Review Article

In Vitro Models of the Canine Digestive Tract as an Alternative to In Vivo Assays: Advances and Current Challenges

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Abstract

Dogs occupy a full place in the family, and their well-being is of paramount importance to their owners. Digestion, a complex process involving physicochemical, mechanical, and microbial parameters, plays a central role in maintaining canine health. As in vivo studies in dogs are increasingly restricted by ethical, regulatory, societal, and cost pressures, an alternative option is the use of in vitro models simulating the different compartments of the canine gastrointestinal tract. This review introduces digestion and gut microbiota as key factors in dog nutrition and health under both healthy and diseased conditions (obesity and inflammatory bowel disease) and highlights similarities and differences between the human and canine digestive tract and processes. We provide the first in-depth description of currently available models of the canine digestive tract; discuss technical and scientific challenges that need to be addressed, and introduce potential applications of in vitro gut models in the food and veterinary fields. Even if the development of some in vitro models is still limited by a lack of in vivo data in dogs that is necessary for relevant configuration and validation, translation of long-term expertise on human in vitro gut models to dogs opens avenues for model optimization and adaptation to specific canine digestive conditions associated with various dog ages, sizes, breeds and/or diets, in both physiological and diseased states.

1 Introduction

1.1 Dogs in familial and economical contexts

Canis lupus familiaris, the domesticated dog, belongs to the Canidae family. The dog is thought to have been the first animal domesticated by humans around 12,000 years ago (Axelsson et al., 2013). Dogs were initially strict carnivores, but they probably acquired the ability to digest starch during the agricultural revolution to become facultative carnivores (Axelsson et al., 2013). Nowadays, canine species include approximately 400 breeds with broad morphological and size variabilities and body weight ranging from 1 kg for a Chihuahua to 100 kg for a Saint-Bernard (Grandjean and Haymann, 2010). Dogs occupy a full place in the family, and their health and well-being are of paramount importance to their owners.

There are estimated to be more than 500 million dogs worldwide, which represents a huge market for the petfood and animal health industry. In 2018, the global petfood market size reached $91.1 billion, representing a 31% increase within 5 years (Phillips-Donaldson, 2019). A vast array of foods, snacks, and nutritional supplements have been developed to support well-being, health, improve aging, or prevent disease. According to the increased interest of owners in maintaining their dogs’ health, petfood has been adapted to fit each dog’s lifestyle, for example specific products for puppies or lactating bitches, sedentary or active animals, and maintenance or hypocaloric diets.

Different types of canine food are available and can be classified into three categories: dry food, canned food, and alternative food (biologically appropriate raw food (BARF), homemade food, and feedstuffs). At the interface between petfood and veterinary compounds, nutritional supplements represent an expanding market with a huge product range. As an example, micornutrients like selenium, taurine or polyphenols can be added for old dogs, calcium, phosphorus, omega-3 fatty-acids and vitamin E for lactating bitches, or L-carnitine for athletic dogs. Supplements for specific nutri-
tional purposes such as prebiotics or probiotics are also developed to decrease food sensitivities or for digestive or articular care.

In 2019, pet medicine, including vaccines, antiparasitic treatments or antibiotics, represented a $17.5 billion market\(^1\). Drugs can be administered in different forms: topically, by injection, or as oral formulations. Oral formulations can have liquid (e.g., solution or suspension), semi-solid (oily or aqueous formulations), or solid (e.g., powder, capsules, conventional or sustained-release tablets) forms. Specific treatments have been developed to avoid lipid absorption in obesity or decrease pain in inflammatory bowel disease (IBD). Of note, vulnerability to specific diseases depends on a dog’s size and breed, with for instance obesity and dental trouble common in small dogs and digestive problems more common in larger animals.

1.2 Regulatory context

In Europe, the European petfood industry federation (FEDIAF) represents 16 national petfood industries and provides a framework for the production of safe, nutritious, and palatable petfood. Heads of medicine agencies or national agencies must evaluate the quality and safety of medicinal products for animals, consumers, users, and the environment as well as the effectiveness of the medicinal product before it can be marketed.

To assess the digestibility of petfood or bioaccessibility of active compounds (including drugs) in the canine gastrointestinal tract (GIT), in vivo studies remain the gold standard. In vivo experiments in dogs are also performed to model the human gut due to similarities in digestive physiology (Lui et al., 1986; Akimoto et al., 2000). More than 17,000 experiments were carried out on dogs in 2018 in the EU28 and Norway\(^2\). However, in vivo assays are increasingly restricted by regulation, ethical and societal constraints, and high associated costs.

The 3Rs proposed by Russel and Burch (1959) widely encourage a reduction in the number of animals used in research and promote the development of alternative in vitro approaches. Among in vitro alternatives, models simulating the canine digestive environment (intestinal cell culture, organoids, or in vitro gut models) can help to answer many scientific questions associated with food and drug behavior during canine digestion, especially on mechanistic aspects. However, this approach requires a comprehensive understanding of the canine digestive process.

1.3 Digestion and gut microbiota as key parameters in dog nutrition and health

Canine digestion involves physicochemical (e.g., pH, digestive secretions, transit time), mechanical, and microbial parameters. These digestive components affect food digestibility, nutrient absorption, and energy release, but also drug metabolism and absorption, and survival of probiotic microorganisms. Thus, the development of new petfood or veterinary products needs to consider these multi-faceted aspects of canine digestion to answer important questions such as: How do physicochemical parameters modulate food digestibility? What is the importance of gut microbiota in canine digestion and drug metabolism? Where are drugs released and absorbed? How is drug bioaccessibility impacted by food matrix, galenic form, physicochemical parameters, or microbiota? How do probiotic strains survive along the GIT? However, recommendations for petfood and drug intake are currently based only on dog body weight or metabolic weight. Development of new products should consider the variations in the digestion process associated with different canine sizes and breeds to move towards personalized nutrition and veterinary medicine.

2 Methods of literature research and aim of the review

Our literature search was performed using PubMed\(^3\) and Google Scholar\(^4\) using the keywords “dog” OR “canine” AND “digestion”, “ph”, “enzyme”, “digestive secretions”, “absorption”, “microbiota”, “bile acids”, “transit time”, “short chain fatty acids”, “fermentation”, “gas”, “mucus”, “in vitro”, “model” in all available years. The online database search was last performed in August 2021 on titles, abstracts, and key words including original articles, reviews, theses, and books. Relevant studies were identified after consultation of the main text, figures and supplementary materials, and information regarding involved dogs (i.e., number of dogs, age, weight, breed, sex, reproduction state, living environment), alimentation (i.e., type of food, feeding frequency, principal components of food), health (i.e., healthy, obese or IBD dogs only), and analysis methods was extracted.

This review paper aims to give a state-of-the-art on canine digestive physiology regarding both physicochemical and microbi-al parameters that can be reproduced in in vitro gut models. Then, we explain how these parameters change under diseased conditions associated with obesity and IBD. In a third part, we provide an in-depth description of all available in vitro models of the canine digestive tract before discussing their limitations and challenges associated with the development of in vitro gut models of healthy or diseased dogs and their applications in the food and veterinary fields. This paper considers the entire canine GIT and associated microbiota and highlights similarities and differences between dog and human digestive physiology to provide new opportunities for canine in vitro gut simulation.

3 Canine digestion

3.1 Digestive anatomy

Because dogs are facultative carnivores, their GIT is adapted to high-protein and high-fat diets, i.e., relatively short and simple

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\(^1\) Animal medicine global market opportunities and strategies to 2023. https://www.globenewswire.com/news-release/2020/01/13/1969734/0/en/Global-Animal-Medicine-Market-Opportunities-Strategies-to-2023-Veterinary-Pharmaceuticals-Will-Gain-20-7B-of-Global-Annual-Sales-by-2023-A-6-6B-Opportunity-for-Veterinary-Parasitic.html

\(^2\) https://webgate.ec.europa.eu/envdataportal/content/alarues/section1_number-of-animals.html (accessed 03.02.2022)

\(^3\) https://pubmed.ncbi.nlm.nih.gov

\(^4\) https://scholar.google.fr
length is correlated with shoulder height at a 6:1 ratio (Morris and Rogers, 1989). Digestion in dogs starts in the mouth with the mechanic action of mastication, using 42 teeth and only 2000 taste buds. The carnines can cut meat into pieces. The molars have a larger crown than in humans and allow grinding of bones. The mastication step compared to herbivores and even omnivores like humans or pigs, reflecting the lower retention time required for meat in comparison to grass digestion (Moon et al., 2018) (Fig. 1). A dog’s digestive tract weight in relation to total body mass is inversely correlated to canine size, representing 7% and 2.8% of the total body weight in 5 and 60 kg dogs, respectively (Weber, 2006). The GIT length is correlated with shoulder height at a 6:1 ratio (Morris and Rogers, 1989). Digestion in dogs starts in the mouth with the mechanic action of mastication, using 42 teeth and only 2000 taste buds. The carnines can cut meat into pieces. The molars have a larger crown than in humans and allow grinding of bones. The mastication step

Fig. 1: Canine digestive compartments and associated mechanical, physicochemical, and microbial processes
Key parameters of the oral, gastric, intestinal, and colonic compartments of medium-sized healthy dogs are summarized and compared to in vivo data in healthy humans. Lack of in vivo data is represented by “?” . BA, bile acid
is less important than in humans as most dogs do not really chew but swallow large pieces of food. After swallowing, the food bolus crosses the esophagus, which has a length similar to that of the human esophagus, i.e., around 30 cm for a medium-sized dog (Kararli, 1995; Freiche and Hernandez, 2010). Like in humans, the canine stomach has the shape of a J-elongated bag. It is located in the abdomen, starts with the cardia and extends to the pylorus, and is composed of the antrum and the fundus (Kararli, 1995). Its dilatation capacity (0.5-8 L volume) is larger than that of the human stomach, and the gastric wall measures between 3 and 5 mm in thickness (Kararli, 1995; Freiche and Hernandez, 2010).

Digestion continues along the small intestine, which is anatomically divided into the duodenum (10%), jejunum (85%) and ileum (5%), measuring from 1 to 3 m total length. In comparison, the human ileum represents 60% of the total small intestine length (Kararli, 1995; Oswald et al., 2015). This suggests that ileal function may differ between dogs and humans. Canine small intestine diameter is smaller than in humans (1 cm versus 5 cm). Duodenal thickness reaches 6 mm, whereas intestinal loops measure around 2-3 mm. The large intestine or colon is 20-80 cm in length with a diameter of 2-3 cm in medium-sized dogs, while it is 90-150 cm in humans with a diameter of 5 cm (Kararli, 1995). The large intestine is composed of the ascending, transverse, and descending colon. Finally, the rectum forms the terminal section of the large intestine ending in the anus. The three parts of the canine colon are less well defined than in humans, with the particularity of being non-sacculated and devoid of a sigmoid colon (Kararli, 1995).

As for humans, peripheral organs are involved in canine digestion. The pancreas secretes pancreatic juice into the duodenum and is involved in protein (trypsin, chymotrypsin, elastase, and carboxypeptidase), carbohydrate (α-amylase), and lipid (lipase and phospholipase) digestion. Pancreatic juice also contains antimicrobial agents that contribute to microbial balance. The liver is coupled with a gallbladder and located near the stomach. The liver plays a central role in digestion, including vitamin and glucose storage (glycogen), detoxification and excretion of toxic substances (e.g., urea), and lipid digestion by saponification of lipids by bile acids (BA). Bile is produced by the liver, stored in the gallbladder, and discharged into the duodenum.

Depending on body size and breed, dogs show a diversity in anatomical features such as intestinal length and volume, intestinal villus morphology, and intestinal or colonic permeability (Zentek and Meyer, 1995; Oswald et al., 2015). Because of the relationship between anatomy and digestive physicochemical parameters, these differences may affect dog digestion and key parameters such as pH, digestive secretions, transit time, and gut microbiota. To avoid making this discussion too complex, the following sections (3.2 and 3.3) focus on digestion processes in medium-sized dogs (from 10 to 30 kg).

### 3.2 Physicochemical parameters

#### pH

Gastrointestinal pH changes along the dog’s digestive tract (Fig. 1). The mean salivary pH of medium-sized dogs is 7.3-7.8 (Smeets-Peeters et al., 1998). In the stomach, the arrival of a food bolus induces the secretion of gastrin, which in turn stimulates hydrochloric acid (HCl) production. The gastric pH of Beagles under fasted conditions is around 1.5 (range 0.9-2.5), similar to that of humans (range 1.4-2.1) (Dressman, 1986; Kararli, 1995; Mahar et al., 2012). Several studies have observed a higher gastric pH in fed dogs, ranging from 2 to 5.5 (Smith, 1965; Dressman, 1986; Kararli, 1995; Shinichi et al., 1996; Martinez, 2002; Duysburgh et al., 2020). In the small intestine, the pH increases to values close to neutrality because of the buffering capacity of pancreatic juice and bile (Kararli, 1995). The pH of the small intestine also increases from the proximal to the distal part, from 6.5 to 8 in medium-sized dogs (Koziolek et al., 2019). The few studies that have investigated the canine jejunal pH measured a mean pH of 6.8 for medium-sized dogs (Mentula et al., 2005; Kalantzis et al., 2006), which is similar to that of humans (value of 6-7) (Kararli, 1995; Martinez, 2002). Colonic pH is, like in humans, more acidic with values of 5.6-5.8 (Smith, 1965; Koziolek et al., 2019). The fecal pH of medium dogs has a pH range of 6-6.9 (Eisenhauer et al., 2019; Nogueira et al., 2019).

#### Gut motility and transit time

Digestive secretions

Dog digestion is accelerated by digestive secretions containing various enzymes (Fig. 1). Amylase, lactate dehydrogenase and adenosine deaminase are found in dog saliva (Lavy et al., 2012; Iacopetti et al., 2017; Ricci et al., 2018). The gastric mucosa secretes gastric juice containing proteolytic (pepsin, chymosin) and lipolytic (lipase) enzymes (Aspinall, 2004; Durand, 2010). Canine pancreatic juice contains amylase, lipase, phospholipases, cholesterolases, proteases, and nucleases (Kienzle, 1988; Robin, 2007). Of note, gastric and pancreatic juices are poorly characterized in dogs, in contrast to bile, for which more data are available. Bile is discharged into the duodenum during postprandial phases. Bile is up to 10-fold more concentrated in the gallbladder than in the liver, with a total concentration around 50 (40-90) mmol/L in dogs versus 3-45 mmol/L in humans (Nakayama, 1969; Kararli, 1995; Kakimoto et al., 2017; Nagahara et al., 2018; Larcheveque, 2019). Canine bile contains up to 15 BA, but the three major BAs (taurocholic, taurodeoxycholic and taurochenodeoxycholic acid) found in healthy dogs make up 99% of the total BA pool (Washizu et al., 1994). 99.04% of BA are conjugated to taurine, 0.15% to glycine and the remaining 0.81% are unconjugated (Nagahara et al., 2018). Fecal samples show concentrations of 5.8-7.5 µg total BA per mg of dry feces (Schmidt et al., 2018; Blake et al., 2019; Manchester et al., 2019). Lastly, as in various mammals including humans, mucins are produced by goblet cells all along the canine GIT (Kararli, 1995). Mucin thickness has been evaluated in dogs only in the stomach, measuring 425 and 576 µm respectively in the fundus and antrum in agreement with human values (Bickel and Kauffman, 1981; Kararli, 1995; Etienne-Mesmin et al., 2019). The mucin layer protects the epithelium from the acidic pH of the stomach and withstands bone fragments (Moon et al., 2018). In the large intestine, the mucous layer is colonized by a microbial biomass, as described in Section 3.3.
intestine (199 mmHg*sec/min) compared to the small intestine (134 mmHg*sec/min) and stomach (55 mmHg*sec/min) (Farmer et al., 2018). Canine motility is quite different to that of humans. Maximum pressure under fed conditions is higher in humans than in dogs (241 and 119 mmHg in the human stomach and small intestine, respectively, versus 52 and 75 mmHg for dog), but with a similar maximum frequency of 3.7 contractions/min in the gastric compartment (Boscana et al., 2013; Farmer et al., 2018). Data on transit time in the different digestive compartments of dogs (Fig. 1) vary widely depending on the method used (e.g., radiopaque markers, plastic beads, 13C-octanoic acid breath test, sulfasalazine-sulfapyridine method, or wireless motility capsule), breed, age, feed (composition, energy density or viscosity) and environment (laboratory or owners’ home, stress context or sedation). Gastric emptying time varies from 2-19.8 h in fed medium-sized dogs (Hinder and Kelly, 1977; Theodorakis, 1980; Ehrlin and Prøve, 1982; Meyer et al., 1985; Lui et al., 1986; Gupta and Robinson, 1988; Hornof et al., 1989; Arnberg, 1992; Carrière et al., 1993; Cullen and Kelly, 1996; Sagawa et al., 2009; Boillat et al., 2010; Mahar et al., 2012; Boscana et al., 2013; Kozioler et al., 2019). Small intestinal transit time has been reported to have a range of 1.1-3.6 h (Boillat et al., 2010; Oswald et al., 2015). Large intestine transit time in dogs ranged from 1.1-49.1 h measured in dogs from a wide range of weights and breeds (Boillat et al., 2010; Warrit et al., 2017).

3.3 Canine gut microbiota
Composition
Microorganisms colonize the entire GIT of dogs from mouth to rectum. Like in humans, there are longitudinal variations (i.e., along the GIT) in gut microbiota composition due to changes in pH, substrate concentrations (including oxygen and nutrient availability), and transit time (Hooda et al., 2012; Friedman et al., 2018; Enenne-Mesmin et al., 2019). Figure 2 gives an overview of available data on gut bacteria composition of the different gastrointestinal regions in dogs.

The stomach is the least colonized compartment with 10^3 to 10^5 colony forming units (CFU) per gram of content in medium-sized dogs, mainly composed by Proteobacteria, including Helicobacter spp. as in humans, which are potential pathogenic strains (Benno et al., 1992; Mentula et al., 2005; Hooda et al., 2012).

The small intestine contains 10^6 to 10^7 CFU/g of content (Benno et al., 1992; Mentula et al., 2005). The duodenum is colonized by Firmicutes (calculated median of the three available publications: 47%), Proteobacteria (27%), Bacteroidetes (9%), Fusobacteria (3%), and Actinobacteria (1%), whereas the jejunum is characterized by a higher abundance of Proteobacteria (37%), Actinobacteria (11%), and Fusobacteria (10%), together with lower percentages of Firmicutes (33%) and Bacteroidetes (7%) (Xenoulis et al., 2008; Suchodolski et al., 2009; Garcia-Mazcorro et al., 2012). A single study on 6 dogs investigated ileal microbiota and observed 31% Fusobacteria, 24% Firmicutes, 23% Bacteroidetes and 22% Proteobacteria (Suchodolski et al., 2008).

The colon is the most colonized part of the GIT, with up to 10^8-10^11 CFU/g of content (Hooda et al., 2012). According to a unique publication using 16S Illumina sequencing to investigate microbiota composition, colonic digesta is dominated by 37% Firmicutes, 33% Bacteroidetes, 29% Fusobacteria and 1% Proteobacteria (Suchodolski et al., 2008a).

The majority of taxa colonizing the colon are also found in canine feces (Pilla and Suchodolski, 2020), which seems to be rather different from the human situation, where a significant number of mucus-adherent bacteria from the colon are not found in feces (Pilla and Suchodolski, 2020). In addition to bacteria (98%), canine fecal microbiota contains 1.1% archaea, 0.4% fungi and 0.4% viruses, mainly bacteriophages (Suchodolski, 2011; Swanson et al., 2011). Fecal microbiota of healthy dogs is dominated by three main bacterial phyla: Firmicutes, Bacteroidetes and Fusobacteria (Pilla and Suchodolski, 2020). Bacteria from Actinobacteria and Proteobacteria phyla are also found in canine feces but in a lower proportion.

Fusobacteria and Proteobacteria seem to be more abundant in dogs than in humans, probably related to a carnivorous versus omnivorous diet (Fig. 1) (Simon, 2019). Unlike in humans, where Fusobacterium is frequently associated with diseases, in dogs this genus is related to non-stressful conditions and therefore probably a healthy state, especially because its abundance increases when dogs have access to the outdoors (Oswald et al., 2015).

In addition to longitudinal variations, scarce data suggest variations in microbiota composition from the digestive lumen to the surface of the intestinal epithelium covered by a mucus layer. Only two studies have investigated the mucus-associated bacteria on the outer mucus layer in the colon of healthy dogs (Simpson et al., 2006; Cassmann et al., 2016). Cassmann et al. (2016) demonstrated that free colonic mucus is mainly colonized by Bacteroides spp. and Eubacteria. Of interest, Akkermansia muciniphila, a well-known mucus-degrading bacterium in humans, which is inversely correlated with obesity, was not yet identified in canine feces (Garcia-Mazcorro et al., 2020).

Metabolic activities
Gut microbiota is known to play a key role in host homeostasis and health maintenance, as it is implicated in many nutritional (e.g., fiber degradation and vitamin synthesis), immunological (immune system maturation) and physiological processes (e.g., vascularization, epithelium integrity, barrier effect against pathogens, and lipid digestion via the metabolism of primary BA into secondary BA) (Durand, 2010; Andoh, 2016). Gut microbiota metabolic activity leads to short-chain fatty acid (SCFA) and gas production as mean fermentation products resulting from degradation of soluble fibers. In dogs, like in humans, the three main SCFAs are acetate, propionate and butyrate, with relative percentages in fecal samples of 60:25:15 (Mondo et al., 2019). Non-digested proteins from diet and endogenous proteins (e.g., from mucins) are also metabolized by gut microbiota, leading to the production of branched chain fatty acids (BCFA), ammonia, indoles and phenols (Weber et al., 2017). Total fecal SCFA and BCFA were investigated, with values widely varying between studies, i.e., 91-423 and 4.7-36.1 μmol/g of lyophilized stools, respectively (Beloshapka et al., 2012; Minamoto et al., 2015; Alexander et al., 2019; Detweiler et al., 2019; Eisenhauer et al., 2019; Nogueira et al., 2019). To our knowledge, there is no i
Dogs are considered clinically obese when their body weight is at least 20-30% above ideal weight, and a universal body condition score (ranging from 1 to 9) defines overweight dogs at 7 and obese dogs at 8/9 (Apper et al., 2020). Both sexes have a similar incidence and all dogs are affected whatever their size, even if certain breeds seem to be more predisposed, like labrador, retriever, Bernese mountain dog, Cavalier King Charles or beagle (Osto and Lutz, 2015). Canine obesity is generally associated with insulin resistance, altered lipid profile, hypertension, orthopedic and cardiorespiratory disease, and development of low-grade systemic inflammation (Tvarijonaviciute et al., 2012). While gene mutations are associated with increased body weight in beagle and labrador, diet is clearly a determinant of obesity through excess energy intake (Zeng et al., 2014; Raffan et al., 2016).

Several mechanisms may implicate gut microbiome in the onset and evolution of dog obesity. Based on human and murine data, this includes higher energy utilization from non-digestible carbohydrates, manipulation of host gene functions, and exacerbation of inflammation (Hamper, 2016). Five studies have compared fecal microbiota composition of obese and lean dogs (Fig. 3). Using 16S rRNA gene pyrosequencing in companion and laboratory dogs, dominance of *Firmicutes* (> 90%) was observed viva data on gas production in dogs, and the two available studies on gas composition focused on malodorous compounds such as hydrogen sulfide (Collins et al., 2001; Giffard et al., 2001).

### 4.1 Obesity

In Western countries, obesity is considered the most common nutritional disorder in pets due to an imbalance between energy intake and expenditure (Osto and Lutz, 2015). Dogs are considered clinically obese when their body weight is at least 20-30% above ideal weight, and a universal body condition score (ranging from 1 to 9) defines overweight dogs at 7 and obese dogs at 8/9 (Apper et al., 2020). Both sexes have a similar incidence and all dogs are affected whatever their size, even if certain breeds seem to be more predisposed, like labrador, retriever, Bernese mountain dog, Cavalier King Charles or beagle (Osto and Lutz, 2015). Canine obesity is generally associated with insulin resistance, altered lipid profile, hypertension, orthopedic and cardiorespiratory disease, and development of low-grade systemic inflammation (Tvarijonaviciute et al., 2012). While gene mutations are associated with increased body weight in beagle and labrador, diet is clearly a determinant of obesity through excess energy intake (Zeng et al., 2014; Raffan et al., 2016).

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in both obese and lean groups, but mean abundance of *Actinobacteria* and *Roseburia* was greater in obese dogs (Handl et al., 2013). Using the same method, Salas-Mani et al. (2018) showed that *Proteobacteria* was predominant in obese dogs (76%), whereas fecal microbiota from lean dogs was mainly composed of *Firmicutes* (85%). In addition, *Clostridiales* appeared to be less abundant in obese compared to lean dogs, while the opposite result was found for *Pseudomonadales*. However, in a recent study involving 17 healthy and 22 obese companion dogs, no significant difference in any taxa was highlighted when comparing the two groups using Illumina 16S rRNA gene sequencing (Forster et al., 2018). With the same method, Bermudez Sanchez and colleagues (2020) described a relative abundance of 92% of *Firmicutes*, 2% *Fusobacteria*, 1% *Bacteroidetes* and a median *Firmicutes/Bacteroidetes* ratio of 0.123 in 20 obese dogs (Bermudez Sanchez et al., 2020). Lastly, in a very recent study, a positive association between *Fusobacteria* level, especially *Fusobacterium perfoetens*, and body score condition in 24 overweight laboratory beagles was established using metagenomic analysis (Chun et al., 2020). In dogs, like in humans, microbial diversity seems to decrease in obese compared to lean dogs, with a Shannon index of 1.3 and 2.3, respectively (Park et al., 2015). However, more investigations are required to characterize canine obese microbiota, in feces but also in other digestive compartments, to determine if some species should be used as obesity markers. As an example, *Proteobacteria* were recently correlated with increased weight in overweight dogs, as has been suggested in humans (Apper et al., 2020).

In addition to microbiota changes, other physiological modifications have been demonstrated in obese humans compared to healthy individuals, including an increase in BA production by liver (coupled with high amounts of primary BA in stool), and a higher SCFA production, leading to a decrease in colonic pH (Rahat-Rozenbloom et al., 2014). Due to similarities between dog and human GIT and lifestyle, it would be interesting to establish whether similar phenomena occur in obese dogs. Mean fecal pH of obese companion beagles (6.6) was not significantly different from that of lean dogs (6.8) whatever the diet, i.e., high-fat or low-fat diet (Xu et al., 2017). In the same study, total fecal SCFA concentration was also equivalent in the two groups, whereas fecal BCFA such as isovalerate and isobutyrate were significantly more concentrated in obese compared to lean dogs. To our knowledge, no study has investigated BA production in obese versus lean dogs. However, recent studies recommend to monitor fecal BA concentrations along with microbiota in lean and obese dogs as they appear to be interesting markers of glucose homeostasis failure in obese dogs (Forster et al., 2018; Apper et al., 2020).

### 4.2 Inflammatory bowel disease

In dogs, IBD is classified as a chronic enteropathy, but defining its prevalence remains difficult because diagnosis of the disease is challenging. IBD is characterized by an irregular alternation of clinically active (with pain and diarrhea) and insignificant phases. Outside active phases, there are recurring gastric symptoms with histopathological changes in mucosa of the small and large intestines (Malewska et al., 2011). The predominant causes of canine IBD include bacterial and environmental factors, genetic predisposition of selected breeds, food allergies, and some drugs (Malewska et al., 2011). Pathogenesis of IBD includes loss of tolerance for endogenous microbiota, chronic inflammation of the GIT associated with an increase in intestinal permeability, and immune cell infiltration in the lamina propria (Junginger et al., 2014). Clinical scenarios include a decline in activity level and appetite, vomiting, increase in stool frequency, loss of stool consistency (increase in fecal water content), and weight loss.

Recent reviews on IBD in dogs (Fig. 3) report modifications in gut microbiota structure (compared to healthy animals) similar to those found in humans (Xenoulis et al., 2008; Hooda et al., 2012; Suchodolski et al., 2008, 2010, 2012a,b; Cassmann et al., 2016; Pilla and Suchodolski, 2020), although Xu et al. (2016) did not observe significant differences between healthy and IBD groups. Minamoto and collaborators (2015) showed an overall decrease in fecal diversity in IBD compared to healthy dogs, as also de-
scribed in humans (Minamoto et al., 2015). Furthermore, a dysbiosis index designed using qPCR targeting of eight bacterial groups to assess fecal microbial changes associated with canine chronic enteropathies allowed discrimination between healthy and diseased dogs with 95% confidence range (AlS amphqeh et al., 2017). Fungal DNA was more frequently detected in dogs with chronic enteropathies than in healthy animals. FISH analysis of colonic biopsies from boxers with granulomatous colitis revealed mucosa colonization by an unknown adherent and invasive E. coli strain (Simpson et al., 2006). The data indicate that global microbial structure and diversity, more than a single taxa, should be followed to discriminate healthy and IBD dog microbiota (Scarsella et al., 2020).

Concerning functional activity, there is no significant difference in fecal SCFA concentrations between IBD and healthy dogs, but a lower indole concentration was measured in diseased dogs (Xu et al., 2016; Pilla and Suchodolski, 2020). This is an important point because indoles have well-known anti-inflammatory effects, strengthen epithelial barrier, and decrease E. coli attachment to the epithelial wall (Chévreton, 2018). Alterations in microbial functions associated with IBD were estimated using a prediction tool (PICRUSt) from 16S rRNA gene data, highlighting a significant increase in secretion system pathways and transcription factors (Minamoto et al., 2015). Other parameters modified in humans during IBD (Duboc et al., 2013; Rana et al., 2013; Fitzpatrick and Jenabzadeh, 2020) such as transit time and BA dysmetabolism (increase in fecal primary BA) have not been investigated in dogs yet.

5 In vitro canine models as an alternative to in vivo assays in dogs

5.1 Generalities on in vitro gut models: static versus dynamic and comparison with in vivo

A wide range of in vitro gut models has been developed, from simple static mono-compartmental models to more complex dynamic and multi-compartmental models (Guerra et al., 2012; Payne et al., 2012). These in vitro models have been primarily developed to mimic human digestion but have also been adapted to simulate animal digestion, mainly that of pig or piglet (Meunier et al., 2008; Tanner et al., 2014; Fleury et al., 2017; Dufourmy et al., 2019), cat (Sunvold et al., 1995a,b; Van den Abbeele et al., 2020) or dog (Sunvold et al., 1995a,b; Smeets-Peeters et al., 1999; Tzortzis et al., 2004; Hervera et al., 2007; Bosch et al., 2008; Cutrignelli et al., 2009; Panasevich et al., 2013; Lee et al., 2017; Vierbaum et al., 2019; Oba et al., 2020; Van den Abbeele et al., 2020a; Verstrepen et al., 2021), as described in Section 5.2.

Simple static models of the upper gut (Minekus et al., 2014) reproduce the successive oral, gastric and/or small intestinal phases of human digestion in a single vessel maintained at body temperature by changing pH conditions and adding appropriate digestive secretions (e.g., α-amylase in the oral phase, pepsin and/or lipase in the stomach, and bile and/or pancreatic juice in the intestinal phase). The simplest models of the colon compartment are thermostatic batch culture systems. These models are inoculated with feces to simulate colonic fermentation and are maintained under anaerobic conditions by flushing them with nitrogen or carbon dioxide without renewal of nutritive growth medium. Such approaches are therefore limited in the time they can be run by substrate availability (24 to 72 h), and parameters like pH or redox potential are not regulated.

Compared to static systems, dynamic models reproduce changes in at least one parameter such as pH kinetics, variation in digestive secretions, or chyme transit. They can be mono-compartmental or are composed of sequential vessels simulating the successive digestive compartments. Dynamic mono-compartmental models of the upper gut are only gastric digesters (Kong and Singh, 2010; Thuenemann et al., 2015), while multi-compartmental models include gastric and small intestinal compartments, most frequently simulating the duodenal section (Tompkins et al., 2011; Ménard et al., 2015). All these models only reproduce physicochemical parameters of the upper digestive tract, such as temperature, gastric and intestinal pH, gastric and ileal deliveries, transit time, digestive secretions and passive absorption of nutrients and water. Dynamic large intestine models are based on the principle of continuous or semi-continuous fermentation and are inoculated with fecal samples. Such models are maintained under anaerobiosis and reproduce colonic temperature, pH, and transit time, and redox potential can be monitored. Moreover, a nutritive medium aiming to mimic ileal effluents and composed of various complex sources of carbon and nitrogen, electrolytes, BA, and vitamins is continually added to the bioreactor while fermentation medium is regularly removed. This allows maintaining functional microbiota for up to several weeks (or even several months with specific adaptations) (Fehlbau et al., 2015). Several configurations of these colon models include the use of three-stage bioreactors in series to mimic the different sections of the human colon (Gibson et al., 1988; Cinquin et al., 2006; Van de Wiele et al., 2015) or the addition of mucin beads to distinguish luminal from mucosal colonic environments and their associated microbiota (Van den Abbeele et al., 2009; Deschamps et al., 2020). Up to now, the TNO gastro-intestinal Model (TIM-1) is probably the most complete in vitro system, with its four compartments reproducing the stomach and small intestine of monogastrics (Minekus et al., 1995; Meunier et al., 2008; Denis et al., 2016), while only two models simulate the whole digestive tract from the stomach to colon: the Simulator of Human Intestinal Microbial Ecosystem (SHIME) (Moly et al., 1993; Rousseau et al., 2020), and the SIMulatator of the Gastro-Intestinal tract (SIMGI) (Barroso et al., 2015).

Despite the obvious limitations of in vitro approaches, i.e., no input from nervous, endocrine or immune systems, artificial gut models have many advantages in terms of low cost, technical flexibility, and reproducibility. Especially, the spatial compartmentalization of bi- and multi-compartmental models allows sample collection over time and in the desired segment of the GIT, while in vivo studies mainly provide endpoint measurements (e.g., in fecal samples), since access to the different segments of the digestive tract (from the stomach to proximal colon) is restricted. Besides, in canine in vivo assays, there is frequently a huge discrepancy between studies due to different diets, life-
style, sizes and breeds. Therefore, as in humans, inter-individual variability is one of the main challenges. In comparison, in vitro approaches enable a high level of experimental control and reproducibility by excluding confounding environmental or dietary factors and therefore allow in-depth mechanistic studies on pharma and food compounds.

### 5.2 Currently available canine in vitro gut models

Since 1995, twelve in vitro models of the canine gut have been developed (Tab. 1). There is no available model of the oral phase, and only three models mimic the upper GIT (Smeets-Peeters et al., 1999; Hervera et al., 2007; Lee et al., 2017).

**In vitro models of the upper gut**

While Hervera and colleagues (2007) simulate stomach and small intestine digestion in batch vessels by adding crushed dog food to pepsin and pancreatin secretions only, a very complete model of the canine upper gut was developed in 2000 based on the TIM-1 technology, initially set up to reproduce human digestive conditions. Though the TIM-1 model is the most complete simulator of the upper gut, it only reproduces physicochemical and not microbial digestive parameters. Another main limitation of this model is that tested food should be finely mixed before digestion, which can influence nutrient digestibility.

FIDO (for Functional gastroIntestinal DOg model) integrates all the upper digestive compartments (stomach, duodenum, jejunum and ileum) and simulates body temperature, kinetics of gastric and small intestine pH, half-time delivery of gastric and ileal compartment, transit time and chyme mixing, sequential delivery of digestive secretions (gastric juice containing *Rhizopus* lipase and porcine pepsin, porcine pancreatic juice, bovine trypsin, electrolytes, and porcine bile), and intestinal passive absorption through hollow dialysis fibers (Smeets-Peeters, 2000). FIDO was set-up to mimic canine digestive parameters of medium dogs based on literature review (Smeets-Peeters et al., 1998). All parameters were therefore adapted to *in vivo* data, except for temperature, which was kept at 37°C like in humans, and parameters of passive absorption, probably due to a lack of data. The model was validated only for nutritional applications, by comparing protein digestibility and calcium bioaccessibility in the FIDO model and in ileal cannulated dogs (5 dogs).

More recently, the Artificial Stomach-Duodenum (ASD) dissolution model was adapted to dog digestion for formulation solubility studies (Lee et al., 2017). This bi-compartmental model, set at 37°C, simulates both the stomach and duodenum with associated pH (6.8 and 6.8-7, respectively), transit time (adapted from *in vivo* data), and pancreatic secretions. In this model, the gastric pH is particularly high compared to the other two (6.8 for the ASD model versus pH 2 for Hervera’s model and 2-6 for FIDO).

**In vitro models of the lower gut: batch and continuous models**

Eight other available devices are *in vitro* static models of the canine colon based on batch fermentation. The simplest model of the colon of adult dogs was developed by Cutignelli et al. (2009). Here, a single vessel was inoculated with diluted feces from adult large dogs and maintained at 39°C under anaerobiosis without any addition of nutritional medium except for the tested carbohydrates. Another batch model set up by Sunvold et al. (1995c) to reproduce the colon of adult medium-sized dogs used a simulated growth medium adapted to dog digestion. This model was validated against *in vivo* data from 30 medium-sized dogs regarding fiber digestibility and production of SCFAs. Another static model, a batch system inoculated with dog fecal samples (no information on age and size of animals), was recently developed by Van den Abbeele et al. (2020a). A nutritive medium was introduced to simulate dog ileal effluents, but this was similar to one used previously for human experiments (Van den Abbeele et al., 2018). Batch models developed by other teams share the limitation that they lack a nutritive medium adapted to the canine situation (Tzortzis et al., 2004; Panasevich et al., 2013; Vierbaum et al., 2019). Bosch et al. (2008) developed a more complete batch fermentation model mimicking the ileum, proximal colon, transverse colon, or rectum compartment in different vessels inoculated with corresponding digestive fluids from 3 adult small or large dogs. In this study, the authors used a nutritive medium previously adapted for piglet fermentation from another medium initially developed for rumen bacteria maintenance without any modification in relation to dog digestion (Williams et al., 2005). The main disadvantage of this model is that it uses digestive fluids collected from dogs to inoculate the vessels, and the inter-individual variability associated with such an approach. Only one static batch model integrates mucin-covered microcosms in order to represent the intestinal mucus, but the associated results are not discussed (Oba et al., 2020). To conclude on these batch models, apart for temperature, fecal inoculation and anaerobiosis, parameters were generally not adapted to canine digestion. Even if *in vitro/in vivo* correlations were established for some of these models, *in vitro* digestion conditions remained far from *in vivo* complexity, and nutrient composition of dog digestive fluids, digestive secretions, or pH variations between small and large intestinal compartments and residence time in each gut section were not considered.

The only dynamic *in vitro* model of the lower dog gut, the SCIME model (for simulation of canine intestinal microbial ecosystem), was adapted from the SHIME system, first set up to reproduce human conditions. The SCIME model is currently unique in reproducing the entire canine GIT from the stomach to the large intestine (Duysburgh et al., 2020). SCIME is composed of four bioreactors simulating the stomach, small intestine, and proximal and distal colon. Only colonic compartments are inoculated with fecal samples from medium-sized dogs. *In vivo* parameters that are reproduced include body temperature, regionalized gastric and intestinal pH, gastrointestinal transit time, digestive secretions (pancreatic juice and bile), and anaerobiosis. The authors mention that most of the parameters were adapted to medium-sized dog digestion, though associated *in vitro* data are not clearly mentioned in the publication. SCIME was validated by comparison with *in vivo* data from ten Beagles digesting fructooligosaccharides regarding microbial composition and SCFA/BCFA production. Of note, *in vitro* results in proximal and distal colon (even if clearly distinct profiles were obtained) were only compared to *in vivo* data in fecal samples. Recently,
| References | Dog's generalities | GI compartment modeled | Adaptations from bibliographic research | Validation processes | Physicochemical parameters | Microbiota |
|------------|--------------------|-------------------------|----------------------------------------|----------------------|---------------------------|-----------|
|            | Age (years) | Dog's size | Stomach and small intestine | In vivo digestibility trial (n=5A) | Temperature | Agitation | pH Value | Transit time | Food reproduction | Digestive secretions | Absorption | Presence of microbiota | Process type | Anaerobiosis | Mucus |
| **UPPER TRACT** | | | | | | | | | | | | | |
| Hervea et al., 2007 | ND | ND | Stomach and small intestine | But no reference to in vivo data | In vivo digestibility trial (n=5A) | 39°C | Magnetic bar | No | Stomach: 2 Small intestine: 6.8 | No | Crushed dry extruded canine food | No | No | N/A | No | No |
| Lee et al., 2017 | ND | Medium | Stomach and duodenum | Adapted from Smeets-Peeters et al., 1998 | In vivo fuma trial (n=6) | 37°C | Paddle | No | Stomach: 6.8 Duodenum: 6.5-7 | Yes | No | Pancreatin solution | No | No | N/A | No | No |
| Artificial Stomach-Duodenum | “Adult” | Medium | Stomach, duodenum, jejunum and ileum | Adapted from Smeets-Peeters et al., 1999 | In vivo digestibility trial (5 feal cannulated dogs) | 37°C | Water pressure on flexible wall | Yes | Stomach: 2.6 Duodenum: 6.2 ± 0.2 Jejunum: 6.5 ± 0.2 Ileum: 7.0 ± 0.2 | Yes, from 1.5 h SITT: T= 5 h | Crushed dry or canned dog food | Yes | No | N/A | No | No |
| Smeets-Peeters et al., 1999 | ND | Large | Stomach and small intestine | Adapted from Smeets-Peeters et al., 1998 | In vivo digestibility trial (5A) | 39°C | Periodic mixing | No | Stomach: 6.8 Duodenum: 6.5 ± 0.2 Jejunum: 7.0 ± 0.2 | No | No | No | No | Yes | No |
| FIDO | 4.3 ± 0.9 | Medium | Large intestine | No validation | 37°C | Continuous shaking at 370 rpm | No | N/A | No | No | No dog food An aerobic sterilized medium | No | No | Yes, from faces | Batch 24 h | Yes | No |
| Viitbaum et al., 2019 | 3 | Large | Large intestine | Based on a literature review | 39°C | No | No | N/A | No | No | No | No | Yes, from faces | Batch 48 h | Yes | No |
| Cutrignelli et al., 2009 | ND | ND | Large intestine | But no reference to in vivo data | In vivo digestibility trial (n=110) | 39°C | Periodic mixing | No | N/A | No | No | No | Yes, from faces | Batch 12 h | Yes | CO₂ gas | No |
| Panasie-vich et al., 2013 | 5-6 | Medium | Large intestine | In vivo fermentation study (n=30) | 39°C | No | No | N/A | No | No | No | No | Yes, from faces | Batch 24 h | Yes | CO₂ gas | No |
| Sunvold et al., 1995 | ND | ND | Large intestine | Based on a literature review | 39°C | Magnetic bar | No | N/A | No | No | No | No | Yes, from faces | Batch 48 h | Yes | N-gas flow | No |
| Van den Abbeele et al., 2020a | ND | ND | Large intestine | Based on a literature review | 39°C | Magnetic bar | No | N/A | No | No | No | No | Yes, from faces | Batch 48 h | Yes | N-gas flow | No |
| **LOWER TRACT** | | | | | | | | | | | | | |
| Hervea et al., 2007 | ND | ND | Stomach and small intestine | But no reference to in vivo data | In vivo digestibility trial (n=5A) | 39°C | Magnetic bar | No | Stomach: 2 Small intestine: 6.8 | No | Crushed dry extruded canine food | No | No | N/A | No | No |
| Lee et al., 2017 | ND | Medium | Stomach and duodenum | Adapted from Smeets-Peeters et al., 1998 | In vivo fuma trial (n=6) | 37°C | Paddle | No | Stomach: 6.8 Duodenum: 6.5-7 | Yes | No | Pancreatin solution | No | No | N/A | No | No |
| Artificial Stomach-Duodenum | “Adult” | Medium | Stomach, duodenum, jejunum and ileum | Adapted from Smeets-Peeters et al., 1999 | In vivo digestibility trial (5 feal cannulated dogs) | 37°C | Water pressure on flexible wall | Yes | Stomach: 2.6 Duodenum: 6.2 ± 0.2 Jejunum: 6.5 ± 0.2 Ileum: 7.0 ± 0.2 | Yes, from 1.5 h SITT: T= 5 h | Crushed dry or canned dog food | Yes | No | N/A | No | No |
| Smeets-Peeters et al., 1999 | ND | Large | Stomach and small intestine | Adapted from Smeets-Peeters et al., 1998 | In vivo digestibility trial (5A) | 39°C | Periodic mixing | No | Stomach: 6.8 Duodenum: 6.5 ± 0.2 Jejunum: 7.0 ± 0.2 | No | No | No | No | Yes | No |
| FIDO | 4.3 ± 0.9 | Medium | Large intestine | No validation | 37°C | Continuous shaking at 370 rpm | No | N/A | No | No | No | No | No | Yes, from faces | Batch 24 h | Yes | No | No |
| Viitbaum et al., 2019 | 3 | Large | Large intestine | Based on a literature review | 39°C | No | No | N/A | No | No | No | No | Yes, from faces | Batch 48 h | Yes | No | No |
| Panasie-vich et al., 2013 | ND | ND | Large intestine | But no reference to in vivo data | In vivo digestibility trial (n=110) | 39°C | Periodic mixing | No | N/A | No | No | No | Yes, from faces | Batch 12 h | Yes | CO₂ gas | No |
| Sunvold et al., 1995 | 5-6 | Medium | Large intestine | In vivo fermentation study (n=30) | 39°C | No | No | N/A | No | No | No | No | Yes, from faces | Batch 24 h | Yes | CO₂ gas | No |
| Van den Abbeele et al., 2020a | ND | ND | Large intestine | Based on a literature review | 39°C | Magnetic bar | No | N/A | No | No | No | No | Yes, from faces | Batch 48 h | Yes | N-gas flow | No |

**Notes:**
- **References:** The references are used to validate and support the described models.
- **Physicochemical parameters:** Temperature, Agitation, pH, Transit time, Food reproduction, Digestive secretions, Absorption.
- **Microbiota:** Presence of microbiota is indicated for each model.
- **Process type:** Static or Dynamic.
- **Anaerobiosis:** Indicates whether the model is designed for anaerobic conditions.
- **Mucus:** Indication of mucus production.

**Generalities:**
- **Age (years):** The age range of the dogs used in the models.
- **Dog's size:** The size category of the dogs.
- **GI compartment modeled:** The specific parts of the gastrointestinal tract modeled.
- **Adaptations from bibliographic research:** Any modifications or adaptations from existing literature.
- **Validation processes:** Methods used to validate the models.

**Characteristics:**
- **Temperature:** The temperature setting for the models.
- **Agitation:** Methods used to agitate the models.
- **pH:** The pH value for the models.
- **Transit time:** The time it takes for food to pass through the models.
- **Food reproduction:** Methods used to reproduce food intake.
- **Digestive secretions:** Indication of digestive secretions.
- **Absorption:** Methodology for absorption processes.
- **Presence of microbiota:** Indication of the presence of microbiota in the models.
- **Process type:** Static or Dynamic.
- **Anaerobiosis:** Indicates whether the model is designed for anaerobic conditions.
- **Mucus:** Indication of mucus production.
SCIME was optimized by the addition of mucin-covered plastic beads, based on Mucosal-SHIME (M-SHIME) technology (Van den Abbeele et al., 2009), to reproduce the luminal and mucosal microenvironments of the canine colon. The resulting model was called M-SCIME (Van den Abbeele et al., 2020b; Verstrepen et al., 2021). Due to a lack of in vivo data, this optimization was not validated compared to bacterial mucosal profiles in dogs, but only compared to previous results obtained in the M-SHIME. Once again, in vitro colonic pH varies widely between currently available models, which could be explained by the fact that authors generally base their model settings on a unique in vivo study, whereas large inter-individual variations are observed in dogs depending on age, size, and breed.

6 In vitro gut models as powerful tools to study canine digestion

6.1 Scientific and technical challenges to be addressed

As described above, in vitro models have not been fully optimized, probably due to the paucity of information on the relevant parameters in dogs. Therefore, many scientific and technical challenges still need to be addressed to improve models of canine digestion and reflect the complexity of this environment (Fig. 4).

First, technical improvements should be considered to simulate canine digestive conditions in each part of the GIT more realistically. Currently, there is no canine chewing simulator, but such a development is not a priority, since most dogs do not chew but swallow large pieces of food. Regarding the upper gut, the FIDO model already shows a high level of complexity. The M-SCIME also possesses a gastric compartment that would merit improvements such as progressive acidification of the chyme (already introduced in the M-SHIME). This change in gastric pH during canine digestion is a key parameter in food disruption and digestion as it influences gastric pepsin and lipase activities (Carrière et al., 1993; Sams et al., 2016). FIDO and M-SCIME both require homogenization of food before in vitro digestion. In dogs, larger food particle sizes seem to reach the stomach. Even if little data on this in dogs is available (unlike in humans), some canine studies showed no correlation between food size and density of particles on gastric emptying time or on the entire upper gut digestion process (Gruber et al., 1987; Meyer et al., 1988; Chen et al., 2008; De Cuyper et al., 2018), while others observed that gastric half emptying time increased with higher meal viscosity and fat content (Ehrlein and Pröve, 1982; Palerm et al., 2020). Recently, a new human gastric and small intestinal model, the Engineered Stomach and Small Intestine (ESIN), was developed to fill this gap and handle both ingested liquids and real-size food particles better in simulated digestion studies (Guerra et al., 2016). Even if this model was primarily set up for human applications, it could easily be adapted to reproduce dog digestion.

Another main issue in upper gut models is the use of porcine, bovine, or fungal secretions/enzymes instead of canine ones. Due to ethical constraints, digestive secretions cannot be collected from dogs for this purpose (and are not commercially avail-
Therefore, further investigations are needed to ensure that digestive secretions from other sources are suitable to represent canine ones (in terms of composition and enzymatic activities) to ensure relevant in vitro simulation. For example, bile composition and conjugation profiles differ widely between species (Thakare et al., 2018a,b). Thus, further analysis is needed to determine whether porcine bile (largely used in in vitro models) is a good model of canine bile (Alvaro et al., 1986).

Another main limitation of current upper gut models is their inability to accurately mimic gastric and intestinal peristalsis. In M-SCIME, peristaltic mixing is reproduced using magnetic stirrers, which is far from the in vivo situation. FIDO more closely reproduces peristaltic movements through gentle mixing with pressurized water jackets, but peristaltic force and frequency have not been adapted for dog models, whereas in vivo data indicate clear differences between humans and dogs (Boscan et al., 2013; Warrit et al., 2017; Farmer et al., 2018). Efforts should therefore be invested to more accurately simulate mechanical deformation resulting from peristalsis in dogs, based on what has already been done in human gastric models (Kong and Singh, 2010; Li et al., 2019).

Regarding nutrient absorption in the small intestine, FIDO incorporates dialysis fiber modules in the jejunal and ileal compartments. In a very recent publication on the M-SCIME (Verstrepen et al., 2021), a dialysis step of ground dog feed was integrated to simulate small intestinal absorption by removing high-molecular weight proteins. Adding such dialysis devices to other upper gut models would be beneficial to better represent the in vivo situation, even if dialysis modules only reproduce passive absorption of small molecules (e.g., fatty acids, oligo- and monosaccharides, small peptides, amino acids, but also drugs and chemicals) and water. Reproducing passive absorption also ensures bile re-

Fig. 4: Main challenges in the development of in vitro gut models of the canine digestive tract and their applications in nutritional and veterinary fields

Overview of the main technical and scientific challenges and applications of canine in vitro gut models as reliable tools to test or develop new products in the food and pharma fields.
absorption in the distal intestine and, therefore, a decrease in bile concentrations from the duodenal to the ileal compartments, which is a key process in both dog and human gut physiology.

To further reproduce absorption phenomena and integrate active transport, canine dog models of the upper gut should be coupled with immortalized canine intestinal epithelial cells (cIEC) (Farquhar et al., 2018), as done in human models with TIM-1 and Caco-2 cells (Déat et al., 2009; Bahrami et al., 2011) or with organoids generated from canine duodenal, jejunal and colonic biopsies (Kramer et al., 2020).

A last key point to be raised is the lack of intestinal microbiota in all the upper gut models, whereas bacterial concentrations up to $10^7$ CFU/g can be found in the distal small intestine of dogs (Benno et al., 1992; Mentula et al., 2005). Even if the role of intestinal microbiota is poorly defined in dogs (Hooda et al., 2012; Deng and Swanson, 2015; Enright et al., 2016; Mondo et al., 2019; Pilla and Suchodolski, 2020), we can assume that intestinal microbiota is involved at least in carbohydrate digestion and may exert a barrier effect against enteric pathogens as observed in humans (Andoh, 2016). This is even more important since, unlike in humans, an appreciable fraction of dietary fibers seems to be degraded in the canine small intestine (Bednar et al., 2000).

This microbial component of the canine digestive tract is integrated in in vitro colon models by inoculation with fecal samples. However, a major issue of all available in vitro colon models is that the nutritive medium used to maintain bacterial growth and activity was not adapted to adequately mimic the composition of ileal effluents entering the colon in vivo. Using digestive fluids as previously done by Bosch et al. (2008) is not a simple and sustainable solution as it makes in vitro models strongly dependent of unstandardized and poorly available in vivo samples. Therefore, efforts are still required to better define a growth medium composition based on available data on dog diet, but also protein, lipid and carbohydrate ileal digestibility (Bednar et al., 2000; Flickinger et al., 2003; Propst et al., 2003). It is also important to consider relevant BA concentrations and profiles reaching the colon, as they may shape gut microbiota composition (Ridlon et al., 2014).

All available in vitro colon models are flushed with nitrogen or carbon dioxide to maintain anaerobic conditions inside vessels or bioreactors, which is unlike the in vivo situation. In the human Artificial Colon (ARCOL) model, anaerobiosis is maintained solely by the activity of resident microbiota to reflect in vivo gut physiology (Friedman et al., 2018; Deschamps et al., 2020; Verdier et al., 2021). Such an adaptation could further increase the relevance of in vitro canine colon models.

Moreover, only bacteria have been followed to date as the main component of gut microbiota. Given the importance of other constituents, such as fungi, methanogenic archaea, or even phages or viruses in gut physiology (Barko et al., 2018), it would be of great interest to extend microbial sequencing to these populations.

Lastly, as mentioned for upper gut models, to further investigate host-microbiome crosstalk, canine in vitro models should be coupled to cell culture assays, as previously described for human models (Bahrami et al., 2011; Chassaing et al., 2017; Geimaert et al., 2017; Defoisy et al., 2018).

Of interest, the Host Microbiota Interaction Module already coupled to the SHIME system (Marzorati et al., 2014) integrates microaerobiosis in close proximity to the epithelium, shaping mucus-associated gut microbiota. This may also be of relevance for the dog colonic ecosystem.

6.2 Future developments and quality requirements
Most available in vitro models, and especially the more complex FIDO and M-SCIME systems, have been designed to reproduce a medium-sized dog’s digestive conditions when ingesting dry food. As widely described, canine digestion is impacted by body weight or breed (Kendall et al., 1983; Bourreau et al., 2004; Hernot et al., 2005; Weber, 2006; Weber et al., 2017). Considering the influence of body weight and/or breed on canine digestive physicochemical and microbial parameters is undoubtedly a promising line of research to develop more relevant in vitro models.

Further, adaptations regarding dog diet (e.g., dry, canned or homemade food, BARF) and age (puppies, adults and elderly dogs) would also bring substantial added value to in vitro canine gut simulation. This has been already performed with success in in vitro human models (Blanquet et al., 2004; Fehlbaum et al., 2015; Denis et al., 2016; Roussel et al., 2016, 2020; Bondue et al., 2020).

Moving forward, we can foresee the development of in vitro gut models simulating not only physiological but also diseased situations, such as those associated with obesity or IBD, as previously done in humans (Bussolo de Souza et al., 2014). In this case, the main objective would be to maintain gut microbiota dysbiosis (considered a typical feature of these pathologies) inside the in vitro models. For this purpose, small intestinal and/or colonic models could be inoculated with feces collected from dogs suffering from obesity or IBD, but also all the associated gut parameters, such as the composition of digestive effluents (including bile profiles), pH and retention time should be modified to fulfill specific diseased conditions.

All these technical and scientific improvements would be possible if corresponding canine in vivo data were available in the literature (or provided by clinical trials), both for setting-up in vitro models and for the validation of their robustness through strong in vivo-in vitro correlations. Such validation is necessary to convince future users of the relevance of in vitro gut models but is unfortunately rarely performed due to cost, time, or technical limitations.

To date, main gaps in in vivo data concern digestive secretions in the upper gut as well as microbial profiles along the digestive tract and characterization of mucus-associated bacteria. Recent developments of non-invasive methods to follow digestive parameters, such as pH, motility, or transit time (wireless motility capsules, e.g., SmartPill) in dogs (Warrit et al., 2017) and in humans (Schwizer et al., 2002; Wang et al., 2005; Zhang et al., 2014) open new avenues and may help to fill these scientific and technological gaps. Regarding such in vivo reference studies, it would be of high importance to standardize the experimental conditions in terms of age, breed, weight, but also diet and lifestyle, which all impact digestive parameters (Mahar et al., 2012; Panasevich et al., 2015; Apper et al., 2020; Pilla and Such-
It is also important to exclude breeds showing well-known specific digestive particularities (like German Shepherd) or specific energy needs (like Husky, Great Danes or Terriers) (Zentek and Meyer, 1995) from such in vivo assays.

Standardization of procedures is also of concern for in vitro studies, and some important guidance is available in the guidance document on Good In Vitro Method Practice (GIVIMP) (OECD, 2018), especially for cell culture practices.

In addition to robustness, canine in vitro model development involves other quality requirements, such as reproducibility and transferability. Reproducibility is considered a key advantage of in vitro studies over in vivo experiments and should be systematically assessed. Transferability of canine in vitro gut models is hampered by their current early stage of development. The simple in vitro systems (such as batch models) can be more easily shared between laboratories than the more complex ones, which require specific technical expertise. Transferability of canine gut models could be accelerated by exchanges between international experts in artificial digestion, as previously done in the frame of the INFOGEST network, which led to the harmonization of static and semi-dynamic in vitro protocols in humans (Minekus et al., 2014; Brodkorb et al., 2019).

6.3 Potential applications of canine gut models in food and pharma

In vitro models represent a powerful platform to study the fate of food and veterinary products in the canine digestive environment, to help elucidate their mechanisms of action and promote innovation in these fields. As parameters can be adjusted in terms of food matrix, age, dog breed or body size, but also by mimicking physiological or pathological situations, we can imagine numerous applications of these systems once developed and validated (Fig. 4). They could provide valuable information to promote the evolution of products already on the market (generic, range extension, etc.) and/or inform the development of new products such as specialized food or innovative drugs. All types of in vitro gut models, from the simplest to the more complex ones, can be useful. Static mono-compartmental models are ideal tools to perform pre-screening of many compounds due to their low cost and easy manipulation. Dynamic multi-compartmental systems, which are more expensive and require expertise but reproduce more physiological digestive conditions, can be applied for a more focused study of selected candidate compounds. The applications already performed under human conditions (Souliman et al., 2006; Cordonnier et al., 2015; Lyng et al., 2016; Bianchi et al., 2019; Blancquaert et al., 2019; Kubbinga et al., 2019) in food and pharma fields can inspire similar studies in canine in vitro gut models.

To date, in vitro models simulating the canine digestive environment have only been used for nutritional applications, mainly to assess fiber digestibility (Sunvold et al., 1995c; Bosch et al., 2008; Cutrignelli et al., 2009; Musco et al., 2018; Van den Abbeele et al., 2020a,b) (Tab. 2). Other applications include evaluation of protein (Kim et al., 2021) or lipid digestibility and bioaccessibility of micronutrients such as vitamins, minerals (van Zelst et al., 2015; Lee et al., 2017) or phytoconstituents like polyphenols. In addition to digestibility, in vitro models can provide valuable information on the effects of fiber on gut microbial composition and activity, through SCFA/BCFA, ammonia or gas measurements (Bosch et al., 2008; Cutrignelli et al., 2009; Van den Abbeele et al., 2020a). This will help to assess the prebiotic status of soluble fibers. In addition to prebiotics, probiotic strains can be tested in the in vitro models to evaluate their interactions with gut microbiota (Ogué-Bon et al., 2011) but also their survivability (and the effect of mode of administration) and their production of active compounds such as bacteriocins.

Regarding veterinary applications, the kinetics of drug release and absorption can be monitored all along the GIT, as well as the influence of oral formulations (Lee et al., 2017), different doses, fed or fasted state, and food matrix (food-drug interactions). Currently, drug posology is only established based on dog body weight or metabolic weight, but many associated variations in digestive parameters that influence drug bioavailability should be considered (Oswald et al., 2015). In addition, in vitro gut models can enhance knowledge of drug metabolism by gut microbiota and/or effects of drugs on gut microbiota composition and function (Sjögren et al., 2014). Hence, gut microbial dysbiosis induced by oral antibiotherapy (El Hage et al., 2019) can be simulated in colonic models and further applied to evaluate the efficiency of prebiotics and/or probiotics in restoring microbiota eubiosis.

Lastly, in vitro models can be employed to assess the efficiency of new microbial restoration therapy strategies such as fecal microbiota transplantation in a safe way before using animals. Fecal microbiota transplantation is based on the observation that transfer of intestinal contents from a healthy donor to a diseased one can improve gut health. This therapy has been recently tested in dogs for the treatment of post-weaning diarrhea, acute diarrhea, IBD, chronic enteropathies, or parvovirus infections and seems to be promising (Burton et al., 2016; Pereira et al., 2018; Niina et al., 2019; Chaitman and Gaschen, 2020; Chaitman et al., 2020).

7 Conclusion

Canine digestion is a complex and regionalized process involving physicochemical, mechanical, and microbial processes. It plays a central role in maintaining dog health and is increasingly recognized as part of the etiology of intestinal and extra-digestive diseases (such as IBD and obesity) in relation to gut microbiota dysbiosis. When testing or developing new products, food and veterinary industries need to consider these multi-faceted aspects of canine digestion and would benefit from relevant in vitro gut models for in-depth mechanistic studies as an alternative to in vivo assays.

Up to now, only a restricted number of in vitro models has been developed to simulate the canine upper or lower digestive tract. These devices show various levels of complexity, i.e., from static mono-compartmental to dynamic multi-compartmental models. The in vitro parameters have not yet been fully
Tab. 2: Application studies of the currently available canine in vitro gut models

| References | Applications | Aim of the study |
|------------|--------------|------------------|
| **Upper GIT models** | | |
| Smeets-Peeters et al., 1999 | ✓ ✓ | Effects of small intestinal transit time on protein digestibility and calcium availability from canned dog food |
| Hervera et al., 2007 | ✓ | In vitro percentage of organic matter disappearance used as a predictor of apparent organic matter and energy digestibility of extruded dog food |
| van Zelst et al., 2015 | ✓ | Identification of dietary factors that affect selenium accessibility in commercial petfood |
| Lee et al., 2017 | ✓ | Evaluation of in vitro dissolution performance of five formulations of an acidic BCS Class II compound |
| Penazzi et al., 2021 | ✓ | Effect of supplementation with black soldier fly (Hermetia illucens) larvae meal in extruded dog food on in vivo and in vitro digestibility |
| Kim et al., 2021 | ✓ | Effect of thermal processing on ileal digestibility of dry matter and crude protein from raw chicken meat |
| **Lower GIT models** | | |
| Sunvold et al., 1995b | ✓ | Effects of cellulose, beet pulp, citrus pulp, and citrus pectin on microbiota fermentation (organic matter disappearance, SCFA, and lactate) |
| Sunvold et al. 1995a | ✓ | In vitro fermentation of selected fibrous substrates: influence of diet composition on substrate organic matter disappearance and SCFA production |
| Sunvold et al., 1995c | ✓ | In vitro fermentation of selected fiber sources and metabolism of fiber-supplemented diets |
| Swanson et al., 2001 | ✓ | Fermentation of vegetable and fruit fiber sources compared to fiber standards following SCFA production, organic matter disappearance, and gas production |
| Tzortzis et al., 2004 | ✓ | Fermentation properties of galactooligosaccharides synthesized by α-galactosidase from Lactobacillus reuteri (SCFA) |
| Biagi et al., 2008 | ✓ | Effects of fiber sources on microbiota composition and activity (SCFA, ammonia, and gas) |
| Bosch et al., 2008 | ✓ | Fermentation kinetics of fibers from canine foods (gas and SCFA) |
| Cutrignelli et al., 2009 | ✓ | Impact of different carbohydrate sources on microbiota fermentation (gas, SCFA, BCFA, ammonia) |
| Ogué-Bon et al., 2010 (39°C) | ✓ ✓ | Evaluation of a symbiotic combination on probiotic growth and microbiota activity (SCFA) |
| Ogué-Bon et al., 2011 (39°C) | ✓ ✓ | Effects of rice bran combined with Lactobacillus acidophilus and Blidobacterium longum on microbiota activity (SCFA) |
| Panasevich et al., 2013 | ✓ | Characterization of potato fiber fermentability by canine microbiota (SCFA and BCFA) |
| Panasevich et al., 2015 | ✓ | Effect of soluble corn fibers on nitrogen-corrected true metabolizable energy and fermentability by microbiota (SCFA) |
| Vierbaum et al., 2019 | ✓ | Effects of Yucca schidigera powder and inulin on protein fermentation metabolites (SCFA, BCFA, phenols and indoles, biogenic amines, ammonia) |
| Donadelli et al., 2019 | ✓ | Effects of different fiber sources used in petfood on organic matter disappearance and SCFA/BCFA production |
| Oba et al., 2020 | ✓ | Potential fermentation and prebiotic effects of GNU100, an animal milk oligosaccharide biosimilar, on microbial communities and metabolite production (gas, SCFA, BCFA, lactate) |
| Van den Abbeele et al., 2020a | ✓ | Effects of yeast-derived formulation on microbial composition and activity (gas, SCFA, ammonium) |
| Traughber et al., 2020 | ✓ | Fermentability of legumes by microbiota and metabolites production (SCFA, BCFA and gas) |
| Duysburgh et al., 2020 | ✓ | Effect of fructooligosaccharide supplementation on microbial community composition and activity (SCFA, BCFA and ammonium) |
| Van den Abbeele et al., 2020b | ✓ | Effect of a S. cerevisiae-based product on microbiota composition and production of fermentation metabolites (SCFA, BCFA and lactate) |
adapted to *in vivo* canine digestion, and some of the models are not yet validated for their application, mainly due to a paucity of *in vivo* data. Therefore, many scientific and technical challenges must be overcome to optimize canine models to represent canine digestive physiology in order to realize their potential in food and pharma studies, e.g., by simulating specific digestive conditions associated with different dog sizes, breeds, or ages, under healthy or diseased conditions.

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Conflict of interest
The authors have no conflict of interest.