to be considered in order to established the desired and adequate system conditions. In addition, the higher level of total dietary fibre fraction caused an increase in fermentation by-product in the gut both for what concerning VFA as well as biogenic amines. Nevertheless, palatability was enhanced for the diet containing LP and the glycaemic and insulinemic curve showed a delayed peak compared to the diet with broken rice as the main carbohydrate source.

8.2.1 Extrusion variables

Pulses are becoming an ingredient of interest for the pet food industry but little is studied about the extrusion processing and kibble formation in the scientific literature (Corsato Alvarenga et al., 2019), and no studies have been performed so far in lentils. Despite this lack of scientific knowledge, grain-free diets, i.e. those formulation using legumes or tubers instead of cereals as the main carbohydrate source in commercial dry pet food, are becoming increasingly popular with pet industry claims for both health and functional properties compared to the traditional cereal grains. Thus, evaluating their inclusion effect in a complete extruded formulation can gave hints on the possible outcome of the extrusion parameters and variables involving the formation of the kibble as well as their characteristics, since few studies in literature evaluate also this aspect (Carciofi et al., 2008; Domingues et al., 2019; Pezzali et al., 2019; Corsato Alvarenga et al., 2020 a).

Taking into account that little variations in processing conditions may influence extrusion variables and product quality (Ding et al., 2005), in order to properly evaluate if the gradual inclusion of LP could have affected the parameters considered, the operational variables feed rate, preconditioner temperature, screw configuration and speed, die design, in-barrel moisture, and knife speed were kept constant. Therefore, any measured variation was related to the food formulation. We observed an increased level in the extruder motor amperage and SME application, when the LP66 diet was processed compared to the other diets. The extruder pressure was also higher in all the diets when the lentil pasta was added (LP33, LP66 and LP100), compared to the CO diet, and the die temperature increased linearly in the diets with LP inclusion. These factors need to be considered

when producing kibbles using pulses as the higher fibre and protein content as well as the lower concentration of starch may increase the resistance to mass flow.

The linear increase in bulk density, following the gradual addition of LP in the formulations, may also be explained by these factors, in particular the total dietary fibre content (Monti et al., 2016). Even if the expansion rate remained similar among the diets (with the exception of LP66), the hardness increased linearly in LP diets and this may be due as well to the lower starch levels and the increment in fibre content of the LP ingredient (Chassagne-Berces et al., 2011; Shevkani et al., 2019). In a previous study, the inclusion of another legume (pea) into rice-based products led to a reduction in the expansion ratio (Singh et al., 2007 a). Similarly, fava bean inclusion in dog formulations (Corsato Alvarenga et al., 2019) led to a linear reduction in expansion ratio and a linear increase in piece density and hardness when increasing the levels of fava beans (0, 10, 20 and 30%). In addition, chickpeas have been reported to have a lower expansion ratio but higher bulk density and hardness compared to cereals (maize and sorghum) when processed at the same extrusion conditions (Wang et al., 2019). Even tough higher fibre inclusion had been related to a reduction in starch gelatinization (Monti et al., 2016), the diets in this study showed a high cooking degree despite the inclusion of red lentil pasta by-product. Furthermore, the inclusion of LP did not appear to affect the amylose:amylopectin ratio.

8.2.2 Nutrient intake, ATTDC, faecal characteristics and fermentation end products

As expected, the nutritional composition varied among the diets due to the different chemical composition of the ingredients tested. However, no differences were observed for DM and fat intakes. The CO diet showed similar digestibility of nutrients compared with the other diets where LP was included, with the exception of CP and TDF fibre digestibility of LP66. In fact, when the replacement of rice with the red lentil pasta by-product was only partial the diet performed better in term of digestibility of these nutrients, showing a quadratic trend. Still, the digestibility of DM, OM and GE decreased when comparing LP100 with LP66. Similarly, in another trial when legumes (pea, chickpea and fava beans) were substituted 100% with rice the nutrient digestibility decreased compared to a partial substitution of 50% (Pacheco, 2020). Nevertheless, the decrease of nutrient digestibility when adding carbohydrate sources rich in fibre have been already reported in another study (Carciofi et al., 2008). Overall, red lentils pasta by-product inclusion appeared to be adequate in term of protein utilization and nitrogen balance.

It is important to notice that the overall nutrient digestibility resulted to be lower compared to other studies using pulses (Pezzali et al., 2019; Corsato Alvarenga et al., 2020 a; Pacheco, 2020; Reilly et al., 2021). For this reason, after the investigation of the possible causes of this discrepancy, the poultry by-product ingredient was found as the main culprit. In fact, the microscopic analysis of the ingredient revealed a certain level of contamination with chicken feet and feathers (source of indigestible collagen and keratin). Further confirmation of this contamination was the *in vitro* digestibility of the crude protein, which showed a lower digestibility of the poultry by-product used in the diets (60.96%) compared to another batch of the same ingredient (90.56%). Due to this unpredictable occurrence also the level of calcium and phosphorus resulted lower compared with the expected recipe, but still above the minimum requirements for dogs according to FEDIAF (FEDIAF, 2020). Despite this unfortunate circumstance the comparison among diets was not affected as the level of inclusion of the poultry by-product was the same for all the diets tested.
When comparing with other studies using lentils in dog food preparation (Carciofi et al., 2008; Quilliam et al., 2021) this study showed a high level of starch digestibility (all the diets were above 99.8%); however, this could be due to the fact that the LP ingredient was previously extruded using a low shear processing method (to produce pasta) and, therefore, pre-cooked, leading to a higher starch digestibility. Recently there have been several publications correlating the dilated cardiomyopathy in dogs with pulses and grain-free diets (Kaplan et al., 2018; Adin et al., 2019; Freid et al., 2021). However, these factors need to be studied carefully before making such statements (McCauley et al., 2020). A recent study (Quilliam et al., 2021) explored the levels of blood taurine in dogs fed 6 diets with different inclusions of legumes (one of which was red lentils) in replacement of rice. After 7 days the plasma concentration of taurine remained within normal ranges for all the diet considered. Even tough long-term studies are still needed, if the diet supply enough levels of taurine precursors (the sulphur-containing amino acids methionine and cysteine), accounting also the reduced digestibility due to the increased fibre content of grain-free diets, so far there should be minimum concern if the content and bioavailability of these amino acids is adequate (Sanderson et al., 2001; Backus et al., 2003; Tôrres et al., 2003). On the other hand, there could be also other factors associated with dilated cardiomyopathy (Smith et al., 2021), and, if this is the case, the causative agent should be identified before correlating it to the disease and the diet.

Faecal quality is an important parameter to evaluate in order to assess pet food quality. In this study, dogs fed diets with higher inclusion of LP showed a linear increase in faecal bulk and a linear decrease in faecal DM and pH. These findings however were expected as the TDF intake increased linearly with LP inclusion in the diet. Similarly, dogs that were fed other grain-free diets, such as

peas and lentils (Carciofi et al., 2008), a combination of peas, potatoes and tapioca (Pezzali et al., 2019) or chickpeas, fava beans and peas (Pacheco, 2020) showed comparable results. The total dietary fibre fraction, especially insoluble fibre, is accountable for increasing faecal output since it is poorly fermented in the large intestine and this may lead to increased peristalsis and water retention capacity (NRC, 2006). Although the increase in faecal bulk and decrease in faecal DM, the median of faecal score of all the diets remained 4 (optimal, well-formed faeces) for all the digestibility trial duration. The decrease in faecal pH is generally associated to fermentation of soluble fibre, possibly inhibiting the growing of pathogenic bacteria. Similarly to Carciofi *et al.* (Carciofi et al., 2008), also in this study the inclusion of lentils lowered the pH when the diet with complete LP substitution (LP100) was fed to the dogs compared to the CO diet. It is important to notice that, in order to make the red lentil pasta, only dehulled lentils were used; as such the effect of this by-product may have been milder than using whole lentils as a large proportion of TDF resides in the hull itself.

The colonic microbiota is responsible for the fermentation of dietary fibres. These fermentations produce a series of metabolic products derived from these microorganisms, such as volatile fatty acids (VFA), gases (H_2, ammonia and methane) and lactate (Middelbos et al., 2007; Holscher, 2017). Since the total dietary fibre intake increased with higher LP inclusion, the increment of faecal VFA content was also expected. In fact, the linear increase in lactate and total VFA as well as the lowering in faecal pH are indicators that the colonic bacterial fermentation in this study changed with the inclusion of LP in the formulation. In particular acetic, propionic and valeric acids linearly increased with the substitution of the LP with rice. The production of VFA (e.g., acetate, propionate, and butyrate) can provide energy for the epithelial cell growth (Sunvold et al., 1995). In fact, the production of SCFA by the microbial fermentations of the colon not only can account for up to 7% of the metabolic energy provision for dogs but also has a major importance for the normal colonic absorptive processes (Herschel et al., 1981). Besides, VFA production reduces pH, encouraging beneficial bacteria growth while inhibiting harmful bacteria development (Maria et al., 2017). Among the VFA, butyrate exerts several different functions, such as cell differentiation, anti-inflammatory effects and enhanced colonic immune response while being the major energy source for enterocytes (Topping et al., 2001; Jiminez et al., 2017; Theodoro et al., 2019). However, not all sources of fermentable fibre induces increases in butyrate, as it was previously observed for beet pulp (Fischer et al., 2012; Maria et al., 2017). Similarly, in this study the inclusion of red lentil pasta by-product did not affect the concentration of butyric acid in the faeces, even though increasing the total VFA. Nonetheless, it is important to remember that propionic and lactic acid can be used as energy sources for hepatocytes, while acetic acid can be an energy substrate for peripheral tissues (Cummings et al., 1987). In addition, in human medicine propionate appears to exert a role in improving glucose

tolerance as well as insulin sensitivity (Venter et al., 1990). Besides, an in vitro study showed the anti-inflammatory effect of propionate on the gut as well as its antioxidant properties on the blood-brain barrier (Hoyles et al., 2018). For these reasons increased faecal propionate concentrations could be considered as potential biomarkers of improved GI functionality for dogs (Félix et al., 2022).

Biogenic amines can be formed by commensal microorganisms in the gut mainly by decarboxylation of amino acids derived from unabsorbed endogenous or undigested protein (Hussein et al., 1999; Jeaurond et al., 2008; Chen et al., 2015; Fan et al., 2017). As a consequence, undigested amino acids may promote the proliferation of microbiota that uses them as energy source (Fan et al., 2015). Functions, benefits and concerns related to the production of these amines in the gut are still debated, as they can have both positive or negative effects depending on the concentration (Fan et al., 2017). However, the threshold level for which we can observe beneficial or harmful effect is still not clear in humans and, for pets, it is even cloudier.

In our study putrescine, phenylethylamine and tryptamine did not show any significant difference among treatments. In other species putrescine has been related to damage prevention of the intestinal mucosa (Girdhar et al., 2006), DNA, RNA and protein synthesis (Ginty et al., 1989) and small intestine development (Peng et al., 2010); as well putrescine has the potential to be used as a possible energy source for the gut (Desury et al., 2002). However, in humans, excessive putrescine supplementation can be toxic and reduce growth, show a reduced anticarcinogenic activity or induce impaired spatial learning and memory (Fan et al., 2017).

The increase in LP inclusion in the tested formulations lead to a linear increase of cadaverine, tyramine, histamine and spermidine; while spermine showed a quadratic effect with higher concentrations for the LP33 diet. Cadaverine showed several mechanisms of protection on the epithelial cells (McCormick et al., 1999; Samartzidou et al., 1999; Fernandez et al., 2001), while spermine plays an essential role in cell growth, differentiation and modulation of the ion channel receptors, mucosal repair and healing processes (Wang et al., 1992; Lew et al., 2007; Hackman et al., 2010; Song et al., 2011; Wośko et al., 2014). Spermidine, besides promoting adipogenesis, also showed similar effects of spermine, even though less efficient (Fan et al., 2017). Histamine interacts with various cellular targets (e.g. epithelial cells or smooth muscle cells) and regulates gastrointestinal functions (such as gastric acid production, intestinal motility, and mucosal ion secretion), using the histamine receptors disseminated throughout the gastrointestinal tract (Rangachari, 1992; Sander et al., 2006). Tyramine stimulates glucose transport in adipocytes through its oxidation by monoamine oxidase (Fan et al., 2017). However, an excessive oral ingestion of spermine and spermidine can have several effects, including the induction of allergic-like reaction

through the gastrointestinal epithelial barrier modulation, mucosal damage and morphological changes in villous height (Fan et al., 2017). Similarly, an excess in histamine in the diet has been related to food poisoning with allergic-like reactions (Taylor, 1986; Schirone et al., 2017), which can be even enhanced with the presence of other biogenic amines such as cadaverine, putrescine, and tyramine (Shalaby, 1996). High concentrations of tyramine can increment susceptibility to enteric infection, as increase the adherence of *E. coli* O157:H7 to the caecal mucosa of mice in a concentration-dependant manner (Fan et al., 2017).

Since the biological consequences of the biogenic amine production in the gut by the resident microorganism are largely unknown, it is difficult to predict if the results of their increase could lead to favouring beneficial or adverse effects (Fan et al., 2017). In another study, an increase in biogenic amines production was associated to the incidence of diarrhoea in weaning pigs fed high protein diets (Wen et al., 2018). Considering this, it is important to notice that in our study, even if the amines increased when increasing the LP inclusion, there was no effect on the stool quality and faecal score. Our findings showed that most of the biogenic amines increased linearly with the gradual increase of CP, and linear decrease of starch, in diets with LP inclusion. As such, the commensal bacterial community in dogs gut, responsible for their formation, may have mutated accordingly to these changes.

8.2.3 Palatability study

Palatability is also an essential aspect for pet food industry since, even if the ingredient may have several nutritional properties, if it is not accepted by dogs and cats, its use is voided. For this reason, it is necessary to test novel ingredients to ensure their acceptance. In the current study, the total substitution of LP with rice lead to a preference of the LP100 formulation compared to the CO diet. In another trial using pulses (chickpea, pea and fava beans) the acceptance was lower or similar for the legume based diets compared to the control diet (rice based) (Pacheco, 2020). Similarly, another study utilizing traditional grain-based diet and legumes- and tuber-based diet did not observe any difference regarding dog food preferences (Pezzali et al., 2019). In addition, in a similar trial, dogs did not prefer kibbles formulated replacing rice with an inclusion of 10 or 30 % of fava bean, while 20 % inclusion showed similar effect on first choice and food intake compared to the control diet (Corsato Alvarenga et al., 2020 a). The results of our study are in contrast with these previous findings; however, we need to take into account that the tested diets (LP100 and CO), differed for hardness and bulk density, since the extrusion parameters were kept constant during the processing. Moisture content is also a parameter affecting palatability but, in this case, we ensured that the two

batches used had similar level of moisture. The composition of the ingredients and the characteristics of the feed are related to the palatability of the food, making its evaluation complex (Challacombe et al., 2011). According to Viera (Viera, 2010) the particle size, shape, density, humidity and size of the kibbles are characteristics that influence the palatability of dog foods. Thus, these parameters were evaluated in the present study and the dry matter, radial expansion and specific gravity did not differ between products ($P>0.05$). Only the specific length differed, lower for LV ($P<0.01$). Therefore, palatability differences may be attributed to the characteristics of LP, acknowledging also that the previous low shear extrusion of the ingredient and the fact that the lentils were dehulled, may have contributed to the organoleptic attributes of the ingredient.

8.2.4 Glycaemic and insulinemic postprandial curve

The response to certain kind of carbohydrates from a glycaemic and insulinemic standpoint is of great value especially for those dogs requiring a low and prolonged response. In fact, obesity and insulin resistance are considered among the major problems in the canine population (German, 2006; Clark et al., 2016). So far, a few studies evaluated the glucose and insulin responses when dogs were fed different kind of carbohydrate sources (Kempe et al., 2004; Carciofi et al., 2008; Adolphe et al., 2012, 2015; Pacheco, 2020; Rankovic et al., 2020). During the postprandial test of this study, dogs in the LP100 group showed a constantly lower glycaemia as well as a lower and delayed insulinemic peak. This is in accordance with a previous study (Carciofi et al., 2008) were the postprandial glucose and insulin response of a lentil-based diet were lower and prolonged compared to commonly used cereal-based diets. Similarly, dogs fed pulses (included lentils) alone or included in a whole diet showed a low glycaemic and insulinemic postprandial responses (Briens et al., 2021). This is also in agreement with Adolphe *et al.* (Adolphe et al., 2015) and Rankovic *et al.* (Rankovic et al., 2020) who found that a diet containing pulses lead to a delayed and lengthened responses in postprandial glucose and insulin, as well as lower peak concentrations and a longer time to peak.

Starch is usually considered the nutrient that mainly affect the insulin and glucose responses in dogs (Nguyen et al., 1998). Besides, several factors influence the digestion and absorption of starch sources, and these can be extrinsic (such as processing) and intrinsic (such as amylose to amylopectin ratio) (Toutounji et al., 2019). Other nutrients (e.g. protein, fat and dietary fibre) do not seem to affect the glucose profiles in dogs such as diet starch content (Nguyen et al., 1998; Monti et al., 2016). Since amylose to amylopectin ratio varies among carbohydrate sources,

according to the botanical origin of the starch (Tester et al., 2004), the diets were tested for these parameters as well. In fact, legumes present generally higher amylose content compared to cereals; as such they should induce lower glycaemic responses as amylose is less susceptible to enzymatic hydrolyzation compared to amylopectin (Hu et al., 2004; Denardin et al., 2012). Even if in this study was reported a lower glycaemic and insulinemic response for the LP100 diet compared to the CO diet, unexpectedly, the levels of amylose and amylopectin were similar between the diets, as well as the amylose to amylopectin ratio. Factors that can have affect the results may be the different level of starch, protein and fibre between the diets, but we also need to take into account that the processing technique may have affected the results. In fact, the red lentils pasta by-product was previously processed using a low-shear extrusion before being part of the formulation, possibly leading to a change in its starch properties. Furthermore, we cannot rule out a possible effect of the higher VFA concentrations as both propionate and acetate appears to have a positive effect on glucose tolerance and insulin sensitivity (Venter et al., 1990; Todesco et al., 1991; Yamashita et al., 2007).

It is also interesting to notice that LP100 diet led to a lower glycaemic blood level (both basal and throughout the trial period) compared to the CO diet, even though the dogs did not show any difference before the trial. This is also reflected in a higher but constant blood insulin concentration of the LP100 group both before and 12 hours after the meal, meaning that the dogs remained with a steadier and smoother insulin curve compared to the CO group. These findings are of importance for those dogs that need delayed and prolonged responses in postprandial glucose and insulin blood levels.

9. CONCLUSIONS

This PhD project focused on the evaluation of innovative ingredients for their possible entrance in the pet food manufacturing. The choice of legumes and insects followed the criteria of nutritional adequacy and sustainability while addressing the current trends in the companion animal sector. Even tough insects industry is still under development for a large scale production, their role in the future of animal (and human) nutrition has been promoted as a possible solution to reduce our dietary wastes and carbon footprint. The assessment of their safety and dietary values is therefore crucial before further investments can be allocated in this field. This project not only evaluated the optimal results in terms of digestibility for a diet based on black soldier fly larvae but also its nutritional safety in a long-term study. However, this can be considered a starting step for the development of further studies, as insects can vary widely from a nutritional perspective depending on their life stages and their feeding. Numerous factors need to be addressed before understanding the potential of this novel ingredient, first of all the role of chitin on gut health and animal metabolism. Nevertheless, this study showed that black soldier fly larvae meal has already the likelihood to become a noteworthy ingredient in the future of the pet food industry. On the other hand, legumes have been commercialized for a longer time compared to insects. However, little has been investigated on their potential benefit a part from their nutritional value. In this project the digestibility parameters of diets based on lentil pasta by-product showed that this ingredient, despite discarded from human-graded food production, retain high bioavailability of nutrients. In addition, lentils can have an effect on the post-prandial glycaemic and insulinemic response, lowering the curve peaks compared to rice, possibly affecting the metabolic pathways of the animal. Nonetheless, the palatability of the ration resulted to be enhanced by the addition of lentil pasta by-product, of which the organoleptic properties appeared to be appreciated by dogs more than rice. However, extrusion processing parameters need to be carefully evaluated due to the reduced level of starch and increased level of fibre and protein of legume-based diets compared to cereal-based ones. In fact, the resistance to mass flow can affect kibble formation and hardness, altering therefore the final product characteristics. As such, this project aimed also to give the tools to understand where to operate on the extrusion processing in order to produce kibbles with similar characteristics depending on the inclusion level of pulses. In general, both of the ingredients selected demonstrated a good outcome in term of possible mass production in commercial products while, at the same time, taking care of the sustainability concerns.

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11. RESEARCH PRODUCTS

11.1 Articles related to the PhD project

Penazzi L, Schiavone A, Russo N, Nery J, Valle E, Madrid J, Martinez S, Hernandez F, Pagani E, Ala U and Prola L (2021) In vivo and in vitro Digestibility of an Extruded Complete Dog Food Containing Black Soldier Fly (Hermetia illucens) Larvae Meal as Protein Source. Front. Vet. Sci. 8:653411. doi: 10.3389/fvets.2021.653411

11.2 Articles not related to the PhD project

Cavallini D*, Penazzi L*, Valle E*, Raspa F, Bergero D, Formigoni A, Fusaro I, When changing the hay makes a difference: A series of case reports, Journal of Equine Veterinary Science (2022), doi:https://doi.org/10.1016/j.jevs.2022.103940

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11.3 Abstracts on international congresses related to the PhD project

1. Penazzi L, Russo N, Prola L, Acrylamide analysis in dry pet food, 24th ESVCN Congress, Munich, 2020

2. Penazzi L, Freire TGB, Frias JL, Prola L, Carciofi AC, Palatabilidade e macroestrutura dos kibbles de formulação para cães com subproduto de macarrão de lentilha vermelha como fonte de carboidrato, 21st CBNA Congress, São Paulo, 2022

3. Penazzi L, Freire TGB, Frias JL, Prola L, Carciofi AC, Lentil pasta by-product as a substitute to broken rice reduces glucose and insulin responses of dogs fed extruded diets, 26th ESVCN Congress, Basel, 2022

4. Frias JL, Penazzi L, Pescuma MG, De Ramos EC, Theodoro SS, Carciofi AC, Glucose and insulin post-prandial responses of dogs fed kibble diets with pea flour, 26th ESVCN Congress, 2022

5. Penazzi L, Freire TGB, Frias JL, Prola L, Carciofi AC, Extrusion parameters and digestibility of complete diet for dogs with lentil pasta by-product, 22nd CBNA Congress, São Paulo, 2023

11.3 Abstracts on international congresses not related to the PhD project

1. Penazzi L, Prola L, Marchese C, Valle E, Nutritional strategies for a horse with recurrent oesophageal obstructions, 25th ESVCN Congress, Vila Real, 2021

2. Freire TGB, Penazzi L, Vasconcerva PB, De Castro A, Dias RS, Menezes MP, Carciofi AC, Relato de caso: hipoparatiroidismo primário em cão, 4th Workshop sobre Nutrição de cães e gatos CBNA, São Paulo, 2021

3. Vasconcerva PB, De Castro A, Freire TGB, Penazzi L, Teran KAH, Dias RS, Da Silva KP, Silveira MV, Dutra CD, Carciofi AC, Uso de dieta comercial e caseira restritas em purinas no tratamento de urólitos de urato: relato de dois casos clínicos, 4th Workshop sobre Nutrição de cães e gatos CBNA, São Paulo, 2021

4. Penazzi L, Valle E, Pagliara E, Ala U, Prola L, Use of free nucleotides supplementation in new-born foals: effects on weight gain and neonatal diarrhoea, 10th EWEN Congress, Cirencester, 2022

5. Penazzi L, Valle E, Pagliara E, Ala U, Nervo T, Prola L, Free dietary nucleotides in newborn foals positively affect fecal VFA production and microbiota, 16th ECEIM Congress, Rome, 2022

6. Freire TGB, Penazzi L, Prola L, Carciofi AC, Case report: adultaration in poultry by-product meal detected by the combination of microscopy and *in vitro* digestibility, 22nd CBNA Congress, São Paulo, 2023

frontiers in Veterinary Science

ORIGINAL RESEARCH
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In vivo and *in vitro* Digestibility of an Extruded Complete Dog Food Containing Black Soldier Fly (*Hermetia illucens*) Larvae Meal as Protein Source

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Growing attention is being directed toward insects as a novel and sustainable source of protein for pet food. The aim of the study was to evaluate nutrient digestibility of a diet containing black soldier fly larvae as its main protein source. Moreover, the purpose of the study was to compare the traditional *in vivo* total collection method with the *in vivo* marker method and *in vitro* digestibility method. Two isonitrogenous and isoenergetic dry diets containing either venison meal (CTRL diet) or black soldier fly larvae meal (BSF diet) as their primary sources of proteins were fed to six adult dogs, according to a Latin square design. The digestibility of nutrients was determined using both *in vivo* ("total collection" and "internal marker" approaches) and *in vitro* methods. The two diets showed similar nutrient digestibility values for dry matter, organic matter, ether extract, ash, and phosphorus. However, a statistical trend ($p = 0.066$) was observed indicating greater protein digestibility in the BSF diet compared with the CTRL diet. Calcium digestibility was higher in the BSF diet compared with the CTRL diet ($p = 0.018$). On the contrary, fiber digestibility was lower in the insect-based diet compared with the venison diet ($p < 0.001$). There was no difference between total collection and internal marker methods in the assessment of *in vivo* digestibility for any of the nutrients considered. The *in vitro* digestibility values for dry matter, organic matter, and crude protein, as well as the estimated *in vivo* digestibility of organic matter and crude protein by the means of the predictive equation, were aligned with the *in vivo* results, although *in vitro* estimations were consistently higher compared with those obtained by *in vivo* analysis. Digestibility analysis of a dog food containing insect meal as the sole source of protein (36.5% inclusion) showed promising results in terms of it presenting similar values as a meat-based diet, indicating its suitability as a sustainable protein source for pet food. Moreover, the study showed that both the *in vivo* marker method and the *in vitro* method could be possible alternatives to the traditional total collection method in digestibility trials.

Keywords: sustainability, pet food, digestibility, protein, novel feed materials, insect meal

INTRODUCTION

With the livestock industry at its limit in terms of sustainable production capacity, and the pet food business in constant growth, new sources of protein are being sought in order to meet the market's demand and the expectations of pet owners (1). Insects may provide a possible solution as an alternative feed, since they can partially replace traditional feed sources, while they also provide a means to bio-converting organic waste (2). Of the various insects being considered, the black soldier fly (*Hermetia illucens*) is showing particular promise due to its immediate potential for large-scale production (3).

The black soldier fly (BSF) has a balanced protein composition and one of the highest amino acid scores compared with other currently reared insects or traditional protein sources (such as fish meal) (4). Compared with crickets and mealworms, BSF boasts a more stable nitrogen and phosphorus composition and has a more advantageous feed conversion ratio (5). It can also be considered a possible sustainable solution due to the possibility of rearing the insects on materials deemed unsuitable for human nutrition, such as alimentary by-products and organic substrates (6).

As pointed out by Böhm et al. (7), insects may constitute an appropriate novel protein source for dogs, presenting cutaneous adverse food reactions. Nevertheless, societal negative opinions about the use of insect meal in pet nutrition have arisen, especially due to insect phobia and concerns about safety. Security aspects about insect consumption were also discussed critically in EFSA Scientific Opinion (8), where uncertainty regarding the risk of non-processed items, due to the lack of data, has been acknowledged. However, EFSA concluded that microbiological risks are expected to be comparable with other food raw materials, provided that insects are fed with allowed feedstuff. Consumers from Western countries still continue to have prejudices regarding the introduction of insects in their diet (9), and, due to the current "humanization trend" (10), this fact could be also translated to their pets. Notwithstanding, public opinion seems to be less concerned about the use of veterinary-prescribed diets based on insects (11). Indeed, veterinarians have expressed interest in hypoallergenic food alternatives prepared using insects (12). According to the Commission Regulation (EU) 2020/354 (March 4, 2020) (13), a product can be claimed to reduce ingredient and nutrient intolerances if it is composed of hydrolyzed proteins or selected and limited protein sources or selected carbohydrate sources. Therefore, according to the current European Regulations, a product composed only of insects as the main source of protein could be considered with the particular purpose of reduction of food intolerance. Concurrently, and reflecting the growing interest in this field of research (14), various recent studies have investigated the possibility of feeding BSF larvae to poultry (15–18), fish (19–21), and swine (22, 23). Recently, a thorough review from Bosch and Swanson (24) explored in depth the palatability, digestibility, and nutritional aspects of the inclusion of insects in dog and cat diet, showing the potential of insects as future pet food products.

The aim of the present study was to evaluate the inclusion of defatted BSF larvae meal in extruded dog food in terms of its *in vivo* and *in vitro* digestibility, in order to assess its suitability for the pet food market. Furthermore, the purpose of the study was to evaluate if the *in vivo* marker method and the *in vitro* digestibility method could be comparable to the traditional *in vivo* total collection method also in these particular diets. The estimated *in vivo* digestibility of organic matter and crude protein calculated by means of predictive equations utilizing data obtained by *in vitro* analysis was also assessed.

Abbreviations: CTRL diet, venison meal-based diet/control diet; BSF diet, black soldier fly larvae-based diet/insect diet; BSF, black soldier fly; ME, metabolizable energy; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract/crude fat; CF, crude fiber; HPLC, high-performance liquid chromatography; ATTDC, apparent total tract digestibility coefficients; TFC, total fecal collection method; SEM, standard error of the mean; D, diet; M, method; D×M, interaction between diets and methods.

MATERIALS AND METHODS

All the experimental procedures were approved by the Bioethics Committee of the University of Turin (Italy) (prot. n. 336595).

Animals and Experimental Design

Six clinically healthy West Highland White Terrier adult dogs [three males and three females, 3 ± 1.8 years old, 7.2 ± 0.8 kg BW, BCS ranging between 4.5 and 5.5 on a nine-point scale (25)] were fed two isonitrogenous and isoenergetic dry extruded diets (control vs. insect diet) according to a Latin square design. During the digestibility experiment, the dogs were housed individually in 3 × 3-m kennels and had *ad libitum* access to fresh water. The dogs were allowed to walk freely for 1 h per day in a concrete outside the pen and play with toys during the adaptation periods.

Diets and Digestibility Protocol

Two diets were tested during the trial. The diets were formulated to be isoenergetic and isonitrogenous. In the control diet (CTRL diet), the protein source was provided in the form of processed [rendering process, method III, according to the EU Reg. 142/2011 (26)] deer (*Cervus elaphus*) protein, whereas the insect diet (BSF diet) provided defatted BSF (*H. illucens*) larvae meal as its sole protein source (Hermetia Futtermittel GbR, Baruth/Mark, Germany). The chemical composition, amino acidic profile, and ingredient composition of both diets are shown in **Table 1**. Diets were formulated and balanced in order to meet nutrient requirements in accordance with the FEDIAF (27) nutrient guidelines for dogs.

Venison was chosen as the primary protein source for this trial since it is one of the protein sources usually incorporated in commercial foods for dogs which show adverse food reactions; similarly, insect meal showed a similar potential (7). Nevertheless, venison meal is more expensive than other common sources of proteins as well as insect meal so far and, for these reasons, was deemed eligible for the comparison of the diets.

The trial was conducted according to the guidelines of Carciofi et al. (28) regarding the use of a marker method and the total collection method for assessing *in vivo* total tract apparent

TABLE 1 | Ingredients and nutritional composition of the experimental diets.

	CTRL[a]		BSF[b]	
Ingredients	**(% as fed)**	**(% of DM)**	**(% as fed)**	**(% of DM)**
Potato meal	51.5		54	
Venison meal	40		-	
Black soldier fly meal	-		36.5	
Vitamin and mineral premix	3		3	
Oils and fats[c]	2.5		2	
Yeast (hydrolysate)	2		2	
Calcium carbonate	-		1.5	
Other ingredients[d]	1		1	
Nutrient and chemical composition[e]				
Dry matter	93.80	-	96.04	-
Organic matter	86.11	91.80	90.21	93.93
Crude protein	16.97	18.09	20.70	21.55
Ether extract	17.42	18.57	15.61	16.25
Crude fiber	5.77	6.15	4.09	4.26
Ash	7.69	8.20	5.83	6.07
Calcium	1.03	1.10	0.87	0.91
Phosphorus	0.93	0.99	0.53	0.55
Collagen	2.72	2.90	0.88	0.92
Hydroxyproline	0.34	0.36	0.11	0.11
Amino acidic profile[e]				
Aspartic acid		1.88		2.09
Serine		0.68		0.79
Glutamic acid		1.98		2.19
Glycine		1.14		1.01
Histidine		0.31		0.49
Arginine		0.86		1.02
Threonine		0.60		0.68
Alanine		0.87		1.15
Proline		1.12		1.07
Cysteine		0.15		0.16
Tyrosine		0.40		0.78
Valine		0.71		1.01
Methionine		0.23		0.39
Lysine		0.80		0.97
Isoleucine		0.53		0.69
Leucine		1.03		1.23
Phenylalanine		0.64		0.79
ME (MJ/kg)[f]	15.66		16.44	

[a]CTRL, control diet; [b]BSF, black soldier fly diet; [c]Poultry purified fat, sunflower oil; [d]Digest (hydrolyzed poultry liver), mineral, and vitamin pre-mix; [e]Analyzed; [f]Estimated according to FEDIAF (27).

digestibility. Chromium oxide (Cr_2O_3) was used as digestibility marker. It was added to a final concentration of 2.5 g/kg of diet. A 5-day test diet adaptation period preceded 5 days of feces collection during the experimental trial.

Food was weighed each day, divided into two equal portions, and given to the animals at 9 a.m. and 5 p.m. in stainless-steel bowls. Food quantity was administered considering maintenance energy requirements according to the FEDIAF equation (110 kcal $\times$ $BW^{0.75}$) (27). Bowls were removed before the next meal, and any uneaten food was weighed and recorded. Feces were collected twice daily, weighed, and kept frozen at −20°C until analysis.

Chemical Analyses

At the end of the collection period, pooled individual feces were thawed, homogenized, and freeze-dried. Feces samples were freeze-dried using a laboratory freeze dryer (5Pascal, Trezzano sul Naviglio, Italy). The process of lyophilization consisted of dry sublimation with water evaporation under low pressure (0.200 mbar) until the samples reached room temperature (25°C). Both the foods and freeze-dried feces were ground to pass through a 1-mm sieve and stored in airtight plastic containers for laboratory tests. The dry matter (DM) of the foods was determined by drying the samples at 103°C to constant weight. The foods and feces were analyzed according to the AOAC (29) standard procedures; thus, ash was determined by muffle furnace incineration (section 942.05), crude protein (CP) was ascertained using the Kjeldahl method (section 954.01), and ether extract (EE) was analyzed following acid hydrolysis (section 954.02). In addition, diet crude fiber (CF) was determined using the method described in section 962.09 (29), and amino acid content by HPLC (Waters Alliance System with a Waters 1525 Binary HPLC Pump, Waters 2707 Autosampler, and Waters 2475 Multi λ Fluorescence Detector, Milford, USA) after pre-column derivatization (30) in samples ground to pass a 0.5-mm sieve. The detection limit ranged from 2.9 to 20.1 pmol/µl depending on the amino acid. Tryptophan was not analyzed.

Samples of foods and feces were burnt to ashes and acid-digested in the microwave (31), prior to the determination of chromium concentrate by inductively coupled plasma optical emission spectrometry (ICP-OES). Calcium and phosphorus were also determined by ICP-OES in the absence of the previous incineration.

Hydroxyproline and the related collagen content were assessed according to the colorimetric method adapted by Kolar (32) and described in the AOAC (29) section 990.26. The acid hydrolysis of the sample was performed under heat; an oxidizing agent was added to the sample, and oxidized hydroxyproline was measured photometrically.

In vivo Digestibility Calculations

Apparent total tract digestibility coefficients (ATTDC) of the individual dietary elements of the two diets were calculated as follows:

a) Total fecal collection method (TFC):

$$\text{ATTDC } X_{diet}\ (\%) = [(\text{total } X_{diet} - \text{total}X_{feces})/\text{total } X_{diet}] \times 100$$

where X is the total contents of DM, organic matter (OM), CP, EE, ash, calcium, or phosphorus in the consumed food or feces produced (X_{diet} and X_{feces}, respectively);

b) Marker method (Cr_2O_3):

$$\text{ATTDC } X_{diet}\ (\%) = \{[(X/Cr_2O_3)_{diet} - (X/Cr_2O_3)_{feces}]/(X/Cr_2O_3)_{diet}\} \times 100$$

where X represents the concentrations of DM, OM, CP, EE, ash, calcium, or phosphorus in the diet or feces;

Cr_2O_3 represents the chromium oxide concentration in the diet or feces;

$(X/Cr_2O_3)_{diet}$ = ratio between nutrient (X) and Cr_2O_3 concentration in the diet;

$(X/Cr_2O_3)_{feces}$ = ratio between nutrient (X) and Cr_2O_3 concentration in the feces.

In vitro Digestibility

The *in vitro* digestibility of DM, CP, and OM of the food was determined (in triplets) employing the methods described by Hervera et al. (33, 34). The methods involve two phases: the first entails incubation for 2 h under conditions simulating gastric digestion (pH 2, 39°C, and inclusion of pepsin), whereas the second phase simulates 4 h of post-gastric digestion (pH 6.8, 39°C, and inclusion of a pancreatin preparation for enzymatic digestion). The resulting residue was filtered, dried, and weighed to determine the remaining DM content and incinerated to determine the residual OM content. Residual CP was determined by ascertaining the nitrogen content of the residue (using the Kjeldahl method) and considering a N:P conversion factor of 6.25. The *in vitro* digestibility of DM, OM, and CP was calculated as the difference between the amount of each initial nutrient in the sample vs. the undigested residue, divided by the initial nutrient content of the sample.

Estimated Digestibility

Data from the *in vitro* digestibility analyses were also used to estimate *in vivo* OM and CP digestibility according to the regression equations reported by Hervera et al. (33, 34):

Estimated digestibility of OM (%) = −9.15 + 1.06 × *in vitro* OM digestibility (%) (33);

Estimated digestibility of CP (%) = 37.91 + 0.52 × *in vitro* CP digestibility (%) (34).

Statistical Analysis

The statistical unit was the individual dog for *in vivo* digestibility trials, and the diet for *in vitro* digestibility trials. The comparisons between diets (CTRL vs. BSF) and methods (*in vivo* TFC vs. Cr_2O_3) were analyzed using two-way ANOVA, considering the diet (D) and the method (M) of *in vivo* digestibility calculation as the source of variation, respectively. Before testing for group and method differences, the normality of the data distribution and the homogeneity of variance were assessed by the means of the Shapiro–Wilk test and Levene test, respectively. The significance level was set at $p = 0.05$. A statistical trend was considered for $p \leq 0.10$. All statistical analyses were performed using R Software (version 3.6.1) (35).

RESULTS

The foods were well-accepted during all the trial lengths, and no episode of nausea or vomiting has been reported. The *in vivo* ATTDC digestibility results are summarized in **Table 2**. The two methods used to estimate *in vivo* digestibility (TFC and Cr_2O_3) showed similar results between the CTRL and BSF groups in relation to DM, OM, EE, ash, and phosphorus. The ATTDC of CF was significantly lower ($p < 0.001$) in the BSF diet compared with the CTRL diet. On the contrary, the ATTDC of calcium was significantly higher ($p < 0.05$) in the BSF compared with the CTRL diet. A statistical trend ($p = 0.066$) was observed for the ATTDC of CP, being higher in the animals fed the BSF compared with the CTRL diet.

No statistical differences were observed between the two ATTDC methods (TFC vs. Cr_2O_3). Furthermore, no statistical interaction between diets and methods was found.

The *in vitro* digestibility data and estimated *in vivo* digestibility results, obtained utilizing the regression equations described in Hervera et al. (33, 34), are reported in **Table 3**. The digestibility values for DM, OM, and CP obtained using the *in vitro* method were higher for both the CTRL and the BSF diet (by an average of +8.43, +5.25, and +6.08%, respectively) compared with those obtained using *in vivo* methods. The estimations of *in vivo* digestibility of OM and CP (based on *in vitro* data) were consistently higher than the data obtained using *in vivo* ATTDC methods: *in vitro* estimation of *in vivo* digestibility overestimated OM and CP digestibility by up to 4.0% and 9.8%, respectively, compared with the *in vivo* methods.

DISCUSSION

This study evaluated the nutritional quality of defatted BSF larvae meal as a potential sustainable novel raw material for pet food, to be integrated into extruded diets as a protein source. In addition, it explored the suitability of the *in vivo* marker method and the *in vitro* digestibility method with the traditional *in vivo* total collection method.

Although the control (containing venison meal) and insect-based diets were formulated to be isonitrogenous, our analysis showed CP content to be almost 4% lower in the former (16.97 vs. 20.70%, respectively); the discrepancy between the diets was nevertheless within the limits stipulated in the EU regulation 2017/2279 regarding "Tolerances for analytical constituents" (36). It is also important to remember that since chitin is a nitrogen-containing polysaccharide, this could also have led to a mild overestimation of the protein content in the BSF diet (6, 37).

We must also acknowledge that the higher crude protein content of the BSF diet compared with the CTRL diet could be an overestimation due to our use of a nitrogen to protein (N:P) conversion factor of 6.25. In fact, several authors recently pointed out that this conventionally used conversion factor may lead to the overestimation of protein content in a variety of feedstuffs (38, 39), including insect meals (40, 41). Furthermore, although Finke et al. (42) estimated that the amount of nitrogen in insect chitin would not significantly affect the total amount of nitrogen, other authors support the hypothesis that the presence of non-protein nitrogen (NPN) in insect CP could cause the overestimation of CP (40, 41).

In our trial, the ATTDC of DM, OM, and EE were similar in both BSF and CTRL groups, whereas the ATTDC of CP were higher in the BSF vs. CTRL group. A similar result was obtained by Lei et al. (43), where increasing levels of BSF meal inclusion (at 0, 1, and 2%) in Beagle dog rations raised nitrogen digestibility, whereas EE digestibility remained similar to that of the control diet. However, Gariglio et al. (18) observed that up to 9% BSF

TABLE 2 | Comparison of the *in vivo* digestibility using the total fecal collection method (TFC) and *in vivo* digestibility with marker (Cr_2O_3) in six dogs (mean values are presented).

	TFC[a]		Cr_2O_3		SEM	*p*-value		
	CTRL[b]	BSF[c]	CTRL[b]	BSF[c]		D[d]	M[e]	D × M[f]
***In vivo* digestibility (%)**								
Dry matter	82.11	82.17	83.05	83.83	0.52	0.698	0.241	0.740
Organic matter	86.23	85.04	86.98	86.46	0.45	0.358	0.247	0.719
Crude protein	72.41	75.80	74.04	78.22	1.01	0.066	0.311	0.842
Ether extract	96.58	96.40	96.72	96.75	0.14	0.800	0.411	0.717
Crude fiber	43.13	18.83	45.78	23.60	3.18	<0.001	0.393	0.798
Ash	32.73	35.76	35.88	41.39	1.95	0.292	0.280	0.757
Calcium	12.16	24.88	19.19	31.62	2.61	0.018	0.162	0.976
Phosphorus	20.77	21.46	26.17	25.83	2.00	0.946	0.280	0.908

[a]*TFC, total fecal collection;* [b]*CTRL, control diet;* [c]*BSF, black soldier fly diet;* [d]*D, diet;* [e]*M, method;* [f]*D×M, diets and method interaction.*

TABLE 3 | Comparison of the *in vitro* digestibility of the two diets (CTRL vs. BSF) and estimated *in vivo* digestibility based on the *in vitro* results.

	CTRL[a]	BSF[b]
***In vitro* digestibility (%)**		
Dry matter	90.65	91.79
Organic matter	90.82	92.04
Crude protein	80.06	82.33
Estimated *in vivo* digestibility (%) based on the *vitro* results		
Organic matter[c]	87.12	88.41
Crude protein[d]	79.54	80.72

[a]*CTRL, control diet;* [b]*BSF, Black soldier fly diet;* [c]*According to Harvara et al. (33) for OM estimation;* [d]*According to Harvara et al. (34) for CP estimation.*

meal inclusion in the diet of growing Muscovy ducks did not change diet digestibility, with the exception of the ATTDC of EE, which was improved in BSF groups. In line with these data, Biasato et al. (23) observed no change in the ATTDC of BSF diets (up to 10% inclusion) in growing piglets. Similarly, Freel et al. (44) did not notice any difference in ATTDC of DM, CP, and EE in a trial involving 56 Beagle dogs fed with diets containing graded levels of BSF meal (5.0, 10.0, and 20.0%) and BSF oil (1.0, 2.5, 5.0%). Furthermore, in a study where BSF meal completely replaced soybean meal in the diet of laying hens, Cutrignelli et al. (45) found BSF to correlate with lower crude protein digestibility, whereas lipid digestibility remained unaffected. Likewise, Kröger et al. (46), in a study involving 12 Beagles, observed a decrease in ATTDC of CP in the BSF group compared to the control group, while the ATTDC of DM was increased when dogs were fed the diet containing the BSF meal (at 20.0% of inclusion). This result could be explained by differing levels of chitin, which can negatively affect protein digestibility (47). Indeed, the reported difference in fiber digestibility between the diets supports this result and explanation, since chitin gets recognized as part of the crude fiber fraction during the analysis (48). Furthermore, the mean values of crude protein ATTDC (for BSF-based diets) observed in our study were in line with those found in Kröger et al. (46) but below those recovered in Freel et al. (44).

Hydroxyproline can be used as an index of protein quality (49), due to its being a marker of collagen content (50). The levels of collagen and of hydroxyproline were higher in the control diet compared with the BSF diet, probably due to the fact that collagen is limited in insect meal compared to that in vertebrate protein meal. This could also explain the higher level of digestibility of the BSF diet compared with the control diet, at least with regard to crude protein digestibility, since the net protein utilization of collagen is zero (51). Collagen content also influences the N:P ratio of protein sources, and consequently the real CP content of the diets, in particular that of the control diet (39). It may also be speculated that the control diet had a decreased crude protein digestibility due to the higher ash content; however, high levels of crude ash did not appear to decrease protein digestibility, as previously reported by Bockskopf and Kamphues (52).

The difference in calcium digestibility could be due to the use of different ingredients to adjust the calcium level of the diets. Indeed, calcium carbonate was added to the BSF diet to obtain the minimum requirements for dogs, whereas in the CTRL diet the calcium requirements were satisfied by the presence of ground bone in the venison meal (thus avoiding the need for any calcium salt addition), and this could have led to the discrepancy. Interestingly, Lei et al. (43) noticed significant increases in the level of calcium in the blood of beagles as the BSF larvae meal content of their food was increased. This result points toward a potential increase in the bioavailability of this macro-element that depends on the inclusion of BSF larvae meal in the diet; however, further investigations are required to confirm and understand the basis of any possible relationship.

It is important to note that no statistical differences were observed between the ATTDC values determined using the marker method and the total collection method for both CTRL and BSF diets, confirming the validity of the marker method as an alternative to the total collection method (28). The values of *in vitro* DM, OM, and CP digestibility were also similar to the results obtained with the two *in vivo* methods, despite being, in line with the previous literature (33, 34), slightly overestimated in the former. We also evaluated whether the equations for the

estimation of *in vivo* crude protein and OM digestibility, utilizing *in vitro* digestibility data, as described in Hervera et al. (33, 34), fitted with the results obtained in this study (shown in **Table 3**). Since the predictive equations proposed were only used to assess feedstuff based on vertebrates and, to our knowledge, no other study inspected if they could be applicable to invertebrates, we decided to include these findings. For both the venison and insect diet, the predictive equations gave slightly overestimated values compared with the mean of the *in vivo* digestibility results, even though they were substantially similar from a nutritional perspective. Indeed, the discrepancy between the crude protein digestibility estimated using the equation and the *in vivo* crude protein digestibility results ranged from 3.2 to 9.8%, whereas the overestimation of the OM digestibility ranged from 0.2 to 4.0%, with lower deviations and a narrower range. According to these results, predictive equations utilizing *in vitro* digestibility values appear to constitute a valid tool for the analysis of feedstuff digestibility and therefore offer a means to reduce, if not avoid, the use of live animals.

CONCLUSIONS

The present study suggests that the inclusion of BSF in extruded diets for dogs (at 36.5%) offers a promising alternative source of dietary protein for this species, in particular in relation to the digestibility profile of crude protein, crude fat, and OM. Our findings also highlight the need for further studies in order to understand the effect of chitin on fiber digestibility and mineral absorption in a BSF-based diet.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethic Committee of Turin University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AS, EP, LPe, and LPr conceived and designed the experiment. EP and NR collected the experimental data. EV, FH, JM, JN, and SM carried out the chemical analyses. AS, LPe, and UA performed the statistical analysis. All the authors interpreted the data. AS, LPe, and LPr wrote the first draft of the manuscript. All the authors reviewed the manuscript for intellectual content and gave approval for the final version to be published.

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