Department of Companion Animals and Horses
University of Veterinary Medicine Vienna

Division of Small Animal Internal Medicine
(Head: Univ.-Prof. Dr.med.vet. Iwan Burgener, PhD, Dipl.ECVIM-CA Dipl.ACVIM)

Canine Organoids for Gastrointestinal Disease Modelling

PhD thesis submitted for the fulfilment of the requirements for the degree of

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Georg Csukovich, MSc

University of Veterinary Medicine Vienna

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PhD Advisory Committee

First Supervisor:
Univ.-Prof. Dr.med.vet. Iwan Burgener, PhD, Dipl.ECVIM-CA Dipl.ACVIM
University of Veterinary Medicine (Vetmeduni), Vienna
Austria

Second Supervisor:
Bon-Kyoung Koo, PhD
Center for Genome Engineering, IBS, Daejeon
South Korea

Third Supervisor:
Dipl.-Ing ^{in} Dr ^{in} nat.techn. Priv.-Doz ^{in} Sabine Brandt
University of Veterinary Medicine (Vetmeduni), Vienna
Austria

Declaration

I hereby declare that the work included in this thesis, entitled “Canine Organoids for Gastrointestinal Disease Modelling”, was performed following the rules of Good Scientific Practice in all aspects. In addition, I certify that this work has not been submitted to other academic institutions for the fulfilment of any other degree or qualification.

Georg Csukovich

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Summary

The aim of this PhD thesis was to explore the potential of canine intestinal organoids as a meaningful three-dimensional disease modelling approach that is superior compared to classical two-dimensional cell culture models. We successfully established a model, which can be used to evaluate the effects of bacterial toxins on the canine intestinal epithelium. We used basal-out and apical-out organoids to analyse both the effect on the basolateral as well as the apical cell surface and found surprising differences.

Toxins of *Clostridioides difficile* did not only show different effects depending on the exposed cell surface, but also depending on the intestinal section of which the organoids were derived from. Apart from toxin effects, the effects of controlled polarity reversal to make the apical cell surface accessible were analysed. We found that apical-out organoids stop proliferating and present higher apoptosis rates compared to basal-out controls. Furthermore, we explored the potential of organoids derived from either healthy donors or dogs diagnosed with inflammatory bowel disease to cope with reactive oxygen species. Chronic intestinal inflammation leads to excessive production of reactive oxygen species *in vivo*. To comprehensively model inflammation in organoids, the presence of immune cells would be inevitable. Despite this, we did find certain differences between organoids derived from intestinally healthy and IBD patients. Unexpectedly, we observed that healthy organoids seem to be slightly more sensitive to reactive oxygen species.

Intestinal organoids can help to reduce the number of animals used for research purposes according to the 3R and can be used for more reliable drug discovery approaches and disease modelling *in vitro* in the future.

Zusammenfassung

Das Ziel dieser PhD-Arbeit war es, das Potential von caninen intestinalen Organoiden als dreidimensionales Krankheitsmodell zu untersuchen, das diverse Vorteile gegenüber klassischen zweidimensionalen Zellkulturmodellen bietet. Wir konnten erfolgreich ein Modell entwickeln, mit dem die Effekte bakterieller Toxine auf das Darmepithel von Hunden untersucht werden können. Hierzu wurden sogenannte basal-out und apical-out Organoide verwendet, um zu untersuchen, welche Unterschiede davon abhängig bestehen, welche Seite des Epithels mit den Toxinen in Berührung kommt.

Wir konnten zeigen, dass das canine Darmepithel unterschiedlich auf Toxine des Bakteriums *Clostridioides difficile* reagiert, wenn unterschiedliche Zelloberflächen mit den Toxinen in Berührung kommen. Zusätzlich zeigen diese Toxine unterschiedliche Effekte auf Organoide aus verschiedenen Abschnitten des Hundedarms. Bei der Verwendung von apical-out Organoiden konnte gezeigt werden, dass diese, im Gegensatz zu herkömmlichen basal-out Organoiden, eine verringerte Proliferationsrate aufweisen. Jedoch sind sie nötig, um die apikale Zelloberfläche Toxinen auszusetzen. Des Weiteren erforschten wir das Potential intestinaler Organoide, reaktive Sauerstoffspezies zu eliminieren, da chronisch entzündliche Darmkrankheiten zu vermehrter Bildung reaktiver Sauerstoffspezies im Darm führen und so das Gewebe schädigen können. Um ein Entzündungsgeschehen in Organoiden vielversprechend zu modellieren ist die Präsenz von Immunzellen unabdingbar. Trotz deren Abwesenheit konnten wir bestimmte Unterschiede zwischen Organoiden von darmgesunden Hunden und jenen von IBD-Patienten nachweisen. Entgegen den Erwartungen zeigten wir, dass Organoide von gesunden Hunden empfindlicher auf reaktive Sauerstoffspezies reagieren.

Intestinale Organoide tragen einen wesentlichen Beitrag dazu bei, Tierversuche und die Anzahl der verwendeten Tiere im Sinne der 3R zu reduzieren. Weiters sind sie ein sehr nützliches Modell für die zukünftige Entwicklung von Medikamenten und das Modellieren bestimmter Krankheitsprozesse *in vitro*.

Introduction

Animals in research

It is without doubt that animal models have greatly advanced biomedical research over decades and led to several breakthroughs. Without the use of animals, virtually none of the drugs we use today would exist. By using animal models for modern research, investigators can gain insight into fundamental processes of both physiological and pathophysiological mechanisms within human and animal bodies. Since many of these processes rely on highly conserved signalling mechanisms, many findings can be extrapolated to humans. Thus, research on animals does not only contribute to basic research and animal health, but also to human health as the generated knowledge is key to develop new therapeutic approaches. The use of animals for research, however, is coupled to strict regulations that provide guidelines for the use of animals and their welfare[1,2].

Despite the advantage of using animal models, there is one big pitfall: the huge translational gap between basic research and the successful development of new therapies, sometimes referred to as the "valley of death"[3,4]. Too many potential drug candidates fail to be translated into efficacious drugs that end up in clinical use. Some of these failures can be explained by the very low genetic heterogeneity of mouse models, which are by far the most used animal models in today's research. Compared to humans or other animals, which are genetically very diverse, inbred mouse models sometimes show entirely different outcomes due to (epi-)genetic and/or metabolic differences between the organisms[5,6]. To complement classical research methods that rely on animal models, the U.S. Food and Drug Administration (FDA) has passed its modernization act 2.0 in 2022. This enables researchers in the United States of America to jump ahead and skip animal models before entering clinical trials on humans with newly developed drugs. Instead, the newly established *in vitro* approaches of organoid cultures and microfluidic systems (e.g., organ-on-chip) may be used for toxicologic assessment of novel drug candidates[7]. This opens up new possibilities for researchers to make the process of drug development not only faster due to higher throughput, but also cheaper. Additionally, fewer animals are used for research, which is one of the most important tasks according to the 3R principle (i.e., Replace, Reduce, Refine)[8].

An important part of today's research is the focus on more personalised medicine but also the emphasis on One Health, the integration of human, animal and environmental health into one holistic approach and the concomitant representation of diversity. One noteworthy animal in this regard is man's best friend – the dog – living in the same environment as its owner. Dogs often develop similar diseases to humans and their physiology is much closer to that of humans compared to mice. Therefore, dogs are still used as laboratory animals for

preclinical toxicological studies[9]. However, much less is known about the canine intestine compared to that of humans and mice. Thus, it is important to gain more insight into the dog's gastrointestinal system and set up new models such as organoids for the development of new therapies.

The intestinal tract of mammals

The mammalian intestine can be divided into the small and large intestine. The small intestine can be further subdivided into duodenum, jejunum and ileum, and the large intestine into the caecum, the colon and the rectum. While every part has its own specific function in regard to digestion and absorption of nutrients (e.g., in the duodenum, food coming from the stomach mixes with bile fluids and digestive enzymes from the pancreas), a common function for all the intestinal compartments is the maintenance of a functional barrier between the submucosal tissue and the lumen of the intestine. If the barrier integrity is damaged from either side, for instance by inflammation or physical injuries, microorganisms can translocate into the tissue and blood stream and cause systemic infections potentially leading to the death of the patient. However, the constantly abundant bacteria, viruses and fungi within the intestines are a challenge for the body even in physiologic settings, as commensal bacteria have to be tolerated while possible pathogenic microorganisms have to be eradicated as soon as possible or at least contained to low levels[10]. As described above, research in this field has relied on animal models for a long time and is only slowly shifting to more advanced *in vitro* approaches.

Organoids

A crucial development for more near-nature *in vitro* research has been the establishment of organoid culture systems in 2009[11]. Intestinal organoids allow the culture and expansion of the intestinal epithelium from either adult stem cells, embryonic stem cells or induced pluripotent stem cells in three dimensions (3D). This approach has many advantages: amongst others, the more complex micro-architecture of 3D organoid cultures compared to common 2D cell cultures allows a better recapitulation of the tissue *in vitro* to study the interaction between the intestinal epithelium of the host and different microorganisms[12].

In general, the intestines of all mammals are very similar in gross anatomy and function. However, as mentioned above, many drugs showing promising effects in mice fail to reproduce these effects or are even detrimental in humans, although many characteristics of the murine and human intestine seem to overlap. For example, the leucine-rich repeat-containing G-protein coupled receptor 5 (*LGR5*) represents an intestinal stem cell marker in both species. Yet, there are many functional pathways that substantially differ[13]. To cite a few, even though toll-like receptor 4 (*Tlr4*) is expressed in the colon of mice and humans, it appears to be non-functional in the latter species. Sequencing data revealed that a number of required accessory

proteins are not expressed in the human colon and thus prevent this organ from reacting to lipopolysaccharides, a key component of Gram-negative bacteria[14]. Another main difference is that mice are herbivores, while humans as well as dogs are omnivores, which has enormous effects on the microbial composition within the intestine[15,16]. One characteristic of the canine intestine is that it seems to lack Paneth cells[17,18]. Paneth cells are interspersed within intestinal stem cells in the intestinal crypts of many mammal species and produce vast amounts of antimicrobial peptides and essential factors for the maintenance of the stem cell niche[19,20]. However, they are only found in the small intestine, while in the large intestine so-called "Paneth-like cells" take over their function[21,22]. It is tempting to speculate that the canine intestine likewise adheres to this rule, especially because it harbours all other main cell types (enterocytes, stem cells, goblet cells and enteroendocrine cells)[23,24].

Intestinal disorders in dogs

Many canine patients suffer from gastrointestinal discomforts, which is reflected by the fact that around every 6th dog at the veterinary clinic is a diarrhoea patient[25,26]. The diseases leading to diarrhoeal incidences are manifold and range from bacterial (e.g., *Campylobacter spp.*)[27], parasitic (e.g., *Giardia spp.*)[28] or viral infections (e.g., canine parvovirus, CPV)[29], over food allergies[30] to more complex and possibly multifactorial syndromes such as inflammatory bowel disease (IBD)[31,32], acute haemorrhagic diarrhoea syndrome (AHDS)[33] and protein-losing enteropathy (PLE)[34]. What IBD, AHDS and PLE have in common is that their underlying pathomechanisms are not yet fully understood. While IBD seems to have many different contributing factors such as a genetic predisposition and diet[35–37], AHDS appears to be caused by an infection with *Clostridium perfringens*. However, this is not yet fully verified as a manifestation of *C. perfringens* may also be the result of AHDS and not the underlying cause[33]. In the search for meaningful *in vitro* models for these diseases, organoids may represent a valuable tool. Adult stem cells can be isolated from healthy donor patients as well as from patients affected by different diseases. However, disease modelling in intestinal organoids is still very complex.

One factor suspected to have an imperative role in IBD are reactive oxygen species (ROS)[38,39]. ROS have essential physiologic functions as signalling molecules in several different signalling cascades. However, a dysregulated redox balance can lead to the oxidation of lipids and proteins, and damage cells[40]. The main molecule involved in the elimination of potentially harmful levels of ROS is glutathione, which can be found in its reduced form (GSH) and its oxidised form (GSSG)[41,42]. Upon excessive production of ROS, the physiologic balance of GSH/GSSG in IBD may be shifted due to the chronic inflammation leading to excessive ROS production[38,39].

Despite IBD being characterised by severe diarrhoea in dogs and humans, both species seem to deal with it differently, and activate specific compensatory mechanisms. Humans seem to lose more sodium leading to hyponatremia[43], while canine patients develop hypokalemia[44]. These differences might be caused by a distinct activation of the renin-angiotensin-aldosterone-system (RAAS) that differs between dogs and humans, and leads to the specific loss of certain electrolytes. Species-specific adaptive activation of RAAS might represent a therapeutic target in the future treatment of IBD. This approach can be well investigated in intestinal organoids in the future as they only consist of one layer of epithelial cells. This in turn allows the analysis of electrolyte transport and activation of the immune system after application of RAAS inhibitors[45].

Disease modelling approaches using organoids

A common problem arising when trying to model diseases using organoids is that cells may be polarised as observed for example in lung and intestinal organoids. In this case, the apical cell surface is hidden as it faces the lumen of the organoid. However, it may be crucial to have access to this surface since this is the one exposed to extrinsic factors *in vivo*, i.e., air or luminal contents of the digestive tract. To gain access to the apical cell surface, researchers have developed different approaches. These include the physical fragmentation of organoids, microinjection, the generation of organoid-derived monolayers and apical-out organoids. Despite these advances aiding the development of *in vitro* disease models, each method also has its specific drawbacks. The advantages and disadvantages have been previously reviewed in more detail[12,46].

Ethical considerations, as well as the limitations of animal models - notably rodent systems - to authentically recapitulate human disease and drug efficacy, call for the establishment of potent *in vitro* models. With the advent of 3D miniature organs termed organoids, it is possible to dissect various physiological and pathobiological mechanisms in accordance to the principle of the 3R.

Dogs share the same environment with humans and develop similar diseases. This also applies to gastrointestinal disorders. As a consequence, canine intestinal organoids represent a promising research tool to study intestinal diseases for the benefit of dogs and humans. However, this *in vitro* model is still in its infancy and requires in-depth characterisation to uncover its merits and limitations. This work aimed at providing a relevant contribution to this field.

Research Questions and Aims

The high anatomical and physiological similarities between the canine and the human digestive tract point to canine intestinal organoids constituting a promising *in vitro* model for the study of physiological and pathobiological conditions in both species. The overall aim of the herein presented research work was to establish canine intestinal organoids and characterise this 3D-model to address this theory.

Research Questions:

I. Do canine intestinal organoids authentically recapitulate intestinal barrier function and present a model to analyse the cytotoxicity of bacterial toxins?

To answer this question, canine intestinal organoids established for this purpose were exposed to different concentrations of *Clostridioides difficile* toxins, and resulting effects were thoroughly analysed. This study also allowed the establishment of several technical procedures contributing to further improvement of organoid technology.

II. Are apical-out canine intestinal organoids an appropriate solution to make the apical cell surface accessible?

To answer this question, apical-out intestinal organoids were generated and assessed in terms of proliferation and cell death.

III. Do intestinal organoids derived from canine IBD patients reflect characteristics of disease?

To answer this question, intestinal organoids were established from healthy donor dogs as well as dogs confirmedly suffering from inflammatory bowel disease. Then these organoids were used to comparatively analyse differences between health and disease with a special focus put on reactive oxygen species.

Publications

Publication I

The World of Organoids: Gastrointestinal Disease Modelling in the Age of 3R and One Health with Specific Relevance to Dogs and Cats

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Review

The World of Organoids: Gastrointestinal Disease Modelling in the Age of 3R and One Health with Specific Relevance to Dogs and Cats

Georg Csukovich *, Barbara Pratscher and Iwan Anton Burgener

Small Animal Internal Medicine, Vetmeduni, 1210 Vienna, Austria
* Correspondence: georg.csukovich@vetmeduni.ac.at

Simple Summary: One Health is a concept that describes the interplay between humans, animals, and the environment. This interaction is becoming increasingly important as researchers try to address it in a laboratory setting. This has led to the development of new and highly sophisticated research methods paving the way for animal-free research methods. Within this context, the development of mini-organs, so-called 'organoids', is of great significance. These organoids represent entire organs on a laboratory scale and can be established from stem cells. Subsequently, organoids are used to model certain disease states and the interaction of the host with specific harmful organisms. With this review, we give an overview of what disease modelling approaches have already been carried out in the past and where the field might be heading in the future. In the context of One Health, we consider animal models whenever possible, putting a focus on gastrointestinal diseases.

Abstract: One Health describes the importance of considering humans, animals, and the environment in health research. One Health and the 3R concept, i.e., the replacement, reduction, and refinement of animal experimentation, shape today's research more and more. The development of organoids from many different organs and animals led to the development of highly sophisticated model systems trying to replace animal experiments. Organoids may be used for disease modelling in various ways elucidating the manifold host–pathogen interactions. This review provides an overview of disease modelling approaches using organoids of different kinds with a special focus on animal organoids and gastrointestinal diseases. We also provide an outlook on how the research field of organoids might develop in the coming years and what opportunities organoids hold for in-depth disease modelling and therapeutic interventions.

Keywords: one health; 3R; organoids

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1. Introduction

The concept of 'One Health' has become increasingly important over the last few years. In contrast to specific scientific disciplines such as human medicine, veterinary medicine, or environmental sciences, One Health is an approach taking more than one of these factors into account [1]. This also includes the political implications of the surveillance of diseases and the prevention thereof and not only scientific research on pathogens and their interaction with host organisms. COVID-19, for instance, is a very prominent and current example. SARS-CoV-2 infections are diagnosed in humans as well as many different species of animals [2], and viral particles can be found in wastewater [3]. Referring to the global problem of SARS-CoV-2 infections for humans, animals, and environmental contamination, one can appreciate the importance of One Health in a global context. In vitro research methods can neither fully model these complex interactions nor entirely replace animal experimentation but are of great importance to reduce the need for animals in today's research.

More than 60 years ago, researchers were looking for ways to reduce pain and distress for laboratory animals. In 1959, Russel and Burch first explained the principle of the three

Rs (3R), i.e., Replacement, Reduction, and Refinement of animal experimentation [4]. Since then, the 3R principle has been implicitly included in animal welfare laws in the United States of America [5] as well as in Europe [6], and researchers are obliged to consider these laws when planning and carrying out experiments involving live animals. Recently, the Max Planck Society for the Advancement of Science e.V. has taken the next step and expanded the classic 3R principle to the 4R principle, also taking 'Responsibility' into account. Researchers commit to using their knowledge in order to further promote animal welfare by engaging in public discourse, improving the social structure of housed experimental animals and expanding the knowledge about the experience of pain, intelligence and consciousness in animals [7]. Animal experimentation is not limited to laboratory mice and rats but also includes other vertebrates such as fish, rabbits, cats, dogs, pigs, and others.

Dogs, for instance, are mainly used for toxicology studies. In the European Union, the number of dogs used for any scientific purpose for the first time accounted for 17.711 in 2018, adding up to 25.717, including dogs already in use [8]. By far, the number is exceeded by the United States, with them having used 58.511 dogs for research in 2019 [9]. These numbers clearly demonstrate the need for replacing animal experimentation with meaningful in vitro or in silico methods according to the 3Rs (and 4R concept) principle or at least reducing them to an absolute minimum. This leads to the improvement of the state-of-the-art in vitro methods to reduce the animal numbers used for research and minimise the pain experienced during experiments. These comprise but are not limited to the use of classical cell culture models as well as more advanced methods such as three-dimensional model systems such as tumour spheroids, organoids, organ-on-a-chip technologies, or computer-based models such as prediction methods based on artificial intelligence (AI), as previously applied to diabetes [10], cardiovascular disease [11], tuberculosis [12], and drug discovery [13]. Spheroids pose a model of compact three-dimensional cell aggregates consisting of cells at different states, e.g., proliferating, hypoxic, and quiescent, which are generated on non-adherent surfaces. These do not necessarily represent complex organ architecture on a miniature scale [14]. On the other hand, organoids are three-dimensional models of organ systems reflecting organ microanatomy. Due to their stem-cell-originating nature, organoids are usually indefinitely expandable [15,16]. Modelling different organ systems of various animals will help to replace animal experimentation in accordance with the 3Rs (and 4R concept) principle. This leads to an improved understanding of the biological principles in a broader context, as humans and different species of animals may react differently to various irritants (Figure 1). In-depth knowledge of diverse species and their organs is pivotal for research in a One Health context, taking humans, animals, and the environment into account. Thus, this review deals with the importance of organoids for today's research and provides an overview of different methods for disease modelling and highlights the limitations of organoids, differences between humans and animals and the possible future applications of organoid-based in vitro research.

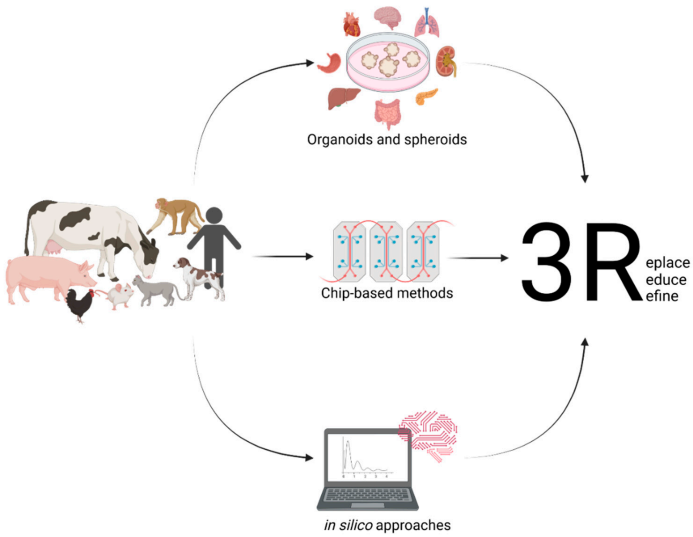

Figure 1. Setting up useful in vitro models from different animals and their various organs and the use of in silico modelling will help to replace the need for animal experimentation.

2. The Importance of Organoids for One Health

The establishment of meaningful in vitro systems to model complex diseases is very important. At the moment, the world is progressing from using classical cell culture models to more sophisticated three-dimensional models to investigate the effects of commensal or pathogenic organisms on certain cells/organs of humans, companion animals as well as farm animals. In humans, many different organs are available as organoid systems, e.g., the brain [17], retina [18], salivary gland [19], thyroid [20], lung [21], blood vessels [22] and the heart [23], mammary gland [24], stomach [25], liver [26], kidney [27], pancreas [28], intestine [29,30], fallopian tube [31], endometrium [32], bladder [33] and the prostate [34]. Many of these can be adapted to cancer organoid cultures, and some have been translated to animal organoid models. There are also very sophisticated air–liquid interface models of patient-derived cancer organoids. One of these models even includes the complex tumour microenvironment with immune cells, making it a very attractive and complex model [35]. A lot of work has been undertaken on organoids from companion animals, including the canine and feline intestine [36–39], the canine and feline liver [40,41], and canine kidney [42], bladder cancer [43], prostate cancer [44], skin [45], and thyroid tissue [46]. These companion animal models are further complemented by organoids derived from farm animals. Among them are primarily intestinal organoids from several species such as pigs, cattle, sheep, horses, and chickens [47], which have recently been reviewed more in-depth elsewhere [48]. In this context, organoids may develop towards a central model connecting the three cornerstones of the One Health concept regarding the physiological and pathophysiological interrelation of human, animal, and environmental health.

Gastrointestinal (GI) diseases do not only affect humans but also constitute a major threat to farm and companion animals and are associated with high costs to healthcare systems and animal owners. Just as in humans, conceivably lethal GI diseases also affect animals. Enteropathogenic viruses and bacteria are frequently responsible for the initiation or further impairment of GI afflictions [49–51]. There are numerous examples of the pathogenic organisms involved in the development of health problems in humans as well as animals. Several reviews have recently highlighted the importance of One Health

approaches putting surveillance, monitoring, and treatment options in a broader context compared to studies investigating only one aspect of potentially zoonotic pathogens. Due to the fact that some pathogens can survive in the environment or animal products consumed by humans, the transmission routes should be examined more closely.

Especially, enteric pathogens are a major threat in a zoonotic One Health context, including parasites such as helminths [52], *Giardia duodenalis*, *Blastocystis*, and *Cryptosporidium* spp. [53], as well as bacteria such as *Clostridioides difficile* (*C. difficile*) [54–56], *Bacillus cereus* sensu lato [57], and *Salmonella* [58], which all affect humans as well as animals. Particularly, the widespread *C. difficile* has been well studied, with the faeces of animals contaminating soil and water with *C. difficile* spores, leading to the spread of the disease to other animals. Alike, the spores from infected humans show up in wastewater, highlighting the importance of *C. difficile* for the environment as well as human and veterinary medicine [59]. This is complemented by reports that animals may be important asymptomatic carriers of toxigenic *C. difficile* [60,61]. Additionally, the co-clustering of isolates from cattle and dogs with isolates from human newborns has been documented, indicating the opportunity for inter-species transmission, either directly or indirectly, via contaminated environments [62]. How food intake shapes gut health has also been reviewed many times. Especially, fermented foods have received a lot of attention because of their ability to substantially change gut microbiota composition and therefore influence physiologic as well as pathologic processes [63].

In recent years, intestinal organoids have become increasingly important in research. They do not only represent a more complex system than classical two-dimensional cell cultures, but their three-dimensional nature also allows for the long-term maintenance and differentiation of many different cell types within one dish. Despite their complexity, intestinal organoids bear the advantage of only consisting of one layer of epithelial cells, thus putting the intestinal epithelial lining at the heart of the research. Intestinal organoids are not only valuable models for the investigation of complex diseases, such as IBD [64,65], but also represent a system which makes it possible to propagate pathogens in vitro, which previously could not be cultured, such as *Cryptosporidium* [66]. Beyond that, organoids even open up opportunities for precision medicine, as any effects can be studied in a patient-specific manner. Organoids can be the missing piece in the puzzle of performing research in a One Health context (Figure 2).

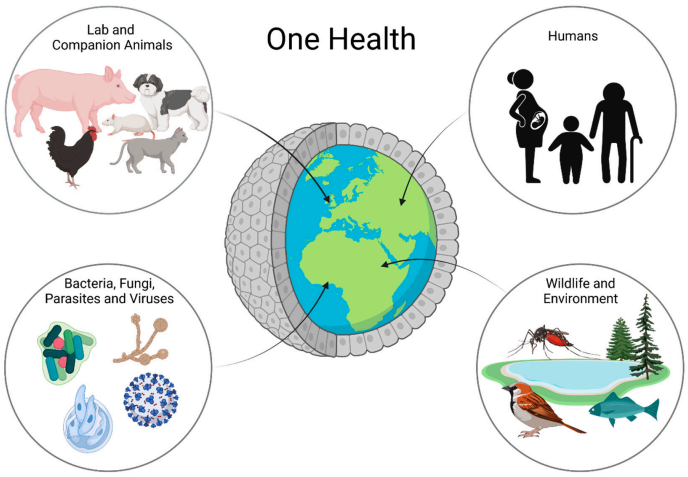

Figure 2. Organoids in One Health Research: Organoids are a possible way to work with all parts of One Health in one platform. Using organoids, one can learn about animal and human health and disease as well as interactions with the environment and bacteria, fungi, parasites, and even viruses.

3. Organoids Modelling the Intestinal Epithelium

The mammalian intestines consist of the small intestine, i.e., duodenum, jejunum and ileum, and the large intestine, i.e., caecum and colon. There are fundamental differences between the small and large intestines, ranging from distinctive cell types over different tissue architectures to different physiological functions as a whole [67,68]. While all sections of the intestine contain certain cell types, such as stem cells, enteroendocrine cells, and goblet cells, and other cell types are only present in specific parts. M-cells, for instance, are only present in the epithelium on top of immune follicles, the intraintestinal lymphatic tissue, also known as gut-associated lymphatic tissue (GALT). There they interact with microbial antigens on their apical cell surface and then present these antigens on the basolateral cell surface to immune cells, thereby initiating an immunologic response [69]. An even more prominent example is Paneth cells in intestinal crypts, where they are intermingled with stem cells and pose an indispensable part of the so-called stem cell niche. These Paneth cells can only be found in crypts of the small intestine but not the colonic epithelium [70]. In 2019, van Es et al. reported that the depletion of Paneth cells from mouse intestines is leading to the adaptation and migration of enteroendocrine cells as well as tuft cells into the crypts in order to supply the stem cell niche with essential growth factors. This may be an alternative also for species in which the existence of Paneth cells has not yet been documented, as is the case for dogs and cats [71,72].

When culturing adult-stem-cell-derived organoids, many of the aforementioned characteristics can be recapitulated in vitro, starting from a single stem cell [30]. Usually, intestinal organoids represent a polarised epithelium of several different cell types, with the basolateral cell surface presented to the outside and the microvilli-bearing apical cell surface oriented towards the lumen side [73]. In 2021, a report highlighted the importance of using organoids from different organisms when it comes to drug toxicity and not simply extrapolating existing results to other species. Anti-cancer drugs have been tested in pig, monkey, and human intestinal organoids and demonstrated differing sensitivities between all three species [74]. Interestingly, Rosselot et al. showed that intestinal organoids even follow a circadian rhythm and that mouse and human organoids react differently to *C. difficile* toxin B depending on their circadian phase, which introduces a whole new level of complexity [75].

Standard intestinal organoids can also be used to model inflammatory bowel diseases. One study shows that human Crohn's Disease (CD) patients have increased interleukin-28A (IL-28A) plasma levels, and organoids were used to model this system and its effects. When they applied IL-28A to human intestinal organoids, their barrier integrity was disrupted in a JAK-STAT-pathway-dependent manner, possibly modelling an important process in CD pathogenesis, as an impaired intestinal barrier is one major aspect of CD. In veterinary science, organoids recently helped to overcome the problem of not being able to propagate serotype I feline coronaviruses (FCoVs). Making this possible now allows for an in-depth functional analysis of the pathogenesis of feline infectious peritonitis and possible treatments [39].

However, to study gastrointestinal diseases using intestinal organoids, many applications depend on the ability to gain access to the apical cell surface on the inside of the organoids, which poses a major hurdle in disease modelling. In order to make the apical cell surface more accessible, several methods have been developed over the last few years:

3.1. Microinjection

Microinjection is a rather laborious method to gain access to the apical cell surface. It may require a lot of training of the experimenter and is not feasible for large-scale screening approaches. However, it is a well suitable method for studying host–microbe interactions. Hill et al. have established a microinjection approach using human intestinal organoids to study the host–microbe interactions of non-pathogenic *Escherichia coli* (*E. coli*). Microinjected *E. coli* were able to colonise the intestinal epithelium and establish a stable interaction between microbes and the host cells. This interaction was characterised by pronounced changes in the transcriptomic profile, epithelial proliferation, improved barrier integrity,

and many more physiologically relevant adaptations [76]. This study was fundamental to a recent follow-up study by Abuaita and colleagues. They used different Salmonella serovars to find out whether known in vivo immune reactions could be modelled in vitro using intestinal organoids. As expected, different serovars led to different levels of immune responses, with the *Salmonella enterica* serovar Typhi infection leading to the weakest response, which is in accordance with its need to induce a weak host response in order to systemically infect the host. Additionally, many transcriptomic alterations induced by the three tested serovars were noticeable, which again highlights the usefulness of organoids for exploring new signalling pathways targetable in disease treatment and prevention [77]. This method cannot only be used to study bacteria–host interactions but is also applicable for the investigation of small parasitic organisms with the host epithelium, as shown by a model using *Cryptosporidium parvum* microinjection for infection and subsequent oocyst harvest [78]. However, as shown elegantly by the microinjection of *Lactobacilli*, when using pluripotent stem cell-derived intestinal organoids, one has to be cautious since the maturation stage of organoids can be increased using different culture media and can drastically influence the success of *Lactobacillus* colonisation of the organoid epithelium [79].

3.2. Apical-Out Organoids

Another useful method to gain access to the apical surface of the epithelium whilst not disrupting the three-dimensional structure of the organoids is the generation of so-called "apical-out organoids". This method was first described in 2019 in a human enteroid model that appealingly demonstrated the importance of turning organoids inside out, providing the example of two different infection models. The rather simple method relies solely on the fact that organoids reverse their polarity once they are cultured floating in the culture medium without being embedded in an extracellular matrix [73]. A slightly modified version of this method was recently provided as a step-by-step protocol [80]. While *Salmonella* were used again to show their potential to infect the apical cell surface, organoids needed to be in their standard basal-out configuration to be infected by *Listeria monocytogenes* [73]. This study also used insights from research from 1994, which already used a three-dimensional model of canine cells (Madin–Darby canine kidney cells), which indicated an inherent function for beta 1 integrin in cell polarity [81]. Co et al. then demonstrated the importance of beta 1 integrin also for enteroid polarity, as applying a beta 1 integrin blocking antibody showed the same effects as the removal of extracellular matrix and led to organoid polarity reversal [73]. This is just one of many examples where first indications from animal cells give rise to novel approaches in more frequently used model systems, clearly highlighting the importance of interdisciplinary research.

Interestingly, intestinal apical-out organoids have been explored intensely in different animal species but not so much in mouse and human organoids over the last few years. A study using pig organoids analysed their potential to form apical-out organoids and set up functional readouts as fatty acid uptake and barrier integrity analyses [82]. There are several groups working on apical-out organoids for disease modelling in different contexts. For instance, porcine apical-out organoids were employed as an in vitro system to analyse the possibility of infecting organoids with the swine-enteric transmissible gastroenteritis virus (TGEV) and their immune response elicited by this virus [83]. Apart from using sheep gastrointestinal basal-out organoids for investigating the host–parasite interaction of *Teladorsagia circumcincta* with the epithelium, ovine apical-out organoids have also been tested in co-culture with *Salmonella enterica* serovar Typhimurium [84]. Meanwhile, chicken apical-out organoids have also proven to be a valuable tool for analysing different host–pathogen interactions. The protozoan *Eimeria tenella* can infect avian apical-out organoids just as well as the influenza A virus. This study also used *Salmonella enterica* as a bacterial example for avian gastrointestinal infection [85]. This is probably due to *Salmonella* being a facultative anaerobic bacterium relevant for the intestinal epithelium, as using obligate anaerobes such as *Fusobacterium* or *Clostridia* with low oxygen tolerance would not be compatible with the cultivation of apical-out organoids. However, especially these bacterial

genera might be of interest for research in the future as they are frequently implicated in gastrointestinal diseases such as colon cancer and ulcerated regions in the human intestine [86] as well as in acute haemorrhagic diarrhoea syndrome (AHDS) in dogs [87]. Specifically, *Clostridia* might bear the risk of being a zoonotic bacterium in the context of One Health, as already outlined above. However, some *Clostridia*, such as *Clostridium hiranonis*, may also exert positive effects on gastrointestinal health. It was reduced in the dysbiosis index of dogs with chronic enteropathy in general [88] and, more specifically, in dogs with IBD [89]. *Clostridium hiranonis* possesses the ability to metabolise bile acids, and the dysregulation of bile acids has been associated with human IBD [90] and in dog enteropathies [91,92], which could potentially be modelled in vitro in the future.

3.3. Organoid-Derived Monolayers

Since handling organoids can be tedious and many standard assays are not adapted to three-dimensional structures, great efforts have been made during the last few years to find a way to reduce the complexity of the organoid system while simultaneously maintaining as many advantages of the organoids as possible. One way to do so is the use of organoid-derived monolayers (ODMs), which serve as a model of an intact intestinal barrier [93]. Classical two-dimensional in vitro models such as the Caco-2 cell system are most frequently used for drug screening and basic research. However, Caco-2 cells are derived from cancer cells and lack some possibly important epithelial enzymes and transporters [94]. Organoid-derived monolayers can be analysed, such as standard two-dimensional cell cultures, and have the advantage that they consist of several different cell types. Additionally, you can prepare them from whatever species you are able to culture organoids from. ODMs can thus be of great help in exploring transepithelial transport of nutrients, damage to the epithelial barrier integrity or similar approaches.

Human intestinal organoid-derived monolayers have been previously used as a model for pharmacokinetics and toxicology. In two-dimensional monolayers, the drug-metabolising enzyme CYP3A4 and several transporters were upregulated compared to Caco-2 cells and intestinal epithelial cells derived from induced pluripotent stem cells and resembled the adult duodenum more closely. These papers also showed the existence of all major differentiated cell types (enterocytes, enteroendocrine cells, goblet cells, and Paneth cells) in these monolayers, while stem cells decreased over time [95,96]. Another study demonstrated the ability of differentiated monolayers to actively transport ions (sodium, potassium, and chloride) and that the hormones serotonin and GLP-1 are produced by epithelial cells [97]. The functional transport of chloride ions has also been shown in porcine organoid-derived monolayers consisting of enterocytes, goblet cells and enteroendocrine cells [98]. Likewise, canine organoids have been used to create transwell-based ODMs that build up a functional barrier that can be used for dog gut research [99]. Aside from functional characteristics, ODMs have also been developed much further as co-culture models with bacteria. Mayorgas et al. used human ODMs as a proxy for the infection with invasive *E. coli* [100]. A slightly more complex system has been introduced by Sasaki et al. [101]. Here, ODMs are produced on transwell inserts. Once these monolayers reach confluence, the transwell chamber is sealed by a butyl rubber plug. This leads to an anaerobic apical chamber, while the bottom chamber, which is in contact with the basolateral cell surface, still has continuous access to oxygen. To test the so-called Intestinal Hemi-Anaerobic Co-culture System (iHACS), the apical chambers were challenged with four different anaerobic bacterial strains (*Bifidobacterium adolescentis*, *Bacteroides fragilis*, *Clostridium butyricum*, and *Akkermansia muciniphila*) and showed the possibility for bacterial survival and propagation over five days of co-culture. This complex example showcases the possibility of using monolayers for bacterial co-culture and possible invasion analyses or co-cultures with commensal bacteria.

4. Limitations

Despite offering outstanding new possibilities for research, e.g., in vitro analysis of physiologic processes, disease modelling and genetic manipulation, organoids also confront researchers with some difficulties and limitations. For example, imaging approaches are more difficult to carry out compared to classical 2D cell culture approaches due to the three-dimensional structure of organoids and the resulting thickness of the sample in whole-mount stainings. However, imaging technology is gradually becoming better, and as confocal laser scanning microscopy (CLSM) is available virtually everywhere, this problem is also becoming smaller. New imaging techniques, such as spinning disk confocal imaging, offer new possibilities, especially for live-cell imaging, as the imaging process itself becomes much faster than in classical CLSM [102]. To overcome the problem of imaging depth, several different tissue-clearing methods have been developed [103,104]. These protocols enable the optical clearing of whole organoids or in vitro 3D tissues for considerably improved clarity and easier imaging of whole-mount samples.

Another difficulty is the batch-to-batch variations of the conditioned media, media supplements, and inhibitors. As organoids require a complex mix of stimulatory and inhibitory components in the medium to simulate the stem cell niche and/or provide the right cues for cell differentiation, all these supplements need to be of high and standardised quality. Chemically synthesised molecules tend not to be a problem as they are of extremely high and pure quality and undergo the appropriate quality checks. However, many labs rely on self-produced conditioned media as supplements for organoid culture media. These conditioned media can substantially vary, depending on the production process, hence skewing the results and hindering reproducibility, even though a report shows that the conditioned media production appears to be reproducible from batch to batch across several different laboratories [105]. To overcome this problem, more cost-intensive, specially designed so-called "surrogate" proteins can be used at defined concentrations [106–108]. Another problem arising from organoid culture media is the variation of media composition between laboratories. While some laboratories still rely on the original culture media [29,30,36,47], certain media are available for driving organoid differentiation while simultaneously ensuring a certain level of stemness in the same dish in human and canine intestinal organoids [37,109] or promoting full differentiation, for example in liver organoids [41,110]. These differences require a highly transparent methodology to ensure reproducibility and highlight the need for standardisation, as outlined by Gabriel et al. [38].

Because organoids are a very complex 3D model, it can be hard to precisely identify the specific factors that provoke the observed changes. Organoids receive various cues from media components and also the extracellular matrix they are grown in that need to be integrated into a physiologic context within the organoid. Therefore, small deviations from standard parameters can provoke drastic changes in the organoids. Matrix proteins are a major part of this dilemma. Matrigel still is the most prominently used extracellular matrix for the cultivation of organoids. However, Matrigel and comparable alternatives are basement membrane extracts derived from Engelbreth–Holm–Swarm sarcomas from mice. Thus, using organoids for research is not necessarily reducing the need for animal experimentation, as large quantities of mice are needed to produce the required extracellular matrix. Additionally, since Matrigel is derived from animals, quality control is rather difficult, no standardised mixture of components is defined, and batch-to-batch variability can be problematic [111]. For the last few years, a lot of money has been invested to produce non-mammalian or even animal-free alternatives to Matrigel. These include but are not limited to peptide-based hydrogels [112], a highly tuneable polysaccharide-based synthetic hydrogel [113], plant-based nanofibrillar cellulose [114,115] and collagen derived from jellyfish [116]. Despite the availability of these mammalian-free matrices, many people have not adopted them in their labs because of time- and cost-intensive procedures.

5. Outlook

Organoids have one major advantage for future research, which is the opportunity to study diseases in a patient-specific manner. Organoids can be established from small biopsies of tissues and expanded in vitro for experimental needs. These can be used for patient-specific drug-screening approaches or the analysis of genetic risk factors for certain diseases [117,118]. However, increasing individuality inevitably leads to less standardised models. In future research, patient-specific models will always have to be analysed with reference to a specific benchmark, i.e., a standardised control sample. Companies such as HUB Organoids in the Netherlands are building large biobanks for human organoids where researchers can apply for licencing agreements in order to use specific healthy or diseased organoids for certain projects [119]. However, for animal research, no such biobank is available, most probably because the research community working with animal-derived organoids is only starting to develop and is still too small.

Organoids may also be used for therapeutic approaches in the future. Kruitwagen et al. showed hepatocyte transplantation in canines with the possibility of curing copper storage disease caused by a mutation of the copper metabolism-domain-containing 1 (COMMD1) gene. Liver organoids were established from COMMD1-deficient dogs, genetically modified to restore COMMD1 function and, subsequently, transplanted back into the dogs of origin. Despite the engraftment percentages being low, the transplanted cells were able to survive for more than two years after transplantation [41]. Sampaziotis et al. used cholangiocyte organoids for direct bile duct regeneration. Importantly, delivering organoids to regenerate damaged bile ducts was demonstrated in mice and humans. While live mice were injected with organoids, normothermic machine perfusion (NMP) was used for human studies, which allows for the physiological perfusion of organs ex vivo. This makes it much easier to control the environmental influences and analyse different parameters. Perfusing these livers with human cholangiocyte organoid cells led to successful engraftment in human bile ducts, demonstrating the proof of principle, that organoid transplantation is feasible in mice as well as humans in the future [120].

Another human/mouse study generated human islet-like organoids to pave the way for diabetes treatment via pancreas islet transplantation. Human induced pluripotent stem cells were differentiated to human islet-like organoids (HILOs) expressing insulin and subsequently transplanted into diabetic mice. These pancreatic island cells could re-establish glucose homeostasis and may be more effective than conventional glucose monitoring and insulin injections as island cells can take on multiple additional roles [121]. In 2020, Meran et al. used organoids from child patients with intestinal failure and expanded them in vitro. They subsequently seeded organoid cells on decellularised small and large intestinal matrices and transplanted these scaffolds into mouse kidney capsules or subcutaneous pockets. These grafts formed luminal structures after transplantation and demonstrated the possibility of re-populating decellularised scaffolds with in vitro expanded cells for transplantation [122]. Similarly, Sugimoto et al. grafted small intestinal organoids onto the surface of the colon. These grafts started to form villus structures and ameliorated the symptoms of small intestinal short bowel syndrome in rats by structurally replacing colon epithelium with small intestinal cells [123].

Scientists are making efforts worldwide to lift organoid technology to the next level, explore new model systems, and generate more meaningful and complex models that mimic in vivo physiology even closer. Recent improvements include organoids with increased complexity, as by Koike et al., who modelled endoderm organogenesis at the foregut–midgut boundary by differentiating human induced pluripotent stem cells. Using this model, they created organoids containing cells from the liver, bile ducts, pancreas, and duodenum organised in one single organoid [124].

Other approaches for combining several organs in one model system mostly go towards using organ-on-a-chip applications. Such a chip incorporates one or many microchannels to connect the chip with a capillary system. This allows for the injection of fluids in a controlled manner that also supports directed flow of a medium, as, for instance,

the intestine also experiences in vivo. Chip technologies can also be upgraded with microsensors and pose an extremely complex system [125]. The advances in organoid technology, microfabrication, cell engineering, and imaging technologies have led organ-on-a-chip to become an innovative technology capable of reproducing physiological cell behaviours in vitro [126]. However, the use of species other than mice and humans for chip-based technologies is very limited, with only two reports. The combination of multiple interconnected organ-on-a-chip systems in a single platform is now bringing this technology to the next level that aims to emulate an entire biological entity that is seldom limited to a single organ termed "body-on-a-chip" [127,128]. Despite these new advancements, there is still a lot to learn from organoids themselves and together with organoids, organ-on-a-chip technologies will take science a step further to replace animal experimentation. The comparison of in vitro organ models from various species will also guide new ways to explore the interconnection of humans, animals, and the environment in the context of One Health and help to explore new treatment strategies for various diseases.

6. Conclusions

Organoids are a promising tool for modern research. The continuous developments of new technologies, co-cultures, and organoid manipulation techniques lead to constant advancement in the field and open up new possibilities for treatments. Organoids of the liver, pancreas, stomach, and intestine are currently the in vitro method of choice for gastrointestinal research. Learning from mouse and human studies, many organoid systems have been adapted to other species. People are just beginning to explore these organoids and their differences from well-characterised models. Animal organoids pose a valuable in vitro method to model and study diseases, test environmental irritants on different organ systems of various species and develop new therapeutics. Keeping this in mind, organoids are becoming increasingly important in regard to the 3Rs (and 4R concept) and One Health research.

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Publication II

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Neutralising Effects of Different Antibodies on *Clostridioides difficile* Toxins TcdA and TcdB in a Translational Approach

Georg Csukovich [1], Nina Kramer [1], Barbara Pratscher [1], Ivana Gotic [2,†], Patricia Freund [1], Rainer Hahn [3], Gottfried Himmler [2], Sabine Brandt [4] and Iwan Anton Burgener [1,*]

[1] Small Animal Internal Medicine, Department for Companion Animals and Horses, Vetmeduni, 1210 Vienna, Austria
[2] The Antibody Lab GmbH, 1210 Vienna, Austria
[3] Department for Biotechnology, University of Natural Resources and Life Sciences Vienna, 1190 Vienna, Austria
[4] Research Group Oncology (RGO), Clinical Unit of Equine Surgery, Department for Companion Animals and Horses, Vetmeduni, 1210 Vienna, Austria
* Correspondence: iwan.burgener@vetmeduni.ac.at
† Current address: Alterras Therapeutics GmbH, 1030 Vienna, Austria.

Abstract: Given the high prevalence of intestinal disease in humans and animals, there is a strong need for clinically relevant models recapitulating gastrointestinal systems, ideally replacing in vivo models in accordance with the principles of the 3R. We established a canine organoid system and analysed the neutralising effects of recombinant versus natural antibodies on *Clostridioides difficile* toxins A and B in this in vitro system. Sulforhodamine B cytotoxicity assays in 2D and FITC-dextran barrier integrity assays on basal-out and apical-out organoids revealed that recombinant, but not natural antibodies, effectively neutralised *C. difficile* toxins. Our findings emphasise that canine intestinal organoids can be used to test different components and suggest that they can be further refined to also mirror complex interactions between the intestinal epithelium and other cells.

Keywords: organoids; *Clostridioides difficile*; sIgA; neutralisation; toxin

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1. Introduction

Functional gastrointestinal (GI) disorders constitute a major, potentially lethal, health problem in humans and animals. It is accepted today that the intestinal microbiome and secretory immune system have a crucial role in maintaining homeostasis between various genera of microorganisms, thereby protecting the GI tract from the detrimental effects of infectious agents [1–3]. Gastrointestinal disorders, including GI cancer, inflammatory bowel disease (IBD) and infectious GI diseases, have a major negative impact on human health and impose a high financial burden on healthcare systems. Infectious diarrheal disease is the second leading cause of death among young children, and GI cancer is responsible for about three million deaths per year worldwide [4].

Potentially lethal GI diseases also affect farm and companion animals, with enterotoxigenic bacteria and enteropathogenic viruses being frequently involved in disease onset and progression [5–7]. One important bacterium in this regard, *Clostridioides difficile* (*C. difficile*), formerly known as *Clostridium difficile*, is an anaerobic, Gram-positive bacterium with zoonotic potential, which is widespread all over the world. Faeces of infected animals contain *C. difficile* spores that contaminate soil and water, leading to the propagation of the infection to other animals and humans. Similarly, spores originating from affected humans end up in wastewater, which makes *C. difficile* a global issue for humans, animals and the environment in the context of "One Health" [8]. The importance of animals as symptomatic and asymptomatic carriers of *C. difficile* has been well documented in recent

times [9,10]. Although isolates of *C. difficile* commonly cluster based on host species, co-clustering of isolates from cows and dogs with those of paediatric human patients recently emphasised the possibility of interspecies transmission via direct contact or a contaminated environment [11].

In humans, it is estimated that breastfeeding would reduce the risk of dying from diarrhoea by twenty times in new-borns, as breast milk represents a rich reservoir of antimicrobial immunoglobulins (Igs) [12]. As with human neonates [13], the immune status of new-born dogs depends greatly on colostrum (and milk) ingestion, since canine neonates are usually agammaglobulinemic [14]. After the closure of the intestinal barrier, 90 to 95% of circulating Igs originate from the colostrum. Inadequate colostrum intake and suckling lead to a deficit in the transfer of passive immunity that is associated with considerably higher mortality and morbidity rates [15].

Secretory IgA (sIgA) is the predominant Ig protecting mucosal surfaces [16]. Polymeric sIgAs consist of two to four IgA monomers, which are linked by the joining (J) chain and a heavily glycosylated secretory component (SC) [17]. The SC protects the sIgA complex from proteolytic cleavage and binds microbial antigens and certain receptors via specific glycan structures [18]. sIgA can be purified in sufficient amounts from milk or whey of animals such as goats and cows and could potentially be used for the prevention of certain GI disorders [19–21]. In particular, glycosylation patterns of caprine and human sIgA show a high similarity [22]. Human colostrum-derived sIgA molecules were previously shown to inhibit the binding of *C. difficile* toxin A (TcdA) to intestinal membranes [18] and partly showed neutralising activity against toxin A and toxin B (TcdB) [23]. Goat milk-derived sIgA isolates from different breeds and lactation periods were investigated for their neutralising potential to TcdA as well as lipopolysaccharide from *Escherichia coli* (*E. coli)* and *Salmonella typhimurium*, the heat-sensitive toxin from *E. coli*, and proteoglycan from *Staphylococcus aureus*. Results obtained by immunoassays reflect a broad spectrum of toxin binding capacity of caprine sIgA with sample-specific variations [24].

Given the high prevalence of intestinal disease in humans and animals, there is a strong need for clinically relevant models of the GI system, since preclinical GI translational research still relies entirely on animals, more specifically, on rodent models that are of limited physiological relevance. As reviewed by Jiminez et al. (2015) intestinal inflammation in rodents differs from naturally occurring diseases in humans, so rodent models are not best suited for the study of this type of disorder [25]. Dogs, however, spontaneously develop chronic enteropathies resembling those in humans, and several subtypes with yet unknown aetiology can be differentiated [26].

Intestinal organoids allow the study of interactions between the gut microbiota and gut epithelium. Hence, exploring ways to mitigate the disease-provoking effects of pathogens and/or boost the health-promoting effects of natural commensals is possible using intestinal organoids, as we have recently outlined [27]. We have previously established and characterised canine intestinal organoid models, such as jejunal and colonic organoids, which can function as epithelial models that faithfully mimic respective intestinal sections [28]. These models have the potential to help close the gap between in vitro screening and in vivo assessment of possible therapeutic drugs, in adherence to the principle of the 3R, i.e., Replacement, Reduction and Refinement, of animal experimentation.

Herein, we report on the use of jejunal and colonic organoids to analyse the neutralising effects of recombinant versus natural antibodies on TcdA and TcdB. Recombinant antibodies comprised the IgG antibody bezlotoxumab, which is in clinical use for the treatment of *C. difficile* infections [29,30], and a recombinant sIgA established by us [31]. Natural antibodies consisted of sIgA purified from pooled goat whey. Analyses of the respective binding affinities of these antibodies to both toxins were carried out by enzyme-linked immunosorbent assay (ELISA). Subsequently, sulforhodamine B (SRB) cytotoxicity and FITC-dextran barrier integrity assays were used to establish the antibodies' respective toxin neutralisation profiles in the organoid model. We show that recombinant sIgA proved equally effective in neutralising the cytotoxic effect of TcdB on canine intestinal organoids

and organoid-derived monolayers (ODM), whilst goat sIgA failed to sufficiently neutralise TcdB in these in vitro systems. This highlights the applicability of canine intestinal organoids for drug testing and the development of treatments.

2. Results

2.1. Recombinant and Natural sIgA Antibodies Were Successfully Purified

Recombinant anti-TcdB sIgA antibodies used in this study were obtained by co-incubating cell culture supernatants from mIgA2-expressing and hSC-expressing recombinant CHO-K1 cell lines described previously [31]. Free dimeric IgA molecules that did not complex with the hSC were discarded by anion exchange chromatography (AIEx) (Figure 1A). Since this purification step failed to also remove free hSC and monomeric IgA (mIgA), size exclusion chromatography was carried out, resulting in pure sIgA (Figure 1B). sIgA from caprine whey was purified by ultra/diafiltration and size exclusion chromatography (Supplementary Figure S1). Most fractions obtained comprised both sIgM and sIgA, with higher sIgA content being noted for later fractions (Figure 1C).

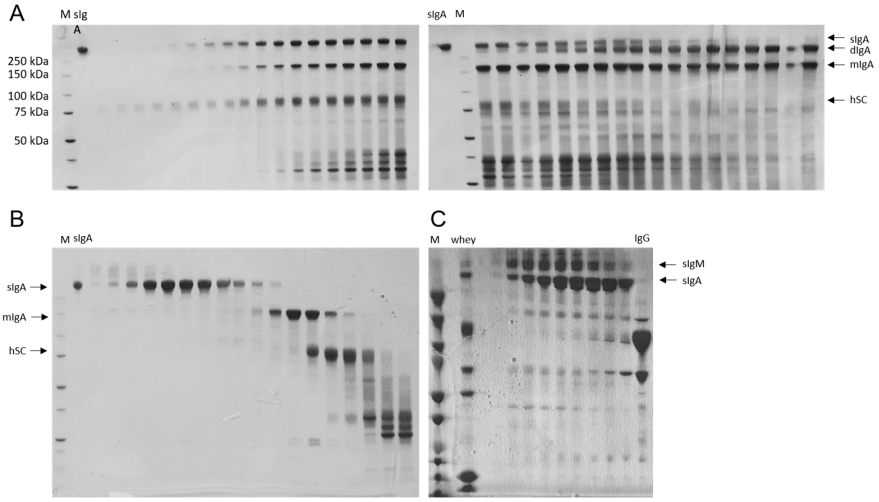

Figure 1. SDS-PAGE of different immunoglobulin-fractions: (**A**) SDS-PAGE analysis of sIgA fractions obtained by anion exchange chromatography. sIgA molecules were eluted under lower salt concentration conditions than dIgA from the AIEx column, allowing their separation. From left to right: subsequently obtained AIEx fractions. (**B**) SDS-PAGE analysis of sIgA obtained by size exclusion chromatography purification. sIgA molecules were efficiently separated by size from mIgA, hSC and other protein contaminants. Fractions were loaded from left to right. (**C**) SDS-PAGE analysis of sIgA-containing fractions extracted from goat whey by chromatography. Fractions were loaded from left to right. M (protein weight marker): BioRad Precision Plus Protein Unstained (A,B), BioRad Precision Plus Protein Dual Color Standard (**C**).

2.2. Recombinant and Natural Antibodies Mainly Bind to TcdB

The binding potential of bezlotoxumab, recombinant monoclonal sIgA and three sIgA fractions from caprine whey with the highest sIgA content according to SDS-PAGE analyses against TcdA and TcdB was analysed via ELISA. All antibodies showed very low binding potential against TcdA compared to TcdB. However, bezlotoxumab and recombinant sIgA were highly specific against TcdB. Caprine whey fractions showed steadily increasing values with increasing concentrations but remained lower in their highest concentration (1600 µg/mL) than bezlotoxumab and recombinant sIgA at 50 µg/mL. Of all whey isolates,

fraction 5B3 scored the best values. Therefore, it was used for all consecutive experiments (Figure 2).

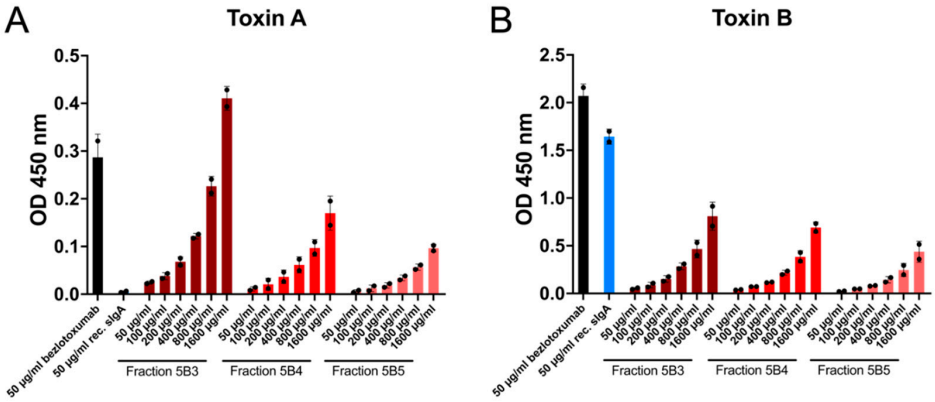

Figure 2. Neutralising potential of different antibodies against *C. difficile* toxin A (TcdA) and toxin B (TcdB). (**A**) All antibodies showed very low binding affinity to TcdA, with caprine whey sIgA fractions slightly increasing at higher concentrations. (**B**) Caprine whey sIgA fractions had a much lower binding potential against TcdB than bezlotoxumab and monoclonal recombinant sIgA. Data are represented as mean ± standard deviation (n = 2).

2.3. Recombinant Antibodies Exhibited Superior TcdB Neutralisation Potential Compared to Natural sIgA Molecules

The cytotoxic effect of TcdA and TcdB on canine intestinal organoid-derived monolayers was addressed by sulforhodamine B assays. Both small and large intestinal ODMs exhibited significantly decreased viability when treated with either toxin alone or a combination of the two. The attempt to neutralise TcdA failed with all three tested antibodies, i.e., viability was still significantly decreased upon TcdA treatment compared to untreated controls (Figure 3A,B). In contrast, pre-incubation of TcdB with 50 µg/mL of bezlotoxumab or recombinant sIgA resulted in toxin neutralisation, with canine epithelial cells remaining unaffected by TcdB. Pre-incubation of TcdB with caprine sIgA did not increase the viability of TcdB-treated ODMs (Figure 3C,D). All ODMs treated with a combination of TcdA and TcdB showed significantly reduced viability compared to untreated controls. Nonetheless, bezlotoxumab and recombinant sIgA were able to mitigate the toxins' negative impact on cell viability to some extent (Figure 3E,F).

2.4. Successful Establishment of Apical-Out and Floating Basal-Out Organoids

In order to make the apical cell surface accessible to assess the effect of bacterial toxins, floating basal-out and apical-out organoids were established. Basal-out and apical-out organoids were of substantially different appearances, as documented by bright field imaging. Whilst basal-out organoids exhibited a large lumen, apical-out organoids appeared more compact and dense and remained relatively small after polarity reversal (Figure 4A). DAPI/Phalloidin co-staining of DNA and F-actin demonstrated the localisation of apical microvilli oriented towards the lumen in basal-out organoids [32]. In contrast, microvilli, i.e., the apical cell surface, were clearly presented away from the organoid lumen, therefore demonstrating successful polarity reversal (Figure 4B,C). Virtually all organoids turned apical-out three days after initiation of polarity reversal.

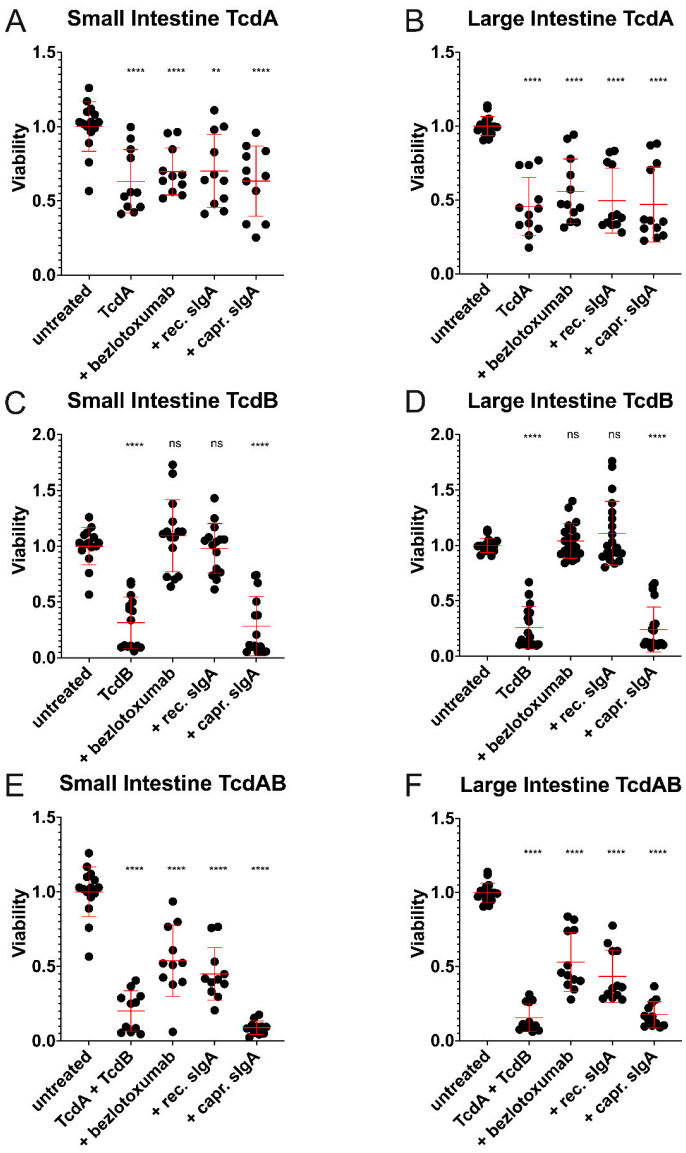

Figure 3. Respective potential of bezlotoxumab, recombinant sIgA and caprine sIgA to neutralise TcdA and/or TcdB, as revealed by sulforhodamine B cytotoxicity assays in ODM culture. Viability of ODMs from canine small and large intestinal organoids treated with TcdA (**A**,**B**), TcdB (**C**,**D**) or a combination of both toxins (**E**,**F**) following their incubation with bezlotoxumab, recombinant sIgA or caprine sIgA is shown. Student's *t*-tests were performed with the viability of untreated control ODMs serving as a reference. Error bars indicate standard deviation from arithmetic means. ** $p < 0.01$, **** $p < 0.0001$, ns = non-significant.

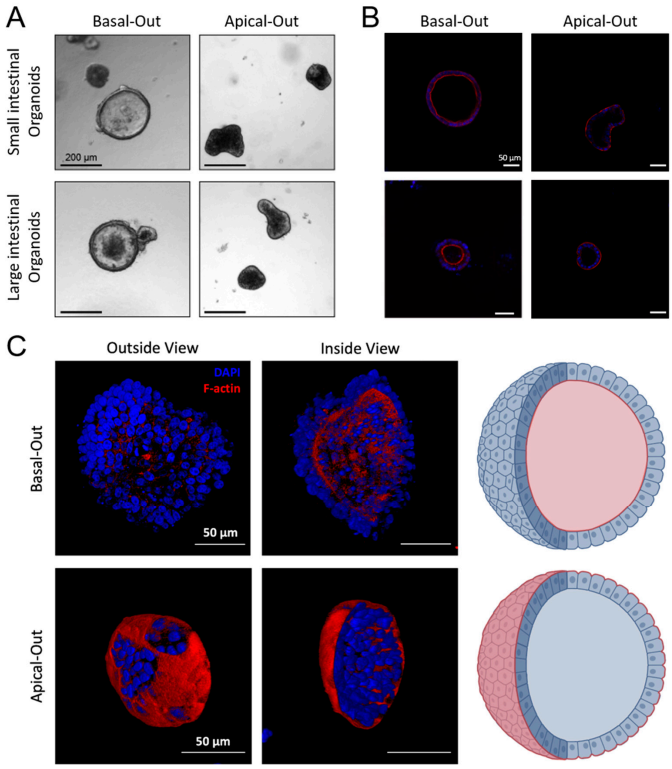

Figure 4. Characterisation of canine small and large intestinal apical out organoids. (**A**) Basal-out and apical-out organoids showed different morphologies as documented by brightfield microscopy. (**B**) Basal-out and apical-out organoids were stained with DAPI (blue) and Phalloidin (red) to visualise F-actin-rich microvilli on the apical cell surface of intestinal epithelial cells. (**C**) Three-dimensional rendering of basal-out and apical-out organoids, including a scheme depicting dense microvilli on the inside in basal-out organoids and on the outside in apical-out organoids.

2.5. Considerably Different Effects of TcdA and TcdB on Basal-Out and Apical-Out Organoids

Since TcdA and TcdB were shown to have a negative impact on cell survival in ODM culture, small and large intestinal organoids were subjected to a FITC-Dextran barrier integrity assay (Figure 5). Toxicity likely depends on the presence of specific receptors on certain cell surface domains. In dogs, these receptors remain to be identified. Hence, we opted for using basal-out and apical-out organoids to study the cytotoxic effects of TcdA and TcdB on either side of the epithelial cells. As shown in Figure 5, TcdA negatively affected cell–cell contacts in all tested organoids, i.e., basal-out and apical-out small and large intestinal organoids (Figure 5B–E). In contrast, TcdB had a negative impact only on barrier integrity in basal-out organoids of the small intestine (Figure 5B). Importantly, this cytotoxic effect of TcdB could be prevented by the pre-incubation of the toxin with bezlotoxumab or recombinant sIgA (Figure 5B,D). This TcdB neutralising effect was also evident in combination with TcdA in small and large intestinal basal-out organoids. Representative confocal microscopy images illustrating these observations are provided in Supplementary Figure S2.

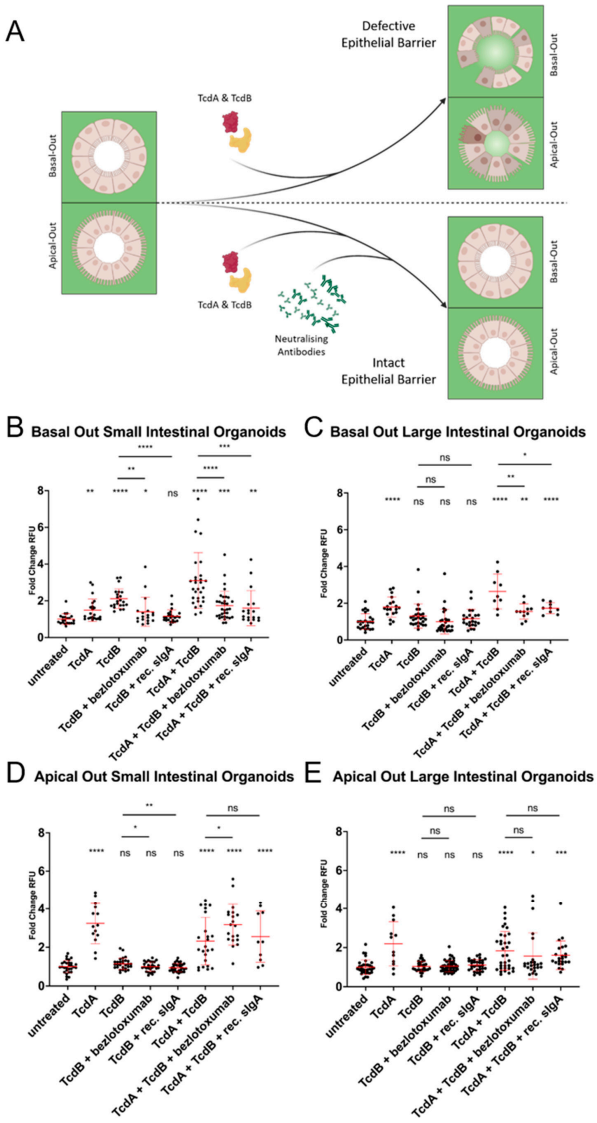

Figure 5. Different effects of TcdA and TcdB on basal-out and apical-out organoids in FITC-dextran barrier integrity assays. (**A**) A scheme representing the experimental approach. Small and large intestinal basal-out (**B**,**C**) and apical-out organoids (**D**,**E**) treated with TcdA, TcdB or a combination of both toxins. TcdB and the combination of TcdA and TcdB were pre-incubated with two different antibodies (bezlotoxumab and recombinant sIgA). Student's *t*-tests were performed using untreated controls as a reference, if not indicated otherwise. Error bars indicate standard deviations from the arithmetic means. RFU = relative fluorescent units, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = non-significant.

3. Discussion

Clostridioides difficile is one of the most important pathogens associated with hospital-associated infections leading to diarrhoea and pseudomembranous colitis. As extensively reviewed by Lim, Knight and Riley, 2020, *C. difficile* poses a major threat in the context of One Health. *Clostridioides difficile* spores can resist severe environmental conditions and be reactivated to germinate in various different hosts, making pet animals a potential source of human infections and vice versa, although exact mechanisms of transmission remain to be clarified. Spores can persist in environmental settings (water, soil, floors, foods) and then infect human beings or animals who have previously undergone antibiotic therapy or reside in different hosts in a latent form [33,34]. Two studies have previously shed light on the importance of asymptomatic carriers of *C. difficile* as an infection source. According to these studies, 10–15% of humans are colonised by *C. difficile* strains, with more than 80% of them constituting toxigenic strains [35,36]. In dogs, apparently, 0–6% of healthy individuals carry *C. difficile* strains, especially those also commonly found in humans [34,37]. However, this percentage might be underestimated, as healthy pets are not as accessible for infection studies as healthy humans.

Given the pathogenic impact of *C. difficile* on human and animal gastrointestinal health, we analysed the potential of two recombinant versus a natural antibodies to bind and neutralise relevant *C. difficile* toxins, i.e., TcdA and TcdB, in canine organoids and ODMs. The studied antibodies comprised the anti-TcdB IgG antibody bezlotoxumab, a recombinant anti-TcdB sIgA antibody established by us, and natural sIgA antibodies isolated from goat whey [31].

In the next step, we addressed the impact of pre-incubation of TcdA, TcdB, or both toxins with the antibody on their individual and cumulative cytotoxic effect on ODMs by employing a sulforhodamine B assay for a more definite outcome from only healthy, living cells attached to the surface and excluded dead cells from the analysis. In agreement with conformed binding affinities to TcdB, bezlotoxumab and recombinant anti-TcdB sIgA effectively neutralised TcdB, thus abrogating its cytotoxic potential, whilst caprine sIgA had no neutralising effect. Similarly, the low binding affinity of recombinant antibodies to TcdA was translated into low neutralisation of the toxin and reduced viability of exposed ODMs. Although binding of caprine sIgA to TcdA could be demonstrated, albeit at a low level, the natural antibody failed to neutralise the toxin and protect ODMs from its cytotoxic effect. This could be due to insufficient amounts of specific sIgA molecules in the whey. However, applying a 20-fold amount of caprine sIgA to the cells was insufficient to neutralise TcdA or TcdB, too, (not shown) despite cow milk sIgA having been already shown to effectively neutralise TcdA [38]. However, this positive effect may depend heavily on the species from which the milk is derived [12]. The combination of both toxins had a more detrimental effect than individual toxins, as reflected by a severe decrease in cell viability. Pre-incubation of the toxin mixture with recombinant antibodies led to a 50% attenuation of this effect. This finding reflected well the poor TcdA reactivity of the three antibody types analysed.

In order to elicit cytotoxic effects, TcdA and TcdB must be internalised into the host cell via endocytosis. Receptor binding is the first essential step in the process of cell entry. In the case of TcdA, a disaccharide harbouring a Galβ1-4GlcNac motif has been identified as a toxin binding structure. This disaccharide is found on the I, X and Y blood antigens present in a variety of cells, and these antigens have been shown to act as receptors for TcdA [39]. The presence of these (and/or other) TcdA receptors on canine epithelial cells remains to be determined. In addition, whilst TcdA has been shown to induce disruption of tight junctions, fluid influx, diarrhoea, inflammation and neutrophil recruitment in human patients [18], several aspects of TcdA and TcdB cytotoxicity in canines are still underexplored.

This motivated us to analyse the toxins' effects on the barrier integrity in three-dimensional small and large intestinal floating basal-out and apical-out organoids. This approach allowed controlled antibody/toxin delivery and subsequent study of toxin-

induced damage without needing to perform laborious microinjections [40,41]. Given that all three antibody types did not sufficiently react with TcdA in ODM culture, and that caprine sIgA failed to neutralise TcdB, 3D experiments were conducted with both toxins, but neutralisation assays exclusively focussed on the TcdB reactivity of recombinant anti-TcdB antibodies.

TcdA reduced barrier integrity in small and large intestinal organoids, irrespective of the intestinal cells' orientation. Since the discovery of I, X and Y blood antigens as TcdA receptors in 2005, more recent evidence points to the existence of several alternative pathways mediating TcdA entry into cells. Various domains of TcdA have been found to interact with numerous cell surface molecules to hijack internalisation into endosomes [42,43].

TcdB only had a cytotoxic effect on basal-out organoids representing the small intestine. Neither small nor large intestinal apical-out organoids appeared to be affected by TcdB. This finding is suggestive of TcdB receptors being predominantly, or even exclusively, expressed on the basolateral surface of cells, which is in accordance to previous literature showing that TcdB mainly effects the basolateral cell-surface [44]. This also indicates that TcdB receptor expression may significantly differ between canine small and large intestine.

Compared to previous studies that used only monolayers to test *C. difficile* toxins [45,46], we have established a system comparing 2D and 3D effects. This clearly shows that 2D experiments cannot be extrapolated well into 3D in vitro settings, as they might be substantially different. Of course, this also holds true to extrapolating data to mouse models or even human treatment, which, in part, explains why the majority of newly developed drugs fail on their way to clinical use. Investigating different species and in vitro setups will aid in the development of new treatment approaches, especially for diseases relevant to a One Health context, where every affected species might react differently to a pathogen and/or the respective treatment.

Studies in animal models and human patients have substantially advanced our understanding of TcdA and TcdB pathogenicity, but also revealed considerable differences between species regarding intestinal vulnerability to these toxins. For example, TcdB is not enterotoxic in rabbit ileal and colonic loops, or hamster and mouse ileal loops, but causes severe jejunal lesions in these species. On the other hand, ex vivo studies conducted in human colonic explants showed that TcdB induces pathobiological changes consistent with enterotoxicity [47]. Of the three previously determined receptors for TcdB (CSPG4, Fzd1,2,7 and PVRL3/Nectin3) [48–51], CSPG4 was found to be absent in mouse colonic epithelial cells [49,52]. Whether a similar absence of particular receptors could be responsible for the finding that canine colonic organoids are less susceptible to TcdB damaging their barrier integrity remains to be determined in future studies.

Species-specific nutrition and environmental conditions make highly divergent demands on the respective gastrointestinal system. This likely explains interspecies variations with respect to resistance/sensitivity of bowel segments to TcdA and TcdB and emphasizes that enterotoxic activities of TcdA and TcdB observed in animal models should be confirmed in the target species to reach final conclusions. In this context, canine intestinal organoids represent a particularly interesting research tool: As "micro copies" of the organ they represent and from which they originate, they allow the recapitulation of many important physiological and pathobiological mechanisms in dogs. In addition, close resemblances between healthy and disease-affected canines and the human gut have been demonstrated [26]. Consequently, canine intestinal organoids can also serve as model for human intestine, thus helping overcome the limitations of rodent systems.

However, one should cautiously evaluate toxin effects in different species instead of extrapolating results from one species to another. Human organoid and/or intestinal epithelial systems have been used previously to assess the effects of TcdA and TcdB [45,46,53]. Using 2D and 3D approaches on canine intestinal organoids, we showed that toxin effects are not the same in small and large intestinal-derived organoids, even from the same species, so results from human studies may not be transferred directly to other organisms. In addition,

our results provided proof of concept that monoclonal sIgA molecules are equally effective as existing IgG antibodies as a more stable treatment option.

4. Materials and Methods

4.1. Recombinant Monoclonal sIgA

Monoclonal recombinant anti-TcdB mIgA2 antibodies and the human SC component (hSC) were produced in recombinant Chinese Hamster Ovary cells (CHO-K1), as described by Bhaskara et al., 2021 [31]. In brief, recombinant anti-TcdB-mIgA2-expressing and hSC-expressing CHO-K1 cells were cultured in a humidified shaking incubator at 37 °C/5% CO_2/160 rpm in 2 L fed batch cultures, each for 10 days in HyClone ActiPro medium (ThermoFisher Scientific, Vienna, Austria). The cultures were fed on days 3, 5, 6 and 7 with 4% (v/v) of HyClone Cell Boost 7a and 0.4% (v/v) of HyClone Cell Boost 7b (ThermoFisher Scientific, Vienna, Austria). On day 10, cell culture supernatants were harvested by centrifugation for 20 min at 1200 rpm, mixed with Sartoclear Dynamics Lab V Kit diatomaceous earth (Sartorius, Vienna, Austria) at 200 g/L and filtered through sterile 0.22 µm filters. Anti-TcdB-sIgA and hSC supernatants were mixed at a 2:1 volume ratio and the mixture was incubated, shaking overnight at room temperature to potentiate the formation of hSC-sIgA complexes.

Then, the antibody-hSC mixture was diluted 1:2 with 20 mM Tris pH 8.0 to reach a conductivity of 8 mSi/cm and loaded onto an Äkta anion exchange column/20 mM Tris pH 8.0 (Cytiva Life Sciences, Vienna, Austria) at 2.5 mL/min. The column was washed with 20 mM Tris pH 8.0 and the samples were eluted with a salt gradient of 0–30% high salt buffer (20 mM Tris/1 M NaCl). Fractionated samples were loaded on SDS PAGE gels (NuPAGE 4–12% Bis-Tris Midi Gel; ThermoFisher Scientific, Vienna, Austria), and stained with InstantBlue (Expedeon, VWR, Vienna, Austria). Then fractions containing sIgA but not free dimeric (dIgA) molecules were pooled, concentrated using Amicon Ultra-15 Centrifugal Filter Units (Merck-Millipore, Darmstadt, Germany) and loaded onto a Sephadex® G-200 size exclusion column in 0.1 M Borate buffer pH 8.0 (Sigma-Aldrich, Vienna, Austria). Following washing with this buffer, protein fractions were eluted with 0.1 M Glycine pH 2.7 into Tris buffer pH 9.0. Finally, protein aliquots were loaded on SDS-PAGE gels. Fractions containing pure sIgA were pooled and filtered through sterile 0.22 µm filters. Borate buffer was substituted by phosphate-buffered saline (PBS), sIgA was concentrated on Amicon Ultra-15 Centrifugal Filter Units to 1.7 mg/mL and kept at 4 °C until use.

4.2. Isolation of Caprine Whey sIgA

Whey was collected from a local dairy farm and pooled from several goats. Then, 1 L of pooled caprine whey was centrifuged at 10,000× g for 1 h to separate large particles. The obtained supernatant was filtered using a Sartoban P0.2 µm depth filter (Sartorius, Vienna, Austria) and a peristaltic pump. Filtration comprised a 0.45 µm pre-filtration and a 0.2 µm filtration step. Using an Äkta flux system (Cytiva Life Sciences, Vienna, Austria), the solution was subsequently concentrated using 2 × 100 kDa membranes with a total filter size of 0.01 m^2. The initial flow rate of 150 mL/min was steadily decreased to keep transmembrane pressure (TMP) below 1.5 bar. Using this approach, 950 mL of starting volume was concentrated by a factor of 29.7 to a final volume of 32 mL, which was subjected to size exclusion chromatography. Finally, preparative chromatography was carried out using the Äkta pure system (Cytiva Life Sciences, Vienna, Austria) with a Superdex 200 26/60 preparative grade column (CV = 320 mL), allowing separation of 10 to 600 kDa proteins. PBS was used as an equilibration and elution buffer for isocratic elution. Ten millilitres of the concentrated sample (9.4% of CV) per run was transferred to a ten millilitres super loop and elution was carried out for a total of one point two millilitres CV. Quantification of sIgA concentrations from isolated goat whey fractions was carried out by competitive ELISA (Antibodies-online, Aachen, Germany).

4.3. ELISA

The binding potential of antibodies to TcdA and TcdB (BioTrend, Vienna, Austria) was assessed by indirect ELISA. The latter was conducted for total volumes of 100 µL/well in high-binding microtitre plates (Greiner bio-one, Kremsmünster, Austria). All washing steps were carried out using PBS-T (PBS with 0.05% Tween 20; Sigma-Aldrich, Vienna, Austria). Plates were coated with TcdA or TcdB at a concentration of 1 µg/mL in PBS and incubated overnight at 4 °C without shaking and then washed three times with PBS-T. After a blocking step of 20 min with PBS-T, the three goat whey fractions showing the purest sIgA profile (5B3, 5B4, 5B5) and control antibodies bezlotoxumab and recombinant sIgA diluted in PBS containing 1% bovine serum albumin (BSA, Carl Roth, Karlsruhe, Germany) were incubated by shaking at 40 rpm for one hour at room temperature. Concentrations tested were 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL and 160 µg/mL for caprine whey sIgA and 5 µg/mL for controls, respectively. Unbound proteins were removed by washing three times. Horseradish peroxidase-conjugated secondary antibodies against goat IgA or human IgG (both BioRad, Vienna, Austria) were diluted 1:10,000 in PBS containing 1% BSA, added to the appropriate wells, and incubated for 45 min at room temperature, shaking at 40 rpm. After washing, substrate (3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma-Aldrich, Vienna, Austria)) was added. Following signal development, the reaction was stopped with 2 N HCl (Carl Roth, Karlsruhe, Germany). The absorbance was measured at 450 nm on a TECAN plate reader (Tecan Life Sciences, Männedorf, Switzerland). Analysis was performed from two independent experiments, each carried out in duplicates.

4.4. Organoid Culture

Canine intestinal crypts were isolated from jejunum and colon according to Kramer et al., 2020 [28]. Tissue sampling was approved by the institutional ethics committee, in accordance with Good Scientific Practice guidelines and Austrian legislation. Based on the guidelines of the institutional ethics committee, the use of tissue material collected during therapeutic excision or post-mortem is included in the University's "owner's consent for treatment", which was signed by all patient owners. The growth medium consisted of 37% basal medium (Advanced DMEM/F12 supplemented with 2 mM GlutaMAX and 10 mM HEPES), 1 × B27 (Invitrogen, ThermoFisher Scientific, Vienna, Austria), 1 mM N-acetylcysteine, 10 nM Gastrin (Sigma-Aldrich, Vienna, Austria), 100 ng/mL Noggin, 500 nM A8301, 50 ng/mL HGF, 100 ng/mL IGF1, 50 ng/mL FGF2 (PeproTech, Rocky Hill, NJ, USA), 10% (*v*/*v*) Rspondin1 and 50% (*v*/*v*) Wnt3a conditioned media. For the first two days of culture, 50 ng/mL mEGF (ThermoFisher Scientific, Vienna, Austria) and 10 µM Rock-inhibitor Y-27632 (Selleck Chemicals, Houston, TX, USA) were added. The growth medium was changed every two to three days. Weekly passaging at 1:4 to 1:8 split ratios was achieved by mechanical disruption using flame-polished Pasteur pipettes. Brightfield images were acquired using a DMi8 microscope (Leica Camera AG, Wetzlar, Germany).

4.5. Generation of Organoid-Derived Monolayers

To analyse the cytotoxic effects of *C. difficile* toxins A and B, organoid-derived monolayers were established. To this aim, organoids were released from the Geltrex matrix via repeated pipetting. Organoids were then trypsinised, and single cells were counted. Subsequently, 15,000 cells/well were seeded in 96-well plates pre-coated with 100 µg/mL Geltrex (ThermoFisher Scientific, Vienna, Austria) diluted in a basal medium at 37 °C for 1 h.

4.6. Sulforhodamine B Cytotoxicity Assay

When almost reaching confluence, cells were treated with 7.5 ng/mL TcdA, 100 ng/mL TcdB, or a combination of both. The applied toxin concentrations were predetermined as the respective IC50. The neutralisation assay was carried out by pre-incubating TcdA and TcdB with 50 µg/mL monoclonal antibody bezlotoxumab (Merck, Darmstadt, Germany), monoclonal recombinant sIgA, or sIgA isolated from goat whey for 2 h at 37 °C

prior to cell treatment. Used concentrations equalled to a 14,000-fold and 930-fold molar excess of bezlotoxumab and a 5300-fold and 350-fold molar excess of sIgA to TcdA and TcdB, respectively. After 24 h, cells were fixed and subjected to sulforhodamine B (SRB) cytotoxicity assays [54]. In brief, cells were fixed with 10% trichloroacetic acid (TCA) for one hour at 4 °C and then rinsed with tap water four times. After air-drying, cells were stained with 0.057% SRB in 1% acetic acid for 30 min. Subsequently, cells were rinsed with 1% acetic acid four times to remove excess SRB before air-drying again. Cell-bound dye was then solubilised in 10 mM TRIS and extinction was measured at 488 nm using a fluoro spectrometer (Glomax® Explorer, Promega, Vienna, Austria).

4.7. Generation of Floating Basal-Out and Apical-Out Organoids

Apical-out and floating basal-out organoids were generated as described previously [55]. Organoids were harvested using Cultrex® Organoid Harvesting Solution (Bio-Techne, Minneapolis, MN, USA) for 1.5 h at 4 °C, under constant shaking. Thereafter, organoids were washed twice with basal medium, resuspended in growth medium and seeded in multiwell plates treated with Anti-Adherence Rinsing Solution (Stemcell Technologies, Vancouver, Canada) to prevent organoid attachment to the surface. To generate floating basal-out organoids, 7.5% Geltrex (ThermoFisher Scientific, Waltham, MA, USA) was added to the culture medium. Organoids were incubated for 72 h in a humidified incubator with 5% CO_2 prior to further use.

4.8. DAPI/Phalloidin Staining

Organoids were fixed with 2% paraformaldehyde (PFA) and stained with Phalloidin (2.5 µg/mL in PBS; Alexa Fluor 546, Invitrogen, ThermoFisher Scientific, Vienna, Austria) to visualise actin filaments, and with 4′,6-diamino-2-phenylindole (DAPI; 4 µg/mL in PBS; Sigma-Aldrich, Vienna, Austria) nuclear staining for 45 min. Images were acquired using a Zeiss LSM880 confocal microscope (Zeiss, Jena, Germany).

4.9. FITC-Dextran Barrier Integrity Assay

Basal-out and apical-out organoids were embedded in 18-well slides with glass bottoms (Ibidi, Gräfelfing, Germany) in Geltrex and treated with 2.5 ng/mL TcdA, 7.5 ng/mL TcdB, or a combination of both with and without neutralising antibodies. This treatment was achieved by replacing the entire medium covering the Geltrex dome with fresh toxins diluted in fresh medium. As in SRB assays, the toxin:antibody:medium mixture was pre-incubated for 2 h at 37 °C prior to cell treatment. Toxin:antibody ratios corresponded to those in the SRB assays, but the concentration of both toxins was reduced to induce damage to the epithelial barrier whilst keeping the organoids alive due to the apparently higher sensitivity of whole organoids compared to ODMs. After 23 h, FITC-dextran 4000 (Sigma-Aldrich, Vienna, Austria) was added to the cultures at a final concentration of 1 mM and images were acquired using a Zeiss LSM880 confocal microscope after 24 h of incubation, as described in Bardenbacher et al., 2020 [56]. Images were analysed using Fiji ImageJ [57] for the calculation of grey values within each organoid.

4.10. Statistics

All statistical analyses were performed using GraphPad Prism Version 9.3.0.

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Publication III

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EDITED BY
Ahmed S. Mandour,
Suez Canal University, Egypt

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Walaa Mohamadein,
Suez Canal University, Egypt

*CORRESPONDENCE
Romy M. Heilmann
romy.heilmann@kleintierklinik.uni-leipzig.de

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Time to eRAASe chronic inflammation: current advances and future perspectives on renin-angiotensin-aldosterone-system and chronic intestinal inflammation in dogs and humans

Romy M. Heilmann[1]*, Georg Csukovich[2], Iwan A. Burgener[2] and Franziska Dengler[3]

[1]Department for Small Animals, College of Veterinary Medicine, University of Leipzig, Leipzig, Germany, [2]Small Animal Internal Medicine, University of Veterinary Medicine Vienna, Vienna, Austria, [3]Institute of Physiology, Pathophysiology and Biophysics, University of Veterinary Medicine Vienna, Vienna, Austria

Chronic idiopathic intestinal inflammation is an increasing worldwide problem that affects companion animals, especially dogs, and human patients. Although these disease entities have been intensely investigated recently, many questions remain, and alternative therapeutic options are needed. Diarrhea caused by dysregulation of intestinal electrolyte transport and subsequent fluid and electrolyte losses often leads to secondary consequences for the patient. Currently, it is not exactly clear which mechanisms are involved in the dysregulation of intestinal fluid absorption, but differences in intestinal electrolyte shifts between human and canine patients suggest species-specific regulatory or counterregulatory mechanisms. Several intestinal electrolyte transporters are differentially expressed in human patients with inflammatory bowel disease (IBD), whereas there are virtually no studies on electrolyte transporters and their endocrine regulation in canine chronic inflammatory enteropathy. An important mechanism involved in regulating fluid and electrolyte homeostasis is the renin-angiotensin-aldosterone-system (RAAS), which may affect intestinal Na^+ transport. While RAAS has previously been considered a systemic regulator of blood pressure, additional complex roles of RAAS in inflammatory processes have been unraveled. These alternative RAAS pathways may pose attractive therapeutic targets to address diarrhea and, thus, electrolyte shifts in human IBD and canine chronic inflammatory enteropathy. This article comparatively summarizes the current knowledge about electrolyte transport in human IBD and canine chronic inflammatory enteropathy and the role of RAAS and offers perspectives for novel therapeutic avenues.

KEYWORDS

alternative RAAS, chronic inflammatory enteropathy, inflammatory bowel disease, electrolyte transport, enteroids, tight junctions

1. Chronic intestinal inflammation—a one-health perspective

Human IBD—comprising mainly Crohn's disease (CD) and ulcerative colitis (UC)—has a high prevalence in industrialized countries, and patients often experience severe distress and significantly reduced quality of life. Healthcare costs to treat IBD in humans are immense, amounting to 15–30 billion US dollars annually in the United States and about 5 billion Euros in Europe (1). The exact prevalence of chronic inflammatory enteropathy (CIE) in dogs is currently unknown, but it is estimated at 1%–2% in referral settings (2). CIE in dogs can range in severity and is subcategorized based on the response to treatment (2). In contrast to canine CIE, different compartments of the intestines are predominantly affected in patients with CD and UC, likely reflecting differences in the disease pathogenesis. Overt inflammatory responses are a common characteristic, resulting from environmental factors (dietary and microbial antigens) combined with a genetic predisposition (3). Dogs have accompanied humans and shared the human lifestyle for over 1,000 years, and it is thus not surprising that they develop similar civilization diseases. The prevalence of idiopathic IBD—either responsive (immunosuppressant-responsive enteropathy, IRE) or not responsive (non-responsive enteropathy, NRE) to immunosuppressive treatment—as a form of chronic inflammatory enteropathies (CIE) in dogs increased simultaneously with the rise of IBD in humans and both diseases share many characteristics, including pathogenesis and clinical signs (4–7). In dogs, CIE is characterized by chronic gastrointestinal signs, exclusion of other underlying diseases, and confirmation of gastrointestinal inflammation together with a response to treatment with either an elimination diet alone (food-responsive enteropathy, FRE) or in combination with immunosuppressant medication (IRE or NRE) (2, 6, 7). The resulting diarrhea and accompanying shifts in plasma electrolytes can severely compromise the dogs' and their owners' quality of life.

A hallmark of IBD is diarrhea due to intestinal hypersecretion and hampered reabsorption of electrolytes and fluid, often accompanied by serum electrolyte changes. Although the clinical signs are similar and largely overlapping, reports suggest different compensatory mechanisms to be activated both in the intestinal epithelium and on the systemic level in affected humans and dogs (8–10), which might also call for different therapeutic approaches. While hyponatremia is the most common electrolyte change in human IBD (11), hypokalemia appears more prevalent in canine CIE (9), suggesting species-specific compensatory mechanisms. A better understanding of the pathophysiologic mechanisms in dogs with CIE is expected to help identify novel therapeutic targets that could ameliorate diarrhea in affected dogs and be valuable for treating human IBD patients. While IBD in people has been under investigation for decades, significantly less is currently known about the pathophysiology of chronic idiopathic intestinal inflammation (CIE) in dogs.

2. Pathophysiology of diarrhea—gastrointestinal electrolyte transport and barrier formation

Central functions of the intestinal epithelium are the formation of a tight barrier to shield the host from luminal microbiota and other noxae and the vectorial transport of nutrients, electrolytes, and water. Uptake and secretion of nutrients and electrolytes are the major driving force for the (mostly paracellular) absorption and secretion of water. The gastrointestinal tract faces large fluid and electrolyte shifts, and the healthy intestinal mucosa absorbs about 98% of that fluid (12, 13). The (passive) movement of water is driven by the (active) uptake or secretion of electrolytes, primarily Cl^- and Na^+. Due to its high absorptive capacity, the colonic epithelium can compensate for an increased secretion and/or defective absorptive capacity in the small intestine (14). Diarrhea develops if the compensatory capacity of the colon is exceeded and is often accompanied by serum electrolyte changes. The highest fecal water output is thus seen with disease involving the colon (12). Not surprisingly, diarrhea is invariably seen in humans with IBD, particularly in UC (15). In dogs, the lesions are typically more heterogeneously distributed in the gastrointestinal tract, and about 80% of affected animals show diarrhea (9). This lower prevalence of diarrhea [80% in dogs vs. 100% in people (9, 15)] might indicate a slightly more efficient compensation of intestinal malabsorption in dogs than in people.

Both increased secretion and reduced absorption of electrolytes cause diarrhea in human IBD patients (16). However, colonic absorption could still compensate for this if the colonic absorptive and re-absorptive transport mechanisms remain intact (17, 18). The main mechanisms for the uptake of luminal electrolytes—and thus the absorption of water—in the mammalian intestine is Na^+-coupled cotransporters, particularly the Na^+/H^+-exchanger family (NHE) and the epithelial Na^+ channel (ENaC). Both are downregulated in human IBD (19, 20) and rodent models of dextran-sulfate-sodium-induced colitis, along with the Na^+/K^+-ATPase that generates the gradient for the effective uptake of Na^+ from the intestinal lumen (11, 20, 21), causing a decreased (re-)absorption of water. A knockout of NHE3, but not of NHE2, leads to diarrhea in a mouse model (22), and NHE3 was demonstrated to be the major isoform for Na^+ absorption across the canine ileum epithelium (23).

This finding is especially interesting in conjunction with reports of increased serum aldosterone levels in human IBD patients (11, 24), suggesting a systemic attempt at a counter-regulation mediated by the renin-angiotensin-aldosterone system (RAAS) as ENaC, NHE3 and Na^+/K^+-ATPase are upregulated by aldosterone (25–27). Other transport proteins might also be involved in the dysregulation of intestinal fluid absorption, such as the anion exchangers putative anion transporter 1 (PAT1), down-regulated in adenoma (DRA), the Cl^- channel cystic fibrosis transmembrane conductance regulator (CFTR) (16, 28), monocarboxylate transporter 1 (MCT1) (11, 21), and anion exchanger 2 (AE2). The $Na^+/K^+/2Cl^-$ cotransporter (NKCC) on the basolateral side of the epithelium might have a pivotal role in regulating the driving force for intestinal secretion [e.g., by CFTR and chloride channel 2 (CLC2)]. Similarly, basolateral K^+ channels might be important in driving colonic secretion. The K^+ channel KCNN4 is specifically upregulated in human IBD patients (29), and additional K^+ channels or pumps may be located in the intestinal epithelial brush border membrane (16), but their role in human IBD (and canine CIE) is poorly understood. The effect of CIE on intestinal electrolyte transport in dogs has not been investigated to date.

Following established electrolyte gradients, the secretion and reabsorption of water mainly take the paracellular route. Therefore, the epithelial barrier formed by tight junction proteins is an important factor in the pathogenesis of diarrhea. Tight junctions and

other cell–cell contacts are essential components located between adjacent epithelial and endothelial cells throughout the mammalian organism. In human IBD, the barrier-forming claudins 3, 4, 5, 7, and 8 are downregulated and disoriented from the plasma membrane, as are occludin and ZO-1, whereas the pore-forming claudin 2 is upregulated (30) along with increased paracellular permeability (20). In dogs with CIE, the expression of claudins or occludin is not altered in the duodenum, but colonic occludin mRNA levels are decreased (31). Apart from these findings, the regulation of tight junction proteins has yet to be investigated in dogs with CIE (32), but a thorough understanding of their role would be a major premise for further studying the pathomechanisms of CIE-related diarrhea in dogs. The colonic expression of occludin and claudin 8 is regulated (along with ENaC) by aldosterone (33), which may imply an additional therapeutic potential for RAAS in IBD and potentially also CIE in dogs.

3. Classical and alternative RAAS pathways—great complexity and far-reaching effects

RAAS has been extensively studied in cardiovascular and renal pathophysiology, and it appears to have much greater non-linear complexity than previously known (34). It acts on intestinal transport and barrier function, as described above. In addition, RAAS is involved in other intestinal functions, including the absorption of glucose and peptides, gastrointestinal motility, and the regulation of mesenteric blood flow (35, 36). Given the differences in electrolyte imbalances between canine CIE and human IBD patients, RAAS pathways might be differentially activated in these conditions.

Classically, renin cleaves angiotensinogen to angiotensin I (Ang I), which is then processed by angiotensin-converting enzyme (ACE) to the vasoconstrictor Ang II that activates aldosterone. This "traditional RAAS" has been well characterized as a circulatory blood pressure regulator (Figure 1A) and has presented a pharmacotherapeutic target for decades. In contrast, the existence of additional peptides derived from Ang I and II that constitute the "alternative RAAS" and their role in cardiovascular physiology and disease pathogenesis has long been neglected. The involvement of these recently discovered factors (Figure 1B) challenges the former simple concept of RAAS but also lends itself to potential novel therapeutic avenues beyond managing cardiovascular pathologies. Recent evidence also supports the coexistence of localized "tissue RAAS" mediating local (paracrine) effects.

Renin, a peptidase, represents the rate-limiting step in the RAAS cascade. After release from epithelioid cells of the renal juxtaglomerular apparatus into the circulation, renin cleaves an N-terminal decapeptide from angiotensinogen, a glycoprotein of the globulin superfamily synthesized in the liver and (though controversial) adipose tissue (37, 38), resulting in Ang I. The biologically active octapeptide Ang II results from the cleavage of Ang I by ACE, which is expressed primarily by pulmonary and renal endothelial cells and has also been detected in other tissues, including the myocardium and intestines (39). ACE is most active when bound to cell membranes. Together with the short half-life of Ang I and II, this indicates localized actions of RAAS (40). Similarly, an effect of renin and/or Ang II at the tissue level, rather than in the circulation, is supported by detecting (pro-)renin receptors in several tissues, such as the heart, brain, placenta, kidney, and liver (41).

The main effect of Ang II is an increase in systemic blood pressure by regulating vasoconstriction and cardiac output (42). As an intermediate effect, increased Na^+ reabsorption in the proximal renal tubules (via NHE3) and induction of thirst and salt appetite, subsequently increasing extracellular volume and, thus, blood pressure, are induced (43–45). As a longer-term effect, Ang II stimulates (a) the expression and secretion of aldosterone, thus increasing the reabsorption of Na^+ in the renal collecting ducts via ENaC on the gene expression level and (b) hypothalamic antidiuretic hormone (ADH, vasopressin) secretion leading to the insertion of aquaporins in the renal collecting ducts (25). Together, these mechanisms increase water reabsorption and thus blood volume and systemic blood pressure (Figure 1). It is important to recognize, however, that the enhanced reabsorption of Na^+ in the collecting ducts causes a concurrent loss of K^+ due to the extrusion of K^+ via apical channels into the lumen of the renal collecting ducts, which is driven by the electrochemical gradient that increases with the reabsorption of Na^+ (46).

Beyond these direct and indirect effects on systemic blood pressure, Ang II also elicits immunomodulatory effects by inducing proinflammatory cytokines and chemokines (e.g., TNFα, IL-6, and TGF-β1) in renal tubular cells and cells of the immune system (47–49). Ang II is also involved in hypertrophic remodeling (e.g., of the myocardium) by inducing cell proliferation and growth, but a direct effect of Ang II on extracellular matrix synthesis has also been observed (24, 47, 50). Thus, Ang II is presumed to be involved in the pathologic process of fibrogenesis (e.g., cardiac, renal, and hepatic fibrosis) (51, 52), which is also a major factor in the pathogenesis of human IBD (53). The binding of prorenin to its tissue receptor further contributes to myocardial fibrosis via the activation of intracellular signaling pathways (54, 55).

Four angiotensin-receptor (ATR) isoforms have been described, AT1R–AT4R. The ATRs are G-protein coupled transmembrane receptors (40) that might dictate the effects of Ang II by spatial differences in tissue abundance. AT1R is the primary receptor mediating the effects of Ang II and is expressed in most tissues, particularly the liver, adipose tissue, and placenta (39, 56). While AT1R is well characterized, the exact functions of the remaining three isoforms of ATR remain currently unknown. AT2R is found primarily during fetal development but may be upregulated under pathological conditions in adulthood (43), especially those affecting the lungs or smooth muscle (56). A vasodilatory effect of AT2R (i.e., opposing AT1R-mediated effects) has also been reported (40, 57, 58) and may provide a "safety net" preventing exaggerated and counterproductive effects of Ang II via AT1R.

Besides these traditional RAAS components, additional enzymes are described to act on Ang I and Ang II, representing the "alternative RAAS" (Figure 1B). To date, the best characterized is ACE2, which can cleave a nonapeptide, Ang (1–9), from Ang I or a heptapeptide, Ang (1–7), from Ang II (59, 60). Interestingly, one of the first observations of an alternative route of Ang I breakdown to Ang (1–7), independent from ACE, was in dogs (61). Ang (1–9) can also be converted to Ang (1–7) by ACE. Ang (1–7) responses can counteract those of Ang II [i.e., vasorelaxant, anti-proliferative, anti-inflammatory, anti-fibrotic,

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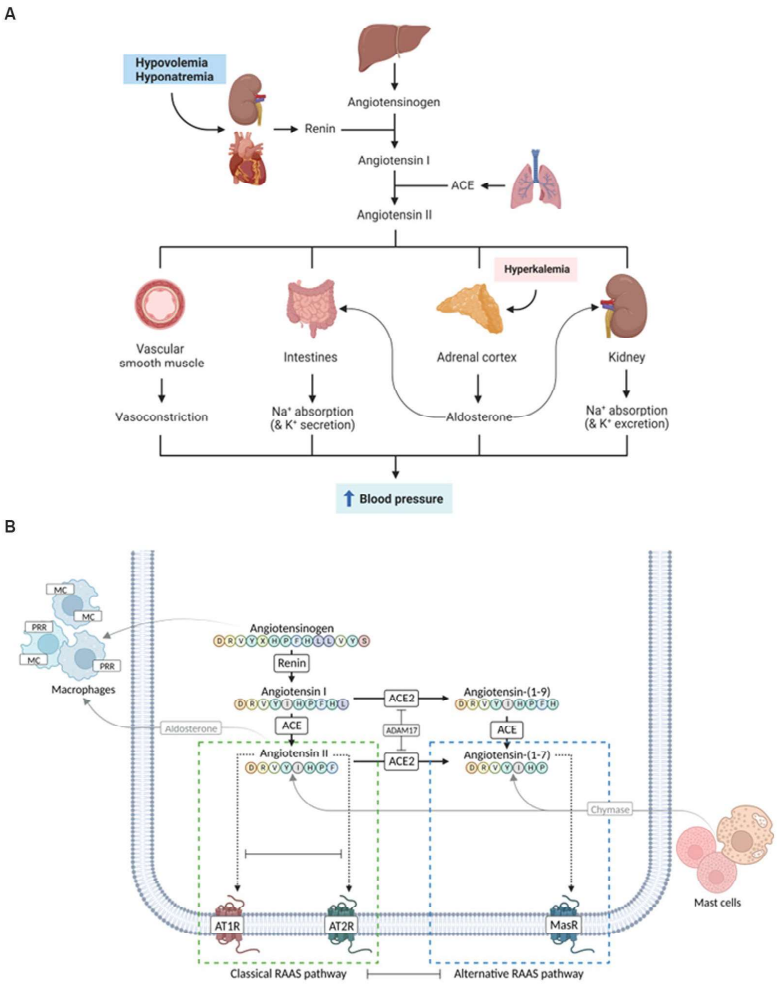

FIGURE 1
Evolution of the complexity of the renin-angiotensin-aldosterone system (RAAS). **(A)** Traditional simple view of the RAAS involving mostly cardiovascular and renal effects. **(B)** More recent complex view on classical and alternative RAAS pathways that might play a role in human inflammatory bowel disease (IBD) and/or canine chronic inflammatory enteropathy (CIE). Whereas the activation of the classical arm leads to vasoconstrictive, proinflammatory, profibrotic, and prothrombotic effects (green dashed box), components of the alternative RAAS pathways result in vasodilatory, anti-inflammatory, antifibrotic, and antithrombotic responses (blue dashed box). ACE, angiotensin-converting enzyme; ATR, angiotensin receptor; MR, mineralocorticoid receptor; PRR, prorenin receptor. Images created with BioRender.com.

and thus likely (cardio-)protective] (59, 62, 63), presumably via binding to AT2R (54, 59). In hypertensive rats, Ang (1–7) reduced the heart rate but not systemic blood pressure (63). Simultaneously, the formation of Ang (1–7) from Ang II is inherent in decreased Ang II concentrations. With the discovery of Mas, an additional RAAS receptor was identified that might act as the main receptor for Ang (1–7) and thus the "alternative arm" of RAAS (59, 62, 64). The pathophysiologic role and effects of Ang (1–7) have raised hopes for a therapeutic application to address the adverse effects of Ang II in various pathologies. However, the pathways and effects of Ang II are currently still controversial and remain first to be clarified (63). Formation of Ang (2–8) (also referred to as Ang III) and Ang (3–8) (also known as Ang IV) has also been described (40). These peptides bind to AT1R and elicit similar effects as Ang II (54).

4. RAAS crossroads between adaptation, disease, and novel therapeutic targets

Components of the RAAS have paracrine and/or autocrine cytokine-like effects and regulate inflammation, tissue repair, and fibrosis (21, 65, 66), all important factors in the pathogenesis of canine CIE and human IBD. In addition to upregulating adhesion molecules, Ang II is chemotactic for inflammatory cells, particularly of the mononuclear lineage. These cells produce RAAS components following activation (mediated by IL-1, TNF-α, NF-κB, and/or PPARγ), resulting in a positive-feedback loop with the potential to perpetuate chronic inflammatory responses (66–68). Ang II also has profibrotic effects via TGF-β, connective tissue growth factor stimulation, and inhibition of matrix metalloproteinase (MMP)-mediated extracellular matrix degradation (69). While conflicting data exist on TGF-β expression in canine CIE depending on the gastrointestinal segment affected (4, 70–73), and unlike in humans stricturing behavior is not observed in affected dogs, intestinal mucosal MMP-2 and -9 activities are increased in canine CIE (74). Toll-like receptor (TLR) and RAGE (receptor for advanced glycation end products) expression are dysregulated in canine CIE (5, 75, 76), and RAAS blockade has anti-inflammatory effects by suppression of TLR2 and TLR4 in humans (77).

Inhibition of RAAS pathways [e.g., Ang II production by ACE inhibitors (ACEIs) or its effects by ATR blockers (ARBs)] could downregulate inflammatory mediators and the innate immune receptors TLR2, TLR4, and RAGE. This concept presents a novel therapeutic strategy that targets the inflammatory response in canine CIE and warrants further study. Classical and alternative RAAS pathways (Figure 1B) are complementary systems with the potential to oppose or compensate for the actions of the contralateral arm (60, 77, 78), and their balance (or imbalance) might play an important role in the pathogenesis of intestinal inflammation. Thus, a (receptor) specific approach is most promising for therapeutically targeting the RAAS. The alternative RAAS has anti-inflammatory properties (59, 60). Ang (1–7) is a promising therapeutic target that attenuated intestinal inflammation in a rodent model of IBD (78). Components of classical and alternative RAAS are expressed in the intestinal mucosa in humans (34, 78, 79), with disparate ACE2 imbalances in the small intestine (downregulation) and colon (upregulation) in IBD patients (59, 62, 79). ACE2, as the main enzyme for cleavage of Ang II to Ang (1–7) which neutralizes the pro-inflammatory and pro-fibrotic effects of Ang II, might be critical for mounting pro- vs. anti-inflammatory responses (80). It is expressed in the gastrointestinal tract in cats (81) but has not been investigated in dogs. Circulating ACE and ACE2 act as decoy receptors, and the plasma ACE2/ACE ratio is increased in people with IBD. Cleavage of ACE2 is controlled by the metalloprotease ADAM17 (34), and ACE2 induction by cardiovascular pathology—shifting the balance between Ang peptides in plasma—is more pronounced in dogs than people (82). MasR is expressed in the canine ileum (83) but remains to be investigated in canine CIE. Likewise, tissue prorenin receptor (PRR) and mineralocorticoid receptor (MR) expression (e.g., by macrophages), as well as chymase activation (e.g., by mast cells), can modulate local RAAS effects (Figure 1B) and inflammatory responses (43) but remain to be studied in canine CIE. ACEIs (decreasing the production of Ang II), Ang II blockade (antagonizing AT1R signaling), MR or PRR antagonists, and/or chymase inhibitors could be useful and inexpensive alternative or adjunct therapeutic options for chronic intestinal inflammation (39, 84, 85) and potentially other autoinflammatory diseases (e.g., autoimmune hepatitis) in dogs.

5. Discussion and conclusions

Humans and dogs are close companions and share several civilization diseases, including idiopathic IBD and CIE. Although the shared Western lifestyle is proposed as a common denominator in the etiology of both conditions, there appear to be some species-specific differences in the disease characteristics, including the primary disease localization and distribution, resulting electrolyte changes, and potentially corresponding (counter-)regulatory mechanisms. While the current body of knowledge and research is more extensive for human IBD than canine CIE, a complete understanding of the underlying pathophysiology and possible mechanistic approach to therapy needs to be improved in both species. Exploration of alternative treatment options for dogs with CIE is needed as currently available drugs—particularly corticosteroids—carry significant side effects and biologicals (e.g., monoclonal antibodies against receptors or inflammatory cytokines) are not currently available (and very unlikely available soon) as a treatment option for canine CIE (86). Understanding commonalities and species-specific differences can be expected to result in the development of improved treatment strategies, and targeting RAAS might be one of these options. A thorough understanding of the role of RAAS pathways in the pathophysiology of canine CIE is needed to assess the therapeutic potential and potential side effects. Novel research methods, particularly canine intestinal organoids (Figure 2) that provide a reproducible and stable *in vitro* system for disease modeling and drug development (87–90), will be vital to further evaluate the effects of RAAS modifiers on epithelial ion transport, inflammatory responses, and intestinal barrier function comparatively. Organoids will allow to implement the 3R principles (6) and pave the way for urgently needed novel disease-specific treatment strategies in canine CIE and human IBD.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

RH, IB, and FD: conceptualization. RH and FD: manuscript draft. RH and GC: figures. All authors contributed to the article and approved the submitted version.

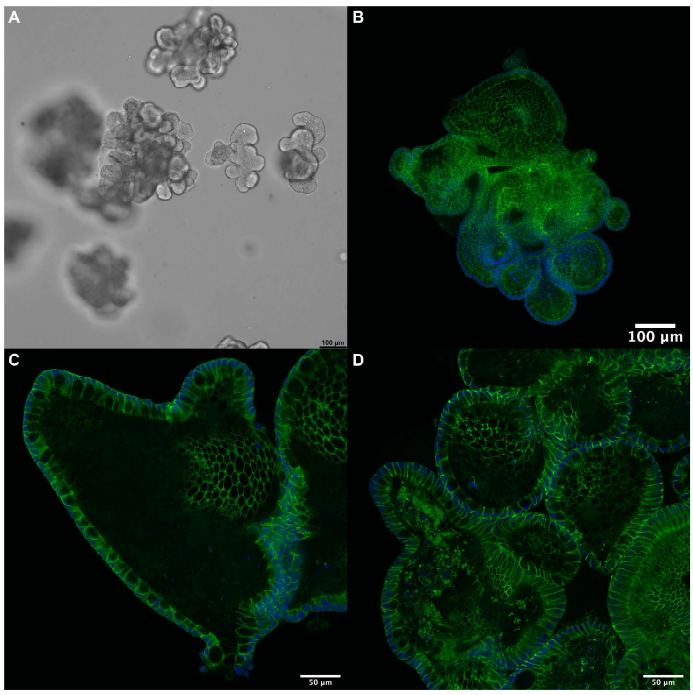

FIGURE 2
3D intestinal organoids for drug discovery. **(A)** Canine enteroids (shown in culture, phase contrast microscopy) pose an advanced *in vitro* model to investigate the pathophysiology of canine CIE further. These organoids allow the study of epithelial transport, inflammation, and barrier function: immunofluorescent staining (green) for **(B)** occludin, **(C)** claudin-1, and **(D)** claudin-7 indicates the formation of a functional polarized epithelium expressing tight junction proteins. Cell nuclei are counterstained with DAPI (blue). Scale bars: 100µm **(A,B)** and 50µm **(C,D)**.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publication IV

The intricacies of inflammatory bowel disease: redox biology in intestinal organoids

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Communication

The Intricacies of Inflammatory Bowel Disease: A Preliminary Study of Redox Biology in Intestinal Organoids

Georg Csukovich [1,†], Janina Huainig [1,†], Selina Troester [2], Barbara Pratscher [1] and Iwan Anton Burgener [1,*]

[1] Small Animal Internal Medicine, Vetmeduni, 1210 Vienna, Austria; georg.csukovich@vetmeduni.ac.at (G.C.)
[2] Institute for Medical Biochemistry, Vetmeduni, 1210 Vienna, Austria
* Correspondence: iwan.burgener@vetmeduni.ac.at
† These authors contributed equally to this work.

Abstract: We evaluated the redox status, precisely glutathione levels, which have a major impact in cellular detoxification and antioxidant defence in IBD-derived and healthy intestinal organoids. Therefore, we wanted to explore the differences in terms of their redox balance and mitochondrial fitness. To this end, we introduced a Grx1-roGFP2 construct into the organoids by lentiviral transduction before performing a stress assay by treating the organoids with hydrogen peroxide and examined the GSH/GSSG ratio using confocal imaging. Using ratio imaging, we could detect statistically significant differences between healthy and IBD-derived samples. To gain more insight, we also performed a GSH/GSSG assay, which directly measured glutathione levels. This analysis revealed that both organoid lines had higher levels of oxidized glutathione due to the stress treatment demonstrated by a lower GSH/GSSG ratio compared to the untreated control. Nevertheless, the results showed no significant difference between healthy and IBD-derived organoids. We further challenged organoids with hydrogen peroxide after incubation with MitoTracker® to see if mitochondrial fitness might be different in IBD-derived organoids. However, these results were also very comparable. In summary, our preliminary findings indicate that both organoid lines demonstrate a well-functioning system in terms of analysis but show no clear difference between healthy and IBD-derived samples.

Keywords: intestinal organoid; redox biology; IBD; glutathione; oxidative stress; ROS; redox imaging

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1. Introduction

In vitro cultured organoids can model the physiological and morphological characteristics as well as cell signaling pathways within humans or animals. Gastrointestinal organoid cultures are most commonly derived from adult stem cells (ASC) that reside in the crypts of the gastrointestinal tract. The stem cells can be isolated from the tissue and retain their function and mutations from their residence site in the gut when maintained under defined cell culture conditions [1]. This important step allows us to create a model that imitates organ function, composition and development. Current research largely comprises the study of normal and pathological circumstances and their application to test possible therapeutics for different diseases [2,3].

One major affliction of the gut is inflammatory bowel disease (IBD), which leads to the infiltration of lymphocytes and macrophages, i.e., inflammation, and to general signs of gastrointestinal dysfunction like weight loss and diarrhea. Genetic and environmental changes have been implicated with the occurrence of IBD and lead to the overgrowth of pathogenic bacteria with the outcome of inflammation and intestinal injury. The exact underlying reasons that trigger this kind of disease are still not well understood [4,5]. However, interestingly many pet animals like dogs develop IBD spontaneously, while rodents like mice require an induction of IBD-like symptoms in order for them to serve as an IBD model [6]. Unlike humans, canine IBD is not classified into Crohn's disease and ulcerative colitis, but is rather a chronic disease diagnosed by the exclusion of common causes (e.g., tumors) and the histologic confirmation of the inflammation of the intestinal mucosa [7,8].

A critical factor contributing to IBD could be reactive oxygen species (ROS) [9,10]. Redox reactions are key functions for the whole mechanism to transport electrons from a donor to an acceptor molecule, and thus have an important role in various signaling processes within our bodies. Different types of ROS include hydrogen peroxide (H_2O_2), nitric oxide (NO), hydroxyl radicals (OH^-) and many more that are usually tightly controlled, but can oxidize lipids and proteins when they accumulate, and therefore harm the cell [11].

The two main sites for ROS production are the cytoplasm and the inner mitochondrial membrane. The formation of mitochondrial ROS (mtROS) happens under physiological respiratory conditions as a consequence of proton leakage and is detoxified by mitochondrial peroxiredoxins [12]. However, this process can be amplified due to cellular stress and high amounts of mtROS can cause the induction of mitophagy and apoptosis [13]. Since H_2O_2 originating from mitochondria can readily diffuse across cellular membranes, it can also be highly toxic to the entire cell if not eliminated immediately. This process is mainly carried out by glutathione, which can be found in its reduced form (i.e., GSH) and its oxidized form (i.e., GSSG) throughout the cell and in various organelles [14,15].

Usually, glutathione is present in larger quantities in its reduced form in healthy and balanced conditions. According to this information, the GSH/GSSG ratio can be used to determine the stress status of the cell, with low GSH/GSSG ratios indicating physiological conditions. If the cell is exhausted because of stress treatment, the oxidized form of glutathione accumulates and the GSH/GSSG ratio is higher. Therefore, the balance of reduced (GSH) and oxidized (GSSG) glutathione is essential for the maintenance of normal homeostasis [15,16]. If these conditions are skewed for whatever reason and the balance between ROS generation and elimination is tilted, the cell is undergoing so-called "redox stress".

The aim of this study was to investigate whether intestinal organoids derived from IBD patients are more sensitive to extrinsically induced redox stress and cannot withstand ROS as well as healthy organoids. Therefore, we analyzed the capability of canine intestinal organoids derived from healthy and IBD dogs to deal with redox stress induced by hydrogen peroxide by determining GSH/GSSG ratios derived from glutaredoxin-1-based redox imaging. In addition, we employed an assay, which directly measures the relative amounts of oxidized and total glutathione. We also considered mitochondria as a major source of intracellular ROS by staining active mitochondria with MitoTracker® to analyze whether IBD-derived organoids might show a mitochondrial defect and therefore produce extensively more ROS upon H_2O_2 challenge. Overall, we could not find any significant difference between healthy and IBD-derived organoids in terms of their redox balance. However, we successfully established a relevant model to analyze the redox system in intestinal organoids, with the potential to expand this system to live cell imaging in the future.

2. Materials and Methods

2.1. Cultivation of Intestinal Organoids

Canine intestinal crypts were isolated from duodenum according to Kramer et al., 2020 [17]. Canine IBD is mostly evident in the duodenum of affected dogs and histologic diagnosis is commonly based on duodenal biopsies. Based on the guidelines of the institutional ethics committee, the use of tissue material collected during therapeutic excision or post-mortem is included in the University's "owner's consent for treatment", which was signed by all patient owners. The growth medium consisted of 37% basal medium (Advanced DMEM/F12 supplemented with 2 mM GlutaMAX and 10 mM HEPES), 1 × B27 (Invitrogen, ThermoFisher Scientific, Vienna, Austria), 1 mM N-Acetylcysteine (ThermoFisher Scientific, Vienna, Austria), 10 nM Gastrin (Sigma Aldrich, Vienna, Austria), 100 ng/mL Noggin, 500 nM A8301 (Bio-Techne Ltd., Minneapolis, MN, USA), 50 ng/mL HGF (PeproTech, Rocky Hill, NJ, USA), 100 ng/mL IGF1 (PeproTech, Rocky Hill, NJ, USA), 50 ng/mL FGF2 (PeproTech, Rocky Hill, NJ, USA), 10% (*v*/*v*) Rspondin1 and 50% (*v*/*v*) Wnt3a conditioned media. For the first two days of culture, 50 ng/mL mEGF (ThermoFisher Scientific, Vienna, Austria) and 10 µM Rock-inhibitor Y-27632 (Selleck Chemicals, Hous-

ton, TX, USA) were added. The growth medium was changed every two to three days. Weekly passaging at 1:4 to 1:8 split ratios was achieved by mechanical disruption using flame-polished Pasteur pipettes.

2.2. Immunofluorescent Analysis of Organoids

Organoids were fixed with 2% paraformaldehyde (PFA) for 15 min and stained according to a previously published protocol including a clearing step after organoid staining [18]. Organoids were stained with the 1:100 Claudin 7 Polyclonal Antibody (Invitrogen, ThermoFisher Scientific, Vienna, Austria) for tight junctions with the secondary antibody AF-488 goat anti-rabbit (Invitrogen, ThermoFisher Scientific, Vienna, Austria) diluted 1:500, 1:200 phalloidin (Alexa Fluor 647, Invitrogen, ThermoFisher Scientific, Vienna, Austria) to visualize actin filaments, and with 4 µg/mL 4′,6 diamino-2-phenylindole (DAPI; Sigma-Aldrich, Vienna, Austria) for nuclear staining. Confocal images were acquired using a Zeiss LSM 880 confocal microscope (Zeiss, Jena, Germany).

2.3. Production of Lentiviral Particles and Transduction of Organoids

In order to generate organoids expressing a redox-sensitive GFP, lentiviral particles carrying the Grx1-roGFP2 plasmid had to be produced and subsequently transduced into the respective organoids. pEIGW Grx1-roGFP2 was a gift from Tobias Dick (Addgene plasmid # 64990; http://n2t.net/addgene:64990, acessed on 8 August 2023; RRID:Addgene_64990).

On day one, the Lenti-X 293T cells (Takara bio, Saint-Germain-en-Laye, France) were split with trypsin and seeded in 10 cm2 dishes. The next day, 4 µg Grx1-roGFP2 plasmid and 2 µg psPAX2 and 1 µg pMD2.G packaging plasmids were mixed with 3 µL Polyethyleneimine HCI MAX (1 mg/mL; PEI; Polysciences Europe) per µg of DNA and incubated for 20 min at room temperature. psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260; http://n2t.net/addgene:12260, acessed on 8 August 2023; RRID:Addgene_12260) and pMD2.G was a gift from Didier Trono (Addgene plasmid # 12259; http://n2t.net/addgene:12259, acessed on 8 August 2023; RRID:Addgene_12259). This transfection mix was added dropwise to Lenti-X 293T cells and incubated overnight. On the third day, the medium was changed to organoid transfection medium, which consisted of Advanced DMEM/F12 (Fisher Scientific), 1 × GlutaMAX (ThermoFisher Scientific, Vienna, Austria), 10 mM HEPES (ThermoFisher Scientific, Vienna, Austria), 1 × B27 (ThermoFisher Scientific, Vienna, Austria), 1 × N2 (ThermoFisher Scientific, Vienna, Austria), 1 mM N-Acetylcysteine (ThermoFisher Scientific, Vienna, Austria), 500 nM A8301 (Bio-Techne Ltd., Minneapolis, MN, USA), 10 µM SB202190 (Selleck Chemicals, Houston, TX, USA), 10 nM Gastrin (PeproTech, Rocky Hill, NJ, USA), 50 ng/mL HGF (PeproTech, Rocky Hill, NJ, USA), 50 ng/mL mEGF (ThermoFisher Scientific, Vienna, Austria), 100 ng/mL mNoggin (PeproTech, Rocky Hill, NJ, USA), 10 µM Y-27632 (Selleck Chemicals) and 5 µM CHIR99021 (Sigma-Aldrich, Vienna, Austria). On the following two days, the lentiviral particles were harvested by filtration through a 45 µm filter (qPORE) using a syringe. The virus supernatant was stored at 4 °C until further use.

For the transduction of the organoids, 1×10^6 cells of trypsinized organoids were seeded in 1.5 mL organoid transfection medium in a 6-well plate. To each well, 1.5 mL virus supernatant and 10 µg/mL Polybrene transfection reagent (Merck, Darmstadt, Germany) were added. The organoids were transfected via spinoculation, which was conducted for one hour at 600 × g and subsequent incubation for three hours at 37 °C. After this step, the suspension was centrifuged at 300× *g* for 5 min followed by two washing steps with DPBS. The cells were seeded in 24-well plates and after a few days sorted by Flow Cytometry (SRT, Beckman Coulter, Vienna, Austria) selecting for GFP positive cells to obtain a pure population of Grx1-roGFP2 expressing organoids.

2.4. Glutaredoxin-Based Redox Imaging

For redox imaging, organoids embedded in Geltrex in 18-well glass bottom µ-slides (Ibidi, Fräfelfing, Germany) were incubated with different concentrations of H_2O_2 (100,

250, 500, 1000 and 1500 µM) for 10 min. Subsequently, organoids were "redox-fixed" with 10 nM N-ethylmaleimide (NEM) for another 10 min, followed by 15 min of fixation with 2 % PFA. After removal of PFA, organoids were washed with PBS and stored at 4 °C until imaging. Organoids were imaged according to Gutscher et al., 2008, being excited at two different wavelengths (405 nm and 488 nm) [19]. Image analysis was carried out in ImageJ. Organoid outlines were generated by adapting the threshold of the image until the organoid outlines could be defined. Only the mean fluorescent intensities within the organoid outlines were measured.

2.5. Glutathione Analysis

To analyze the levels of oxidized and total glutathione, organoid-derived monolayers were established. To this aim, organoids were released from the Geltrex matrix via repeated pipetting, trypsinized, and resulting single cells were counted. Subsequently, 12,000 cells/well were seeded in 96-well plates pre-coated with 100 µg/mL Geltrex (ThermoFisher Scientific, Vienna, Austria) diluted in basal medium at 37 °C for 1 h. The GSH/GSSG-Glo™ Assay was carried out according to the instructions of the manufacturer (Promega, Madison, WI, USA) for both healthy and IBD-derived monolayers in triplicates once reaching confluence. Cells were stressed with 1000 µM H_2O_2 for 10 min.

2.6. Mitochondrial Stress Analysis

For the analysis of mitochondrial fitness, organoids were incubated with 500 nM MitoTracker® CM-H2Xros for 20 min at 37 °C. Subsequently, organoids were stressed with 1000 µM H_2O_2 to simulate ROS production for 10 min and then fixed with 2 % PFA for another 15 min. After washing with PBS, organoids were stored at 4 °C until confocal imaging. Organoids were analyzed in the same way as ratio images from redox imaging above by outlining the organoids and only analyzing the mean fluorescence intensities of the organoids themselves, excluding any surrounding signal.

2.7. Statistical Analysis

All statistical analyses were performed in Graphpad Prism 10 (GraphPad Software, Boston, MA, USA). *p*-values below 0.05 were considered significant.

3. Results

3.1. Immunofluorescent Analysis

To gain insights into how IBD-derived organoids might be different from healthy patient-derived organoids, we combined DAPI staining with phalloidin to visualize microvilli at the apical cell surface and tight junction staining with Claudin 7. As evidenced by Figure 1, both healthy and IBD-derived organoids present their apical cell surface into the organoid lumen. Additionally, both organoids demonstrate intact tight junctions, i.e., well-structured epithelial barriers.

3.2. Redox Imaging

We used our healthy and IBD-derived organoids, which expressed a redox-sensitive roGFP2-coupled Grx1 protein to analyze the redox balance of the organoids based on the ratio of oxidized to reduced glutathione (GSSG/GSH). The fusion of roGFP2 to glutaredoxin allows for the live cell imaging of the intracellular redox balance since roGFP2 can be excited at two different wavelengths (i.e., 405 nm and 488 nm), depending on its redox state, therefore making ratio calculation possible. After splitting the two acquired image channels and calculating their 405 nm/488 nm ratio, images are presented using the *Fire* filter of ImageJ, which nicely illustrates the resulting ratios with higher values appearing lighter. The results indicate significantly elevated ratios with increasing concentrations of H_2O_2 in both groups. We could observe a slight but significant difference between healthy and IBD-derived organoids (*p*-value 0.0438), showing that in IBD-derived organoids, less glutaredoxin, and hence glutathione, seems to become oxidized (Figure 2A–D).

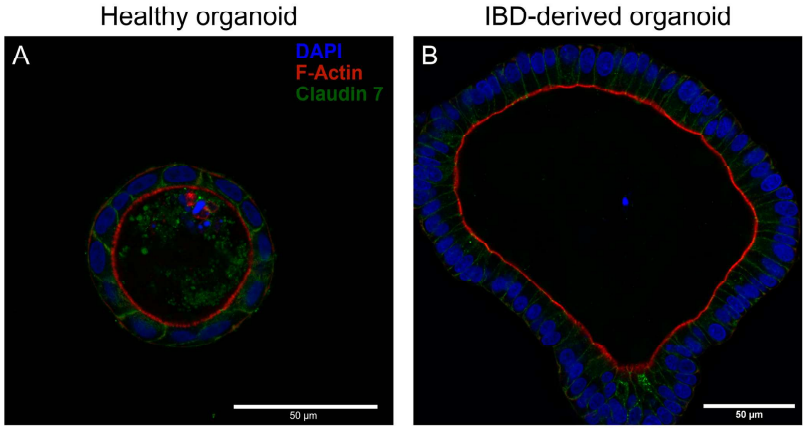

Figure 1. Representative confocal images of Claudin 7/phalloidin/DAPI-stained healthy (**A**) and IBD-derived (**B**) organoids. Both organoids show orientation of phalloidin into the lumen of organoids and well-established cell–cell contacts as evidenced by Claudin 7 staining.

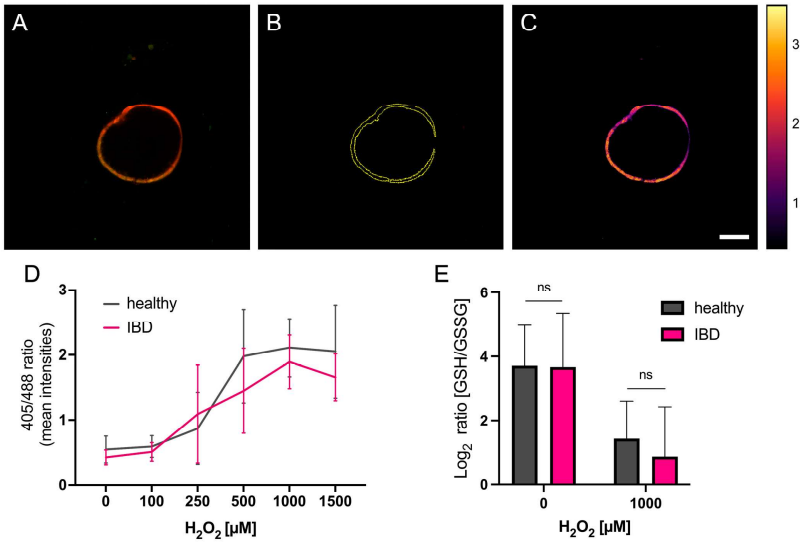

Figure 2. Analysis of ratio imaging with ImageJ. (**A**) Overlay of an exemplary organoid treated with 250 µM H_2O_2 with the two acquired images of both excitation wavelengths (405 nm/488 nm). (**B**) Boundary of the organoid determined by ImageJ. (**C**) Ratio image of the organoid with the *Fire* filter. The boundaries from (**B**) are used to only calculate the ratio of the area of the organoid. Brightness and contrast of (**C**) have been enhanced for visualization purposes only. Scale bar = 100 µm. (**D**) Analysis of ratio imaging from healthy and IBD-derived organoids shows a significant difference between the two organoid lines when increasing H_2O_2 stress treatment (2-way ANOVA: H_2O_2 concentration p-value < 0.0001; health p-value = 0.0438). (**E**) Results from glutathione quantification. GSH/GSSG ratios are not significantly different between healthy and IBD-derived organoid-derived monolayers under both non-stressed and H_2O_2-stressed conditions (Welch t-test). n = three technical replicates including 2–5 evaluated organoids per concentration.

3.3. Glutathione Analysis

To validate the results previously obtained from redox imaging, which only yields a proxy for intracellular glutathione levels and is therefore solely an estimate of the real redox balance, we used a commercial kit to quantify the amount of GSH and GSSG in our samples. These experiments were performed on organoid-derived monolayers (ODM) for standardization reasons, as analyzing a confluent monolayer is easier to define than having the same number of organoids in each well. The results demonstrate that the GSH/GSSG ratios are almost the same in untreated samples. Furthermore, ODMs challenged with H_2O_2 show a clear decrease in the GSH/GSSG ratio. IBD-derived ODMs decrease even further, but not significantly, thus supporting the result from redox imaging (Figure 2E).

3.4. Mitochondrial Stress

Since the mitochondrion is the primary source when it comes to intracellular ROS production, it is crucial to also consider mitochondria when analyzing oxidative stress in organisms. Therefore, we stained active mitochondria in healthy and IBD-derived organoids and looked for differences in stress levels in those samples.

The results clearly demonstrate that cells of both organoid lines appear more stressed after H_2O_2 treatment compared to the untreated controls. With induced oxidative stress, the cells of both organoid lines indicate a higher stress level by increased mean grey values. However, the comparison of both lines demonstrates no significant difference in the level of oxidative stress in the mitochondria (Figure 3).

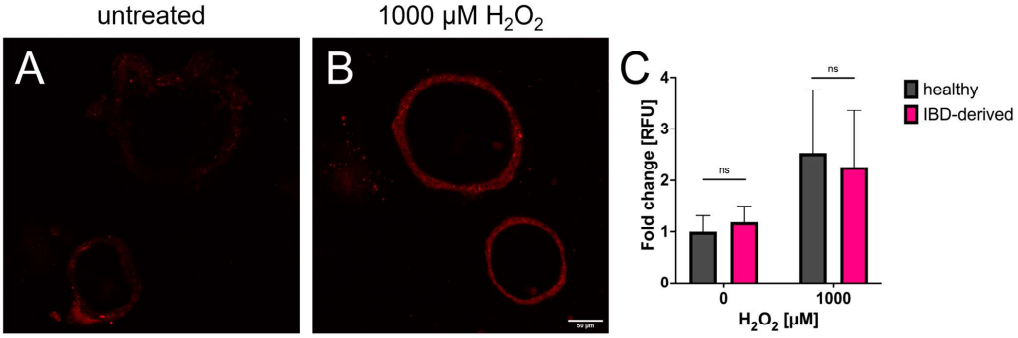

Figure 3. The results from Mitotracker analysis. (**A**,**B**) Representative confocal images of Mitotracker-treated organoids showing the difference between untreated organoids and organoids after being stressed with H_2O_2. Brightness and contrast have been enhanced for visualization purposes only. (**C**) Data from image analysis normalized to untreated healthy organoids. Both healthy and IBD-derived organoids have comparable mitochondrial activity in both stressed and non-stressed conditions (Welch *t*-test). n = three technical replicates including 2–5 evaluated organoids per concentration.

4. Discussion

The aim of this study was to isolate adult stem cells from the duodenum of a healthy dog and one IBD patient to establish a culture of intestinal organoids. Furthermore, we wanted to identify differences between the two organoid samples and test their response to oxidative stress by comparing these two contrary starting conditions.

Several studies have previously analyzed IBD-derived organoids in different regards. For example, d'Aldebert described in 2020 that human IBD-patient-derived organoids present apical-out properties, which means that unlike healthy organoids, IBD-organoids present their apically localized microvilli to the outside. This phenomenon could also be mimicked by incubating healthy organoids with an inflammatory cocktail of three different cytokines (IL-1β, IL-6, TNF-α), which indicates that inflammatory signals are responsible for flipped organoid polarity [20]. However, we could not see that same result in our canine

organoids since both healthy and IBD-derived organoids exhibit proper basal-out polarity with inward-facing microvilli [20]. Other studies focused on the transcriptional profiling of healthy versus IBD-derived organoids and found the overexpression of Paneth cell gene expression in organoids derived from patients with ulcerative colitis (UC) [21] or decreased expression of goblet cell marker gene mucin 2 (*MUC2*) in Crohn's disease patients [22]. These results indicate an atypical apportionment of intestinal cell types that can influence intestinal homeostasis and propagate chronic inflammation. An abnormal distribution of cell types within the intestine can lead to the differential expression of key enzymes responsible for the generation of intracellular ROS including DUOX2 and NOX1, which should be analyzed in future experiments [23].

As mentioned above, intestinal inflammation is accompanied by the production of ROS, which may have further detrimental effects on the healing of lesions and the regeneration of the intestine [9,10,24]. However, there are no reports investigating the role of ROS in IBD-derived organoids in vitro yet. Therefore, we analyzed the antioxidant system of healthy and IBD-derived organoids in several ways to determine whether these organoids have an intrinsically lower antioxidant potential. Furthermore, we show that mitochondrial function is not different between healthy and IBD-derived organoids and is not a probable determinant of chronic intestinal disease.

Our results show only slight differences between IBD-derived and healthy organoids when treated with hydrogen peroxide as a stress inducer. The main purpose was to see how the glutathione ratio changes after stress treatment within different concentrations. The fact that IBD organoids show a significantly lower GSSG/GSH ratio in our ratio imaging approach is interesting and unexpected, as IBD organoids were expected to be more sensitive to redox stress compared to healthy organoids. It is tempting to speculate that IBD-derived organoids might possess upregulated basal levels of glutaredoxin expression to cope with increased levels of ROS in vivo.

The results from the GSH/GSSG assays correlate well with the results obtained from ratio imaging. While an increase in ratio up to about 2-fold could be measured in ratio imaging with increasing concentrations of H_2O_2, GSH/GSSG assays show a greater than 2-fold reduction compared to oxidized glutathione. As the ratios are calculated reversely, both assays show the same result. Therefore, both systems are well-suited for redox analysis. Nevertheless, we could not observe a shift towards more glutathione in IBD-derived organoids.

We would like to point out that the concentrations of H_2O_2 in our assays are likely beyond physiological levels [25]. However, concentrations up to 1.5 mM have previously been used in combinations with the same Grx1-roGFP2 sensor to analyze the antioxidant system of cells [16]. Since we attempted to analyze the organoids' ability to absorb the shock of being stressed, we used higher levels of hydrogen peroxide to analyze the full potential to eradicate ROS. Higher levels of H_2O_2 may also be necessary to achieve quick diffusion through the extracellular matrix (ECM) that will differ from analysis on cells without them being embedded in the ECM [26].

5. Conclusions

In summary, our results indicate neither a drastically imbalanced antioxidant system in IBD-derived organoids nor a malfunction of mitochondria. Despite these results, the redox system should not be ruled out entirely as a factor contributing to IBD, as our IBD-derived organoids behave differently upon H_2O_2 challenge compared to healthy organoids in ratio imaging. Many other parameters in vivo may influence redox balance that remain to be modelled in vitro in future studies. These include the incorporation of other cell types like fibroblasts, endothelial cells, and immune cells into three-dimensional organoid cultures.

In 2015, co-culturing intestinal organoids with lamina propria lymphocytes showed an increased size of organoids, elevated intestinal stem cell regeneration and proliferation dependent on IL-22. In addition, the introduction of monocyte-derived macrophages and an infection via *E. coli* in human colon organoids led to an altered macrophage morphology,

increased migration, cytokine secretion and mirrored inflammatory bowel disease [27]. However, co-cultures with immune cells are still not state of the art in organoid research as it makes intestinal organoid cultures much more complex and more difficult to analyze but could also help to elucidate miniscule differences between healthy and IBD-derived organoids that may have relevant overall effects.

Nonetheless, our study relied on a very small sample size and should be validated with a higher number of organoids derived from different patients as our results are still very preliminary. Additionally, the process of inflammation could be different between canines and humans, which might explain the difference in organoid polarity compared to d'Aldebert et al., 2020. One more reason for the lack of differences between healthy and IBD-derived samples can be the difficulty in the diagnosis of IBD itself. There are multiple symptoms of the disease, which can overlap with symptoms of various other illnesses (e.g., variable stool consistency and frequency) [28].

However, the advancement of this organoid model system may offer a promising approach to promote the goals of One Health and help to further reduce animal testing and optimize animal welfare in the world of science.

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Publication V

Polarity reversal of canine intestinal organoids reduces proliferation and increases cell death

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ORIGINAL ARTICLE

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Polarity reversal of canine intestinal organoids reduces proliferation and increases cell death

Georg Csukovich[1] | Maximilian Wagner[1] | Ingrid Walter[2,3] | Stefanie Burger[2] | Waltraud Tschulenk[3] | Ralf Steinborn[4] | Barbara Pratscher[1] | Iwan Anton Burgener[1]

[1]Department for Companion Animals and Horses, Small Animal Internal Medicine, Vetmeduni, Vienna, Austria

[2]VetBioBank, VetCore, Vetmeduni, Vienna, Austria

[3]Institute of Morphology, Working Group Histology, Vetmeduni, Vienna, Austria

[4]Genomics Core Facility, VetCore, Vetmeduni, Vienna, Austria

Correspondence
Georg Csukovich, Department for Companion Animals and Horses, Small Animal Internal Medicine, Vetmeduni, 1210 Vienna, Austria.
Email: georg.csukovich@vetmeduni.ac.at

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Abstract

Apical-out intestinal organoids are a relatively simple method of gaining access to the apical cell surface and have faced increasing scientific interest over the last few years. Apical-out organoids can thus be used for disease modelling to compare differing effects on the basolateral versus the apical cell surface. However, these 'inside-out' organoids die relatively quickly and cannot be propagated as long as their basal-out counterparts. Here, we show that apical-out organoids have drastically reduced proliferative potential, as evidenced by immunohistochemical staining and the incorporation of the thymidine analogue EdU. At the same time, cell death levels are increased. Nevertheless, these phenomena cannot be explained by an induction of differentiation, as the gene expression of key marker genes for various cell types does not change over time.

1 | INTRODUCTION

Gastrointestinal diseases represent a major health burden all over the world. One of the most important gastrointestinal diseases in humans is inflammatory bowel disease (IBD) with an estimated 6.8 million people living with IBD.[1] Interestingly, IBD does not only occur in humans but also affects animals like dogs and cats, but it is not a naturally occurring disease in common laboratory rodents.[2] Therefore, gastrointestinal diseases do not only cause immensely high costs for health care systems but also to pet owners and require in-depth analysis as commonly used treatments are unsatisfactory and the underlying cause for IBD is multifactorial and still not entirely known.[3]

In the search of developing new in vitro model systems that resemble physiological properties found in vivo, organoids have been around for almost 15 years since the first establishment of intestinal organoids from *Lgr5*$^{+}$ stem cells.[4] In the meantime, organoids comprise a variety of different organs, including several gastrointestinal ones like stomach,[5] liver[6] and pancreas,[7] besides the classical intestinal organoids. The complex three-dimensional architecture of organoids, which consist of several different cell types (e.g., stem cells, enterocytes, goblet cells and enteroendocrine cells), can be established from either adult stem cells or induced pluripotent stem cells (iPSCs) via targeted differentiation. In this regard, canine intestinal organoids are not only necessary for veterinary research, but could also potentially replace live dogs used for research (especially pharmacological testing) in the future.

However, intestinal organoids bear the major drawback of the apical cell surface of epithelial cells being inaccessible and hidden on the inner surface of the organoids. Han et al. provide a very good review on the possibilities one has to access the apical surface.[8] Apart from the possibility to physically disrupt organoids to reach the apical side temporarily, generating organoid-derived monolayers[9] or using microinjection techniques[10] have become alternative approaches. Another feasible method is the generation of so-called 'apical-out' organoids. In regard to this method, we have previously reviewed different disease modelling approaches using intestinal organoids, especially in a One Health context.[11]

Apical-out organoids were first published by Co et al., who have demonstrated that human intestinal organoids that usually grow

embedded in a three-dimensional extracellular matrix (ECM) can reverse their polarity when they are cultured in a floating manner in medium without ECM.[12] This method has now been adopted by many other labs and expanded to different animal species, for example, pigs,[13] chickens,[14] cows[15] and dogs,[16] and also organoids derived from human iPSCs and embryonic stem cells.[17,18] Remarkably, regardless of the species from which the organoids were derived, the majority of these studies utilised their apical-out organoids within the initial 7 days following the induction of polarity reversal.[13,16,17,19–21] Experiences from our lab show, that canine intestinal apical-out organoids produce a lot of cell debris within the culture well, which is most probably due to dying cells being extruded from the epithelial layer of cells directly into the medium. This phenomenon increases until apical-out organoids slowly start to die off completely after approximately 1 week of culture. Taken together, these observations lead to the hypothesis that the polarity reversal of intestinal organoids leads to a change in proliferative behaviour and a higher rate of apoptotic cells in apical-out organoids compared to basal-out organoids.

According to previous reports, whether the induction of polarity reversal is associated with organoid differentiation is to some extent controversial. Two studies show that apical-out organoids do not present immensely different gene expression profiles compared to their basal-out counterparts as long as they are cultured in their standard growth medium.[12,18] However, according to another study using porcine organoids, the expression of the stem cell marker termed leucine-rich repeat-containing G-protein coupled receptor 5 (*LGR5*) is diminished after 3 days of apical-out culture and differentiation markers chromogranin A (*CHGA*; enteroendocrine cells), mucin-2 (*MUC2*; goblet cells) and intestinal alkaline phosphatase (*ALPI*; enterocytes) show strikingly elevated expression.

In this study, we successfully established apical-out intestinal organoids from canine small and large intestines and quantified the rate at which organoids reverse their polarity after ECM removal. Additionally, we show that apical-out organoids have drastically decreased number of actively proliferating cells after 36 h. We then analysed organoid viability and cell death in basal-out and apical-out organoids over a period of 72 h and discovered that apical-out organoids have lower cell viability-coupled with higher cell death rates, which might explain why apical-out organoids die after a few days in culture. However, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) data show that this increase in cell death does not coincide with increased differentiation in canine intestinal organoids.

Overall, we would like to emphasise that newly established model systems require careful characterisation before their utilisation as certain attributes can fundamentally influence outcomes and potentially skew findings.

2 | RESULTS

2.1 | Polarity reversal

We quantified the efficiency of polarity reversal over time, in intervals of 12 h, after inducing polarity reversal. This quantification was based on DAPI/Phalloidin stainings of organoids as can be seen in Figure 1G,H. Successful polarity reversal was also evidenced by transmission electron microscopy (TEM) of basal-out and apical-out organoids (Figure 1A–F). While basal-out organoids present their apical microvilli into the organoid lumen, apical-out organoids are oriented in the other direction. Organoids feature apical desmosomes and a basal lamina, as seen in TEM images. Unexpectedly, nearly 40% of organoids exhibited apical-out polarity at the onset of the polarity reversal (Figure 1I), with the majority of the remaining organoids displaying mixed polarity. This was despite the fact that the vast majority of organoids show a basal-out state before ECM removal. However, we could verify that 72 h after inducing polarity reversal, almost 100% of organoids are apical-out with a small number of mixed polarity organoids and absolutely no basal-out oriented organoids. On the other hand, floating basal-out controls remain in a basal-out state 72 h after harvest.

Treatment with α-ITGB antibody at a concentration of 3 μg/mL for 3 days led to mixed polarity organoids, with no full polarity reversal (Figure 2). Further increasing the concentration of α-ITGB merely led to an increase in cell death. Thus, attempting to reverse the polarity of canine intestinal organoids with α-ITGB is not advisable and does not yield efficiently re-polarised organoids for downstream experiments.

2.2 | EdU staining

To get an overview of the proliferation status of organoids after polarity reversal, we incubated organoids with 10 μM EdU for 1.5 h every 12 h starting at the time point when polarity reversal was induced. While proliferation, as measured by EdU^+ cells, was high right until the end at 72 h in basal-out organoids, apical-out organoids showed a clear reduction of proliferating cells. The decrease in proliferation was most pronounced comparing the 24-h time point to the 36-h time point after polarity reversal, where we noticed a clear drop of EdU^+ cells, exemplified by data from small intestinal organoids (Figure 3A). A very similar pattern can also be seen in organoids from the large intestine (Figure S1). Image analysis using Arivis4D allowed us to quantify the amount of EdU^+ cells over time, highlighting the clear difference between basal-out and apical-out organoids with a significant interaction effect of time × polarity (p value 0.0028).

Although treatment with α-ITGB antibody was unsuccessful in inducing complete polarity reversal, integrin signalling will be reduced in apical-out organoids due to the absence of ECM, with the lack of signalling ultimately impacting the MAPK pathway.[22,23] Therefore, we attempted to re-activate MAPK-signalling using the small-molecule activator Senkyunolide I (SENI), an agonist of Erk1/2. However, boosting MAPK signalling did not lead to higher numbers of EdU^+ cells in apical-out organoids after 3 days of SENI treatment compared to untreated controls (Figure S2).

2.3 | Viability and cell death

After noticing that organoids seemingly stop proliferating after reversing their polarity, we used viability and apoptosis/necrosis assays for

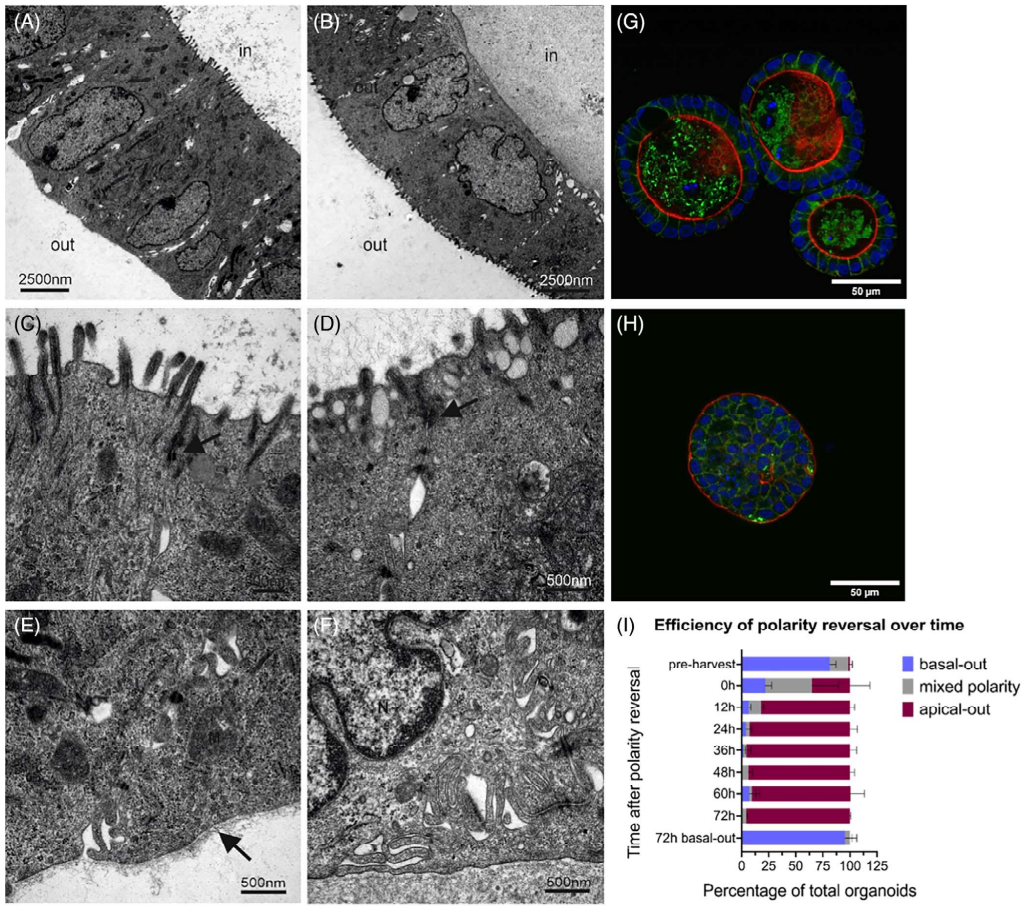

FIGURE 1 Polarity reversal of canine intestinal organoids. (A–F) Electron microscopic images representing basal-out (A, C, E) and apical-out (B, D, F) organoids. Arrows indicate desmosomes in (C) and (D) and a basal lamina in (E). M = mitochondria; N = nucleus. Immunofluorescent stainings of basal-out (G) and apical-out (H) organoids with Claudin-7 (green) and Phalloidin (red) representing the flipped polarity. Nuclei were stained with DAPI (blue). (I) Quantitative analysis of organoid repolarisation over time. Data are presented as mean ± standard deviation.

further analysis of the difference between basal-out and apical-out organoids. The viability assay, serving as an indicator of cell mass, reveals that basal-out organoids continue to increase their cell mass, that is, proliferate, until the final time point at 72 h following polarity reversal. However, apical-out organoids only show a small peak at 12 h post-polarity reversal with subsequently decreasing viability values. This is consistent with apoptosis and necrosis measurements, in which apical-out organoids generally present slightly higher values than basal-out organoids. Overall, all samples show highly significant values when analysing the difference between basal-out to apical-out organoids over a time course of 72 h, except for colonic apoptosis, where polarity did not have a significant effect (Figure 4 and Table S1).

These data were further verified with immunohistochemical staining of Ki-67 (for proliferation) and cleaved caspase-3 (for apoptosis) in both small intestinal (Figure 5) and large intestinal organoids (Figure S3). Immunohistochemical stainings fit very well to the aforementioned data of higher proliferation in basal-out organoids and slightly increased cell death in apical-out organoids. While all organoids show similar properties at time point 0, basal-out organoids are steadily increasing in size, cell number and the amount of Ki-67^{+} cells. Apical-out organoids generally appear more unstructured with the single epithelial layer being less clear at 72 h after polarity reversal (see also Figure 1H). Especially areas in the centre of apical-out organoids show more cleaved caspase-3^{+} cells.

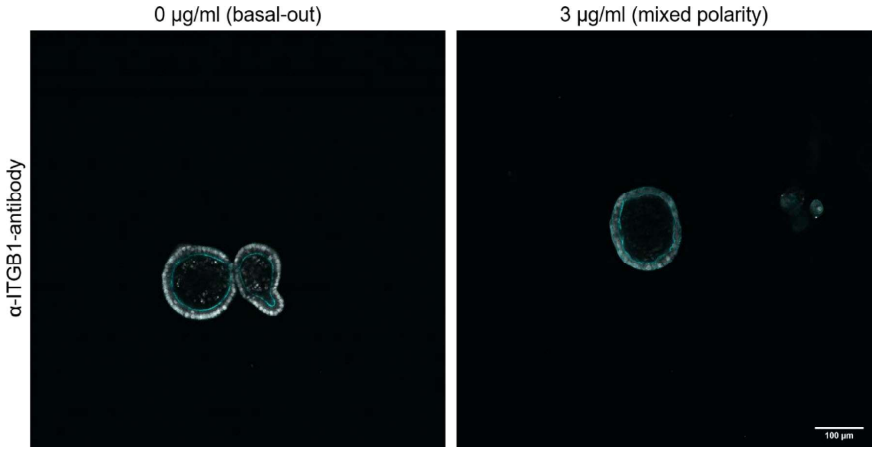

FIGURE 2 Organoids incubated with α-ITGB1-antibody for 3 days partially reverse their polarity. In contrast to control organoids (left), which remain in a basal-out state, ITGB1-antibody treated organoids present a mixed polarity state in which Actin staining (cyan) indicative of microvilli can be seen on both surfaces of the organoids. Nuclei were stained with Hoechst (grey).

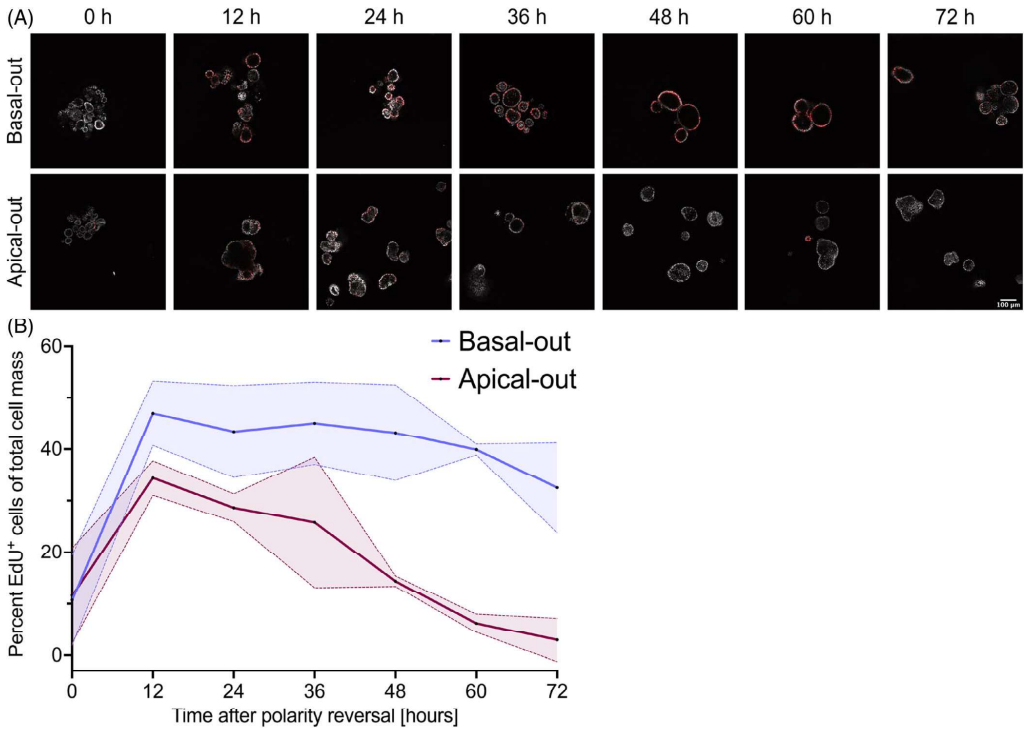

FIGURE 3 (A) Staining of EdU incorporation (red) in small intestinal basal-out and apical-out canine intestinal organoids over 72 h, highlighting the reduced proliferation of apical-out organoids compared to their basal-out counterparts. Nuclei were stained with Hoechst (grey). (B) Quantification of EdU^+ cells of both small and large intestinal basal-out and apical-out organoids. There is a significant interaction effect of time × polarity (p value 0.0028).

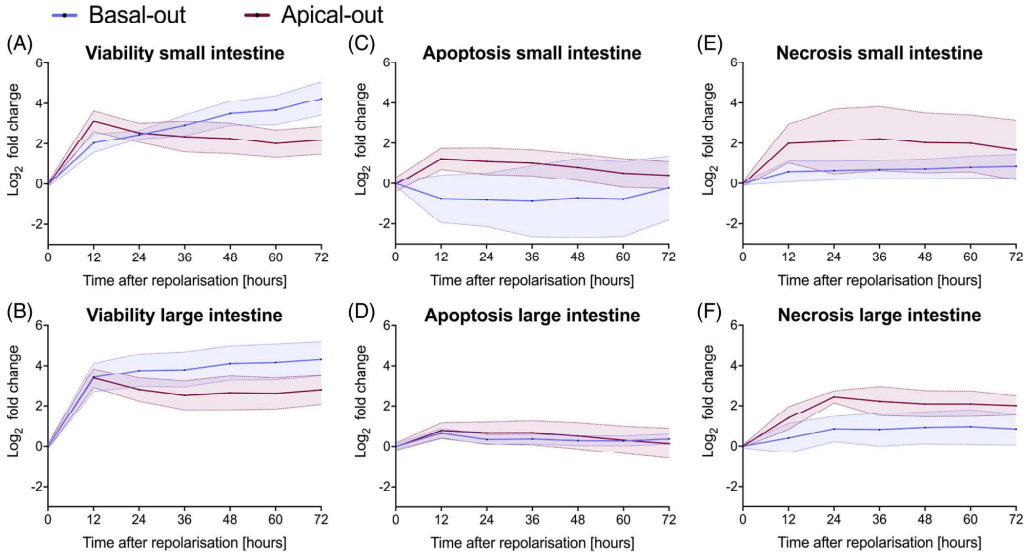

FIGURE 4 Viability, apoptosis and necrosis measurements of small and large intestinal organoids. The effect of polarity was significant over 72 h in all samples according to a two-way ANOVA. Data are presented as mean ± standard deviation of three different small and large intestinal organoids, which were measured in eight individual reactions each. ANOVA, analysis of variance.

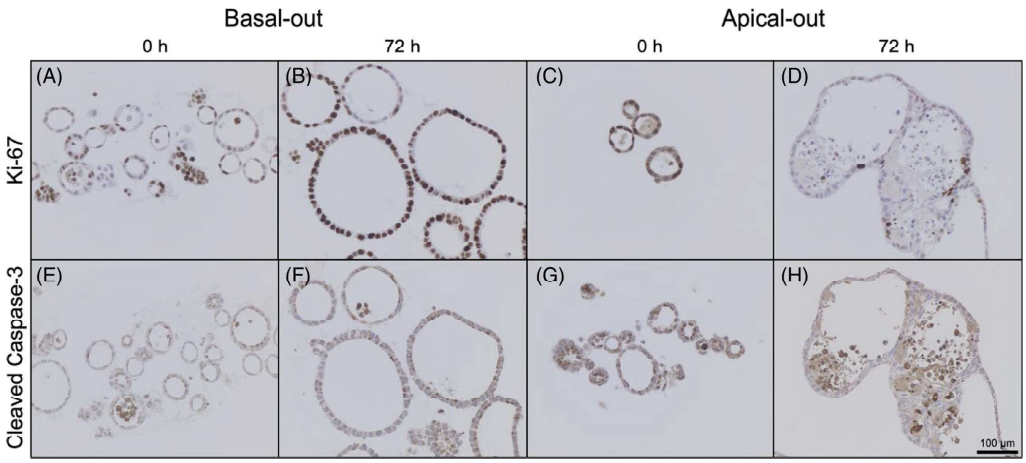

FIGURE 5 Immunohistochemical stainings of basal-out and apical-out small intestinal organoids for proliferation marker Ki-67 and apoptosis marker cleaved caspase-3. Scale bar = 100 µm.

2.4 | Transcript expression

One possible explanation for reduced proliferation concomitant with increased apoptosis could be increased cell differentiation leading to stem cell exhaustion. To define whether cell differentiation after polarity reversal is responsible for reduced proliferation in apical-out organoids, we collected organoids every 12 h after removing the ECM to perform RT-qPCR analysis. Compared to directly after ECM removal

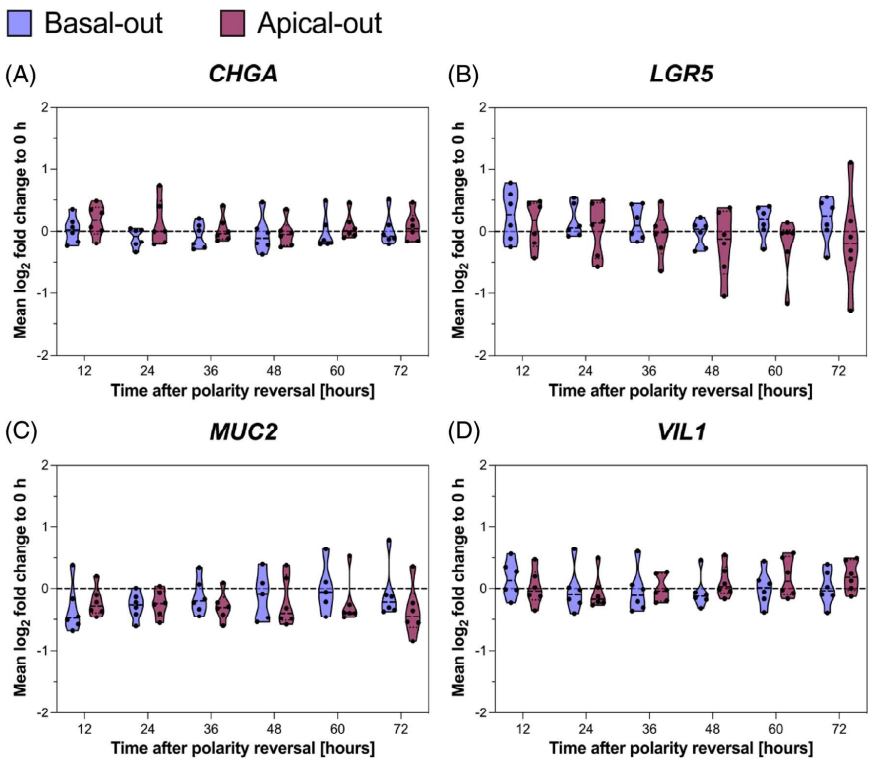

FIGURE 6 RT-qPCR analysis of basal-out and apical-out organoids after inducing polarity reversal. Only minor changes in transcript abundance can be observed indicating stability of culture condition. Normaliser: geometric mean of *DAP3* and *ESD*, calibrator: time point of 0 h (not depicted). $n = 6$ (three small and three large intestinal organoids). RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

(i.e., 0 h time point), there are no significant changes in transcript expression of the key stem cell marker gene *LGR5* or markers for cell type differentiation (*CHGA*, *MUC2* and *VIL1*) over time. However, there is a trend towards *LGR5* and *MUC2* transcript reduction (Figure 6).

3 | DISCUSSION

The efficiency of polarity reversal was very comparable to previously published data with almost no basal-out organoids left after 36–48 h after inducing repolarisation,[12] and seemed to be much more efficient than porcine intestinal organoids, where only about 40% of organoids are in an apical-out state after 48 h.[13] However, astonishingly only after incubation with organoid harvesting solution for 1.5 h, about two thirds of all organoids showed apical-out or mixed polarity based on phalloidin staining. Considering that before the utilisation of organoid harvesting solution, over 80% of organoids exhibited basal-out polarity, it really is remarkable that organoids are capable of rapidly reversing their polarity, even in cold buffered harvesting solution where cellular functions, such as cytoskeletal remodelling, would be expected to operate less efficiently than under normothermic conditions. Even though many organoids do not show basal-out polarity directly after ECM harvest, almost 100% of organoids are in a basal-out state when cultured in medium supplemented with 7.5% Geltrex. Thus, two different scenarios are possible: initially seeded apical-out and mixed polarity organoids could potentially die in basal-out medium and only basal-out organoids remain at 72 h post-harvest or apical-out organoids return to a basal-out state. These findings suggest that selecting the appropriate time point for subsequent experiments may be critical, as the polarity of organoids could impact the results and the utilisation of mixed polarity organoids may not yield meaningful data.

Even though canine intestinal organoids appear to be closer to human than porcine organoids in terms of repolarisation efficiency, we could not reverse organoid polarity using the same α-ITGB1 antibody as Co et al.[12] Even though organoid polarity changed to a more mixed-polarity state, no organoids presented complete apical-out characteristics after being treated with α-ITGB1 for 3 days. Consequently, in the

case of canine intestinal organoids, β1-integrins do not appear to be the sole determinant of proper organoid polarity, and their inhibition does not induce complete polarity reversal, as opposed to human organoids. The exact mechanism for organoid polarity inversion in canine intestinal organoids remains unexplained and will have to be explored more in-depth in follow-up studies. Nevertheless, integrin signalling can potentially influence many other intracellular signalling pathways, for instance, the MAPK signalling pathway, thus directly affecting proliferation, differentiation and cell death. However, the attempt to activate MAPK signalling via activation of Erk1/2 using small-molecule activator SENI was not successful, with very few EdU^+ cells in apical-out organoids, as is the same in untreated organoids. Therefore, we propose that other factors, different from MAPK signalling, determine whether organoids stop proliferating upon polarity reversal.

Viability and cell death measurements make clear, why apical-out organoids die after about 1 week. While basal-out organoids show constantly increasing viability values, apical-out organoid viability rates drop after an initial peak at 12 h after polarity reversal. Together with apoptosis and necrosis values, which are both generally higher in apical-out organoids than in basal-out organoids, this leads to a decreasing number of cells. This indicates that more apoptotic/necrotic cells coupled with a smaller number of proliferating cells, ultimately lead to dying organoids. These results are further validated by Ki-67 (i.e., proliferation) and cleaved caspase-3 (i.e., apoptosis) immunohistochemical stainings. These stainings highlight the significantly higher proliferative potential of basal-out organoids over apical-out organoids. Concurrently, there are fewer cleaved $caspase\text{-}3^+$ cells in basal-out organoids, leading to much higher viability in basal-out compared to apical-out organoids.

In general, intestinal organoids are very stable culture systems over many passages.[24,25] Despite this, there exist conflicting data on cell differentiation in apical-out organoids. In two studies, stem cell markers (*LGR5* and *ASCL2*) remain constant or even increase in expression. Similarly, *CHGA* and *MUC2* show no change. Interestingly, Co et al. show increased expression of enterocyte marker gene sucrose isomaltase (*SI*), while Kakni et al. demonstrate slightly decreasing levels of enterocyte marker gene villin-1 (*VIL1*).[12,18] Using our canine organoids, we demonstrate that overall, key marker genes are relatively stably expressed in our basal-out and apical-out organoids. While *LGR5* and *MUC2* expression decreases slightly, but non-significantly, *VIL1* expression is slightly increased 72 h after polarity reversal. These data are partly in conflict with previous studies, too. We see a slight decrease in *LGR5* and *MUC2* levels but a slight increase in *VIL1* expression in canine organoids. However, since we analysed all samples every 12 h after polarity reversal, we can successfully rule out time-dependent oscillations that might lead to random results and prove the high stability of our system in terms of gene expression. The reasons why other studies might deliver different results are manifold and range from differently composed culture media to different gene regulation in other species and the choice of appropriate reference genes for RT-qPCR data normalisation.

Since polarity reversal shows drastic effects in terms of proliferation, this can have an immense effect on potential disease modelling applications. However, there is no obvious difference between small and large intestinal organoids as organoids from both sections of the intestine change in the same way upon polarity reversal. Given the fact that proliferation may affect various signalling pathways, basal-out and apical-out organoids might be different in many other ways. Therefore, reversing the polarity can not only change the morphology but also signalling within the cell and hence how organoids react to anything you intend to test, whether it is simply a change in medium components or something more complex like toxins or even pathogens like viruses or bacteria. Thus, any system using apical-out organoids should be carefully assessed before use, especially when comparisons to basal-out organoids are made. Our data show that it is critical to choose the correct time point for any experiment to minimise differences between basal-out and apical-out organoids.

4 | CONCLUSION

Given the important differences between basal-out and apical-out organoids within this paper, it must be critically evaluated, whether apical-out organoids can and should be used as a relevant in vitro model. Apical-out organoids undeniably present crucial advantages over basal-out organoids in some respects as the apical cell surface is directly accessible and therefore the epithelium can be easier challenged with all sorts of different toxins/pathogens that usually affect the intestine from its luminal surface. However, apical-out organoids show much lower proliferation and viability and are not as long-lived compared to basal-out organoids despite presenting necessary stability in terms of gene expression and differentiation. Therefore, other options (e.g., organ-on-chip technology) might seem more tedious and costly, but could potentially be more relevant if the basolateral and apical cell surfaces are accessible at the same time.

This leads to the conclusion that the characteristics of apical-out organoids should be critically evaluated before using them for disease modelling or similar approaches, especially if they are subject to comparisons to basal-out organoids. Other options to gain access to the apical cell surface of intestinal organoids should be considered to support findings from studies using apical-out organoids. For instance, organoid fragmentation, microinjection and the generation of organoid-derived monolayers are valid alternative methods that can complement apical-out studies.[8]

5 | MATERIALS AND METHODS

5.1 | Organoid culture

Canine intestinal crypts were isolated from jejunum and colon of three different dogs according to Kramer et al.[26] Based on the guidelines of the institutional ethics committee, the use of tissue material collected during therapeutic excision or post-mortem is included in the university's 'owner's consent for treatment', which was signed by all patient owners. Organoid growth medium consisted of 37% basal medium (Advanced DMEM/F12 supplemented with 2 mM GlutaMAX and

10 mM HEPES), 1 × B27 (Invitrogen, Thermo Fisher Scientific), 1 mM N-acetylcysteine, 10 nM Gastrin (Sigma-Aldrich), 100 ng/mL Noggin, 500 nM A8301, 50 ng/mL HGF, 100 ng/mL IGF1, 50 ng/mL FGF2 (PeproTech), 10% (v/v) Rspondin1 and 50% (v/v) Wnt3a conditioned media. For the first 2 days of culture, 50 ng/mL mEGF (Thermo Fisher Scientific) and 10 µM Rock-inhibitor Y-27632 (Selleck Chemicals) were added. The growth medium was changed every 2–3 days. Weekly passaging at 1:4 to 1:8 split ratios was achieved by mechanical disruption using flame-polished Pasteur pipettes. For experiments where it is indicated, small-molecule inhibitor Senkyunolide I (SENI) was used in a concentration of 5 µM as described previously.[27,28]

5.2 | Polarity reversal

Apical-out and floating basal-out organoids were generated as described previously.[12] Organoids were harvested using Cultrex® Organoid Harvesting Solution (Bio-Techne) for 1.5 h at 4°C, under constant shaking. Thereafter, organoids were washed with basal medium, resuspended in growth medium and seeded in multiwell plates treated with Anti-Adherence Rinsing Solution (Stemcell Technologies) to prevent organoid attachment to the surface. To generate floating basal-out organoids, 7.5% Geltrex (Thermo Fisher Scientific) was added to the culture medium. Organoids were incubated for up to 72 h in a humidified incubator with 5% CO_2 before further use.

5.3 | Transmission electron microscopy

To further analyse polarity reversal of organoids, basal-out and apical-out organoids at day three after induction of polarity reversal were used. All samples were fixed in 3% buffered glutaraldehyde (pH 7.4, Merck). Organoids were then pre-embedded in 1.5% agarose. After being washed in 0.1 M Soerensen buffer (pH 7.4), the samples were postfixed for 2 h at room temperature in 1% osmium tetroxide (Electron Microscopy Sciences). This was followed by dehydration in an ethanol series along with an increasing series of propylene oxide (Sigma-Aldrich) before embedding and polymerisation in epoxy resin (Serva) for 48 h at 60°C. Ultrathin sections (70 nm) were cut for transmission electron microscopic evaluation and contrasted in methanolic uranyl acetate (Fluka Chemie AG) and alkaline lead citrate (Merck). For imaging, a transmission electron microscope (EM 900, Zeiss) equipped with a slow-scan CCD camera (2k Wide-angle Dual Speed, TRS) and ImageSP Professional software (SYSPROG, TRS) were used.

5.4 | Immunofluorescent staining

Organoids were fixed with 2% (v/v) paraformaldehyde (PFA) and stained according to a previously published protocol including a clearing step after organoid staining.[29] Organoids were stained with 1:100 Claudin 7 Polyclonal Antibody (Invitrogen, Thermo Fisher Scientific) for tight junctions with secondary antibody AF-488 goat anti-rabbit (Invitrogen, Thermo Fisher Scientific) diluted 1:500, 1:200 phalloidin (Alexa Fluor 647, Invitrogen, Thermo Fisher Scientific) to visualise actin filaments, and with 4 µg/mL 4′,6-diamino-2-phenylindole (DAPI; Sigma-Aldrich) for nuclear staining. Confocal images were acquired using a Zeiss LSM 880 confocal microscope (Zeiss).

5.5 | EdU staining

To assess cell proliferation, the Click-iT® EdU Imaging Kit (Invitrogen, Thermo Fisher Scientific) was used. Basal-out and apical-out organoids of the small and large intestine were incubated with 5-ethynyl-2′deoxyuridine (EdU) at a final concentration of 10 µM for 1.5 h at 37°C and were then fixed with 2% (v/v) PFA for 15 min at room temperature. Staining was carried out according to the manufacturer's instructions. DNA was counterstained using Hoechst33342 (Abcam). Confocal images were taken using an LSM 880 (Zeiss). The acquired images were further analysed using the Vision4D Software by Arivis/Zeiss. Using this software, organoids chosen for analysis were encircled individually and segmented into their nuclei positive for Hoechst and EdU, respectively. Only nuclei larger than 15 μm^2 were considered for the analysis. A total number of 390 organoids were analysed. Dead cells within the organoid lumen were excluded. After calculating the area of the positive cell nuclei, we summed up all the numbers for each time point at both polarity states before calculating the percentage of EdU^+ cells of the total cell mass (EdU^+ and $Hoechst^+$ nuclei). This number then serves as an estimate of EdU^+ proliferating cells within our sample.

5.6 | Viability and cell death assays

Viability and apoptosis of basal-out and apical-out organoids were assessed using the RealTime-Glo MT Cell Viability Assay (Promega) and RealTime-Glo Annexin V Apoptosis Assay (Promega; referred to as 'apoptosis and necrosis assay'). Equal numbers of organoids were seeded into each well of a white 96-well plate with clear bottom to induce polarity reversal as described above. Detection reagents were prepared according to the manufacturer's instructions and added to the respective wells. Luminescence was measured at 0, 12, 24, 36, 48, 60 and 72 h after adding the substrates at time point 0 h using a GloMax Explorer plate reader (Promega). Experiments were carried out in eight technical replicates of three biological replicates per intestinal section (small and large intestines).

5.7 | Immunohistochemistry

Organoids were fixed in 2% (v/v) PFA for 15 min and embedded in paraffin. Sections from paraffin tissue blocks were cut for standard immunohistochemical staining. All slides were deparaffinised with xylene and rehydrated through a graded series of alcohols followed by endogenous peroxidase blocking with 0.6% hydrogen peroxide in

methanol. Heat retrieval in citrate buffer (pH 6.0) was done with a steamer. Protein blocking was performed with 1.5% goat serum (Sigma-Aldrich) in PBS. Immunolabelling was performed by incubation with a monoclonal mouse anti-Ki-67 antibody (MIB1, DAKO, #M724029) at a dilution of 1:500 in PBS or a monoclonal rabbit anti-cleaved caspase-3 antibody (#9664, Cell Signaling Technology) at a dilution of 1:250 in PBS overnight at 4°C followed by 60 min at room temperature. The next day, sections were incubated with the respective horseradish peroxidase (HRP) labelled secondary antibody (mouse or rabbit Immunologic Bright Vision HRP) and the signal was detected by 3′3′diaminobenzidine (Bright DAB, Immunologic, Arnhem) reaction. 'No primary antibody' controls were used to show that there was no host-specific binding of the secondary antibodies.

5.8 | Reverse transcription-quantitative polymerase chain reaction

At time points 0, 12, 24, 36, 48, 60 and 72 h after induction of polarity reversal, basal-out and apical-out organoids were harvested. Organoid RNA was isolated using the ReliaPrep RNA Tissue Miniprep System according to the manufacturer's instructions (Promega). About 500 ng RNA was subjected to RT with oligo-dT and random hexamer primers according to the manufacturer's recommendations (GoScript Reverse Transcription System, Promega). Dye-based qPCR was carried out using GoTaq® qPCR Master Mix (Promega) and the primer sequences were provided in Table S2. Amplification conditions were as follows: 15 min of initial denaturation at 95°C, 40 cycles of 15 s of denaturation at 95°C, 60 s of annealing/extension at 60°C and a read step, followed by 10 s of dissociation at 95°C and a melting curve from 65°C to 95°C in 5 s per 0.5°C increments. Quantitative data analysis involved adjustment of experimental amplification efficiency (E).[30] The efficiency of each individual sample was calculated in silico from non-baseline-corrected fluorescence values using the Real-time PCR Miner software[31] (http://miner.ewindup.cn/miner/). Experimentally measured Cq values were adjusted by the term $Cq \times (\log_{10}(E+1)/\log_{10}(2))$.[32] Outlying triplicates of samples causing a standard deviation of more than 0.5 were excluded from analysis. Abundance of a target transcript was normalised to the geometric mean of the reference-gene pair *DAP3* and *ESD* (manuscript in preparation) and subsequently calibrated to the 0 h time point and presented as mean $\log_2$ fold-change.

5.9 | Statistical analysis

Data from EdU staining and log-transformed data from viability, apoptosis and necrosis measurements as well as RNA expression data were subjected to statistical analysis by means of a two-way analysis of variance to take time and polarity into account. Statistical evaluation was performed using GraphPad Prism 9 (GraphPad Software).

AUTHOR CONTRIBUTIONS

Conceptualization: Georg Csukovich, Maximilian Wagner and Barbara Pratscher. *Methodology*: Georg Csukovich, Ingrid Walter and Ralf Steinborn. *Formal analysis*: Georg Csukovich and Maximilian Wagner. *Investigation*: Georg Csukovich, Maximilian Wagner, Ingrid Walter, Stefanie Burger and Waltraud Tschulenk. *Data curation*: Georg Csukovich, Maximilian Wagner, Ingrid Walter. *Writing—original draft*: Georg Csukovich and Maximilian Wagner. *Writing—review and editing*: Georg Csukovich, Maximilian Wagner, Ingrid Walter, Stefanie Burger, Waltraud Tschulenk, Ralf Steinborn and Stefanie Burger. *Visualisation*: Georg Csukovich, Maximilian Wagner, Ingrid Walter, Stefanie Burger and Waltraud Tschulenk. *Supervision*: Georg Csukovich, Barbara Pratscher and Iwan Anton Burgener. *Funding acquisition*: Georg Csukovich and Iwan Anton Burgener.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the article and its Supporting Information. Additional raw data supporting the conclusions of this article will be made available by the authors upon inquiry.

ORCID

Georg Csukovich https://orcid.org/0000-0001-5615-666X

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Discussion

Our research highlights that *in vitro* research, especially experiments involving three-dimensional cell culture models such as organoids, is more relevant than ever before in modern research and can answer a variety of open questions. Concurrently, animal experimentation can be reduced by simply using biopsies taken during endoscopic examination or tissue from patient animals *post mortem* to generate adult stem cell-derived intestinal organoids, which contain self-renewing stem cells. Thus, fewer animals need to be sacrificed in accordance with the principle of the 3R initiative, i.e., Replacement, Reduction and Refinement of animal experimentation[8].

Taken together, this thesis allowed deeper insights into the physiology of canine intestinal organoids and the fate of the mucosal epithelium during disease. With a special focus on *in vitro* disease modelling, we analysed specific effects of inflammatory bowel disease and of toxins from *Clostridia* on canine intestinal organoids.

Clostridioides difficile infections

Albeit TcdB of *C. difficile* showed a very drastic effect on organoid-derived monolayers (ODM) of the canine intestine, we obtained conflicting data via three-dimensional barrier integrity assays. Here, we only observed an impairment of the intestinal barrier function when incubating basal-out organoids of the small intestine with TcdB, but the toxin had no effect on basal-out organoids of the large intestine. On the other hand, apical-out organoids remain almost completely unaffected by TcdB. This result is very intriguing as it suggests that the receptor for TcdB is located exclusively on the basolateral cell surface. Moreover, expression of this receptor is clearly site-specific, i.e., not expressed in all sections of the intestine. This result also emphasises previous reports highlighting the fact that many different factors including expression profiles, epigenetic masks and many more are defined by their specific location within the body and that these specificities remain unaltered and rather constant in 3D organoid culture over time[14,47].

While both TcdA and TcdB seem to have the same intracellular targets (Rho, Rac and Cdc42) essential for cell adhesion and migration, the individual receptors are partly unknown[48]. Several different ways to enter the cell have been described for TcdA. They range from broadly expressed Lewis antigens[49] over glycosphongolipids[50] to sulphated glycosaminoglycans (sGAGs). For instance, sGAGs are also expressed on low-density lipoprotein receptors (LDLR), which are known to induce endocytosis upon ligand binding[51]. This may be an important route for TcdA to enter the cell. However, much less is known about the exact receptors of TcdB, especially in dogs. Various different molecules have been identified as receptors for TcdB in the past years demonstrating its very broad mode of action, making many

different cells receptible for TcdB and therefore susceptible for epithelial damage in the gut. Some of these known TcdB receptors are members of the Frizzled (FZD) protein family as well as chondroitin sulfate proteoglycan-4 (CSPG4)[52,53], tissue factor pathway inhibitor (TFPI)[54,55] and nectin cell adhesion molecule 3 (NECTIN3)[56].

To date, human individuals with *Clostridioides difficile* infections (CDIs) are often treated with the monoclonal antibody *bezlotoxumab* that is directed against TcdB and neutralises its effects by preventing receptor binding. This antibody had the same effect in canine intestinal organoids, indicating that the putative binding domains of TcdB to its receptors are likely the same in humans and dogs. In addition, an equal TcdB neutralising effect could be achieved with a novel monoclonal sIgA antibody. The advantage of using sIgA molecules instead of existing IgG antibodies could be the form of administration. While bezlotoxumab is administered intravenously[57], sIgA molecules, in contrast to IgG, have been shown to remain active after being exposed to stomach acid following oral administration [58–61]. Apart from classical neutralising antibodies, there has been a recent report using alpaca-derived nanobodies to effectively neutralise TcdA and TcdB. In this report, the authors also appreciated the huge variety seen in TcdB binding domains depending on different clinical isolates of *C. difficile*[62]. This fact undoubtedly complicates therapies protecting against TcdB damage, which renders the relatively good effects of bezlotoxumab and our newly developed sIgA antibody even more remarkable.

However, there are several new approaches in developing effective therapies against CDIs. One recent study targeted CSPG4 as a main receptor for TcdB. They showed that CSPG4 expression was largely regulated by oestrogen signalling and Hippo pathway signalling. Chemical inhibition of the Hippo pathway led to downregulation of CSPG4, resulting in the protection of the murine intestine from TcdB-induced damage *in vivo*[63]. This route against CDIs is definitely interesting but should be critically investigated. As integral components of Hippo signalling, YAP and TAZ have a very important function in regenerative processes, especially in the intestine[64,65]. Therefore, interfering with this pathway and thus regeneration in the intestine can have unwanted side effects by disturbing normal tissue homeostasis. It has to be evaluated whether long-term application of Hippo inhibitors affect physiologic intestinal functions. This issue could be addressed in intestinal organoids of human origin before envisaging investigations *in vivo*, all the more since current rodent models have limitations in terms of translatability. A different approach has been proposed by Heber and colleagues, who treated cells with the small molecule drug ambroxol and observed decreased cell damage upon incubation with TcdA and TcdB. Ambroxol not only exhibited its known function as inhibitor of endolysosome acidification to release the toxins into the cytosol. The drug also reduced the hydrolytic enzyme activity of TcdB[66]. Combined with its proven safety in clinical applications[67], ambroxol might be a safe option to mitigate CDI and reduce the damage caused

by *C. difficile* toxins. A completely different approach was the design of an oral vaccine against *C. difficile.* It is based on a chimeric protein consisting of TcdA and TcdB peptides derived from a non-toxigenic *C. difficile* strain. This protein induced an effective antibody response against the two toxins, and also against *C. difficile* flagellins and surface layer proteins in mice. Importantly, mice immunised with the protein were also cross-protected from infection by another *C. difficile* strain accompanied by a reduced number of spores in mouse feces[68]. These results are very promising but need further validation and studies on the long-term protectivity. Additionally, the relevance of the vaccine may be questioned since 10-15% of human beings are asymptomatic carriers of *C. difficile*, of which approximately 80% even carry toxigenic strains[69]. Because CDIs are not considered a huge threat by the public, vaccine acceptance might be low as similarly observed for other human vaccines over the past decades[70]. On the other hand, immunocompromised human patients as well as pet animals may greatly benefit from preventive CDI vaccines.

Discrepancies between two-dimensional and three-dimensional models

Interestingly, we observed an effect of TcdB on ODMs in small and large intestinal samples. This is not only a paradox because of the contrasting results from 3D assays, but also because only the apical surface of the cells is exposed to the medium/toxin mixture when grown in 2D. However, we do not see an effect of the toxin on the apical cell side in 3D barrier integrity assays. We cannot explain this contradiction so far, but the latter clearly underscores the importance of 3D models for toxicity screens and the development of new therapies. One explanation could be that neighbouring cells in 3D might show stronger attachment to each other and provide absolutely no intercellular spaces through which toxins overcome the tight junction barrier. Any small intercellular space allowing toxins to reach the basolateral cell surface in 2D culture would result in a sort of domino effect by which this channel would successively enlarge. This in turn would increase the permeability for toxins and thus enhance cell damage.

Despite the merits of 3D organoids, research in 2D deserves to be continued. It should be complemented with the corresponding 3D models whenever feasible to gain a more holistic picture of the processes of interest, which is an opinion shared also recently in a review on using intestinal organoids over classical cell culture models for investigations on the intestinal barrier[71]. The importance of employing 3D models in research is highlighted by various reports that reveal the profound differences between two-dimensional and three-dimensional models. These range from examples from keratinocyte cultures that show different electrical characteristics[72], over the influence of hypoxia in retinal epithelial cells[73] to various examples of cancer cells that respond differently to treatments if they are cultured three-dimensionally[74–77].

Apical-out intestinal organoids

The use of apical-out intestinal organoids yielded a series of interesting data and different infection processes could be modelled in several different species using this approach[78–81]. However, as we outline in our article on the effects of polarity reversal, apical-out organoids show decreased levels of proliferation and elevated levels of cell death[82]. After approximately 48 hours, almost none of the cells retain their proliferative status, while basal-out organoids keep on growing and show increasing cell mass. The reduced viability of apical-out organoids hampers their prolonged propagation. This is in accordance with most other reports on apical-out organoids, which are most commonly used between days 2 and 5 after polarity reversal[78,80,81,83,84]. As of now, one can only speculate on the reasons leading to a complete stop of proliferation in apical-out organoids.

One reason could be that essential niche growth factors such as WNT3A and RSPO, which are supplied with the organoid growth medium, cannot reach the basolateral cell surface anymore and therefore do not bind to their respective receptors FZD and LGR5. This leads to a massive reduction of β-catenin signalling and reduced proliferation. Additionally, the initial description of the generation of apical-out intestinal organoids described the effect of repolarisation as a β-1 integrin dependent process. By supplementing the organoid growth medium with a β-1 integrin blocking antibody, organoids repolarised into an apical-out state, even when embedded in Matrigel[78]. Reduced integrin signalling can in turn influence various different signalling pathways and thus reduce proliferation, too. However, we could not verify this possibility in canine intestinal organoids, where the incubation with β-1 integrin blocking antibodies only led to organoids showing a mixed polarity: Some cells remained in a basal-out state, while others reversed their polarity as indicated by phalloidin staining of actin-rich microvilli on the outside[82].

Another reason for decreasing proliferation rates after polarity reversal may be caused by a completely altered cell geometry. Gjorevski et al. described a method of using micropatterned hydrogels in order to guide the development of crypt-villus structures in intestinal organoids in a predictable way, thus gaining spatial and temporal control over their development[85]. These advancements can help make organoid models more standardised and increase their applicability.

The importance of mechanical cues, shape and stiffness is also presented in various other reports. There is literature describing the importance of ECM stiffness and the interaction of the ECM with different cell types and compartments of organoids. Their modelling describes that surface tension has a pronounced influence on crypt shape – and thus stem cell activity. This report also describes the importance of myosin-dependent constriction of the apical side leading to the typical cone-shape of intestinal stem cells to assure their physiological function[86].

Reduced curvature accompanied by altered cell surface areas can also be observed in older mice, highlighting the influence of cell shape on intestinal function[87]. Intestinal stem cells not only display a distinct cellular shape, but also organise their cytoplasmic content in a highly specific way. Scharaw et al. recently showed that intestinal stem cells exhibit a special type of Golgi apparatus, which is laterally oriented towards neighbouring Paneth cells. Presumably this orientation increases the transport efficiency of cell surface receptors as exemplified by EGFR, and hence results in faster signalling. The group also showed that the Golgi organisation changes with age, while the total Golgi size remains unaltered[88]. The different types of Golgi organisation could be directly linked to the changed cell shape in the aging murine intestine due to the reduced curvature.

Taken together, the surface areas of the basal vs. apical cell surfaces are interchanged when organoids reverse their polarity. This can disturb various signalling processes from focal adhesion to receptor signalling relevant for stem cell proliferation or mechanochemical cues and altered intracellular organisation. Thus, the functional organisation of organoids and their stem cell niche could be the determining factor of reduced proliferation after polarity reversal.

Despite the drawbacks associated with the use of apical-out organoids, organoids are a promising tool for *in vitro* disease modelling. Especially when it comes to the effects of bacterial toxins described earlier, or the epithelial interaction with other pathogens such as viruses or parasites, organoids can help uncovering molecular mechanisms underlying infection and disease.

Modelling inflammatory bowel disease *in vitro*

One disease that urgently requires powerful *in vitro* models is IBD. Despite an increasing incidence in humans and various dog breeds including Yorkshire Terrier, Boxer and German Shepherd, we have limited mechanistic understanding of the disease due to its complexity and the lack of a good model system[89–93]. Single aspects of this disease can be recapitulated in two-dimensional cell culture; however, research has mostly relied on different mouse models to study IBD *in vivo*[94]. All these models employ artificial conditions that differ from those observed in IBD and thereby often have little resemblance to the chronic inflammatory processes that characterise IBD[95]. Consequently, there is an undoubted need for alternative experimental models that overcome existing limitations and more authentically reflect the characteristics of IBD.

To this end, we successfully established organoids from intestinal biopsies of dogs diagnosed with IBD. In these organoids, we wanted to explore the importance of reactive oxygen species on intestinal epithelial health and characterise the differences between healthy intestine- and IBD affected intestine-derived organoids in regard to their respective capability to deal with ROS. Interestingly, compared to healthy organoids, we observed a lower ratio of

oxidised to reduced glutathione after challenging IBD organoids with hydrogen peroxide. We expected to obtain the opposite results. However, this result was not due to a general upregulation of glutathione levels in IBD-derived organoids as we assessed total glutathione levels in both types of organoids and did not find significant differences[96]. The exploration of other factors relevant to IBD requires further investigations, including studies on the involvement of TcdB and its interplay in IBD patients, who seem to be more susceptible to CDIs[97].

Outlook

Building on a previous characterisation of canine organoids in our lab[24], we could further characterise healthy and IBD-derived intestinal organoids in terms of their potential to deal with damage inflicted by ROS. This research sets the starting point for the further evaluation of differences between healthy and IBD-organoids, which is essential for modelling IBD in a dish in the future. Furthermore, the development of apical-out organoids opens up many possibilities for other disease modelling approaches since they allow easy access to the apical cell surface, which is exposed to luminal contents *in vivo*. Thus, *in vitro* disease modelling can be lifted to the next level including certain components of the microbiome into organoid culture to gain further insight into host-microbe interactions. Using toxins of *C. difficile*, we could validate findings from human and mouse studies also in the canine intestinal epithelium. We could set up a useful model and respective analytical procedures for future studies on the canine intestine using different pathogens with higher relevance to canine illnesses. Moreover, we could assess the influence of the renin-angiotensin-aldosterone system (RAAS) in canine chronic enteropathies in the organoid system in the future.

In contrast to IBD that is characterised by chronic inflammation, acute haemorrhagic diarrhoea syndrome (AHDS) is a sudden and often life-threatening diarrhoeal disease in dogs, which leads to a massive loss of fluids and often emesis. Pathologic analyses of dogs that died from AHDS revealed signs of necrosis on intestinal epithelium accompanied by an excessive infection with *Clostridium perfringens*[33]. *Clostridium perfringens* is a bacterium closely related to *Clostridioides difficile*, which is one of the most common causes of nosocomial infections in humans, leading to diarrhoeal and emetic symptoms[98–100]. Since these epithelial lesions could not be reasoned to other causes, *C. perfringens* may be a probable cause for AHDS.

In one study from 2018, all dogs diagnosed with AHDS were positive for *C. perfringens* expressing a novel type of toxin, termed NetF. Furthermore, some dogs with diarrhoea harbour a specific combination of two *C. perfringens* toxins, i.e., NetF and cpe, whereas this combination cannot be detected in any clinically healthy dog[101]. This finding reflects the role of *C. perfringens* and its toxins in canine diarrhoea, especially in life-threatening AHDS. It has been known for a long time that bacteria of the phylum *Clostridium* undergo endospore formation when environmental conditions are unfavourable, e.g., in case of nutrient deficiency. These spores can be commonly found in our environment, ranging from soil to fresh vegetables such as salads[102]. Dangerous subtypes producing harmful toxins such as the novel *C. perfringens* toxins NetE and NetF are of high relevance and need to be avoided in any case. These bacteria are not only dangerous for humans who may consume contaminated food, but also for their pets, as they share our environment. Therefore, *C. perfringens* has a relatively high zoonotic potential, infecting both humans and animals with spores contaminating the environment and posing a universal threat in the context of One Health[103].

Investigating the effect of novel *C. perfringens* toxins NetE and NetF on the canine intestinal epithelium is of great importance for animal research, leading to possible new ways to treat the disease, apart from fighting the symptoms, but rather combatting the disease at its core. However, the mechanisms underlying the damage caused by these toxins need to be explored in more depth, which requires sophisticated *in vitro* models like organoids.

As part of a one-health concept, investigating pathogens that show possible zoonotic potential such as *C. perfringens* or *C. difficile* will become more and more important in the future, as they may negatively affect pets, wild animals, and humans. Any progress within this field will promote the development of more effective therapeutics in human and veterinary medicine. However, it is crucial to understand the composition and functional characteristics of organoids compared to primary tissue to draw maximum benefit from this *in vitro* system. Organoids, as successfully applied in the human setting, can be used for drug testing, the exploration of intercellular interactions, and basic research investigating specific functions of different cell populations in a dish. From an ethical point of view, the use of organoids can significantly help reduce the number of animals sacrificed for research.

Our insights gained so far strongly recommend our organoid models for future studies on the mechanisms of action of *C. perfringens* toxins to elucidate their effects on the canine intestinal epithelium.

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