Research article

Cryptosporidium parvum, a potential cause of colic adenocarcinoma

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Abstract

Background: Cryptosporidiosis represents a major public health problem. This infection has been reported worldwide as a frequent cause of diarrhoea. Particularly, it remains a clinically significant opportunistic infection among immunocompromised patients, causing potentially life-threatening diarrhoea in HIV-infected persons. However, the understanding about different aspects of this infection such as invasion, transmission and pathogenesis is problematic. Additionally, it has been difficult to find suitable animal models for propagation of this parasite. Efforts are needed to develop reproducible animal models allowing both the routine passage of different species and approaching unclear aspects of Cryptosporidium infection, especially in the pathophysiology field.

Results: We developed a model using adult severe combined immunodeficiency (SCID) mice inoculated with Cryptosporidium parvum or Cryptosporidium muris while treated or not with Dexamethasone (Dex) in order to investigate divergences in prepatent period, oocyst shedding or clinical and histopathological manifestations. C. muris-infected mice showed high levels of oocysts excretion, whatever the chemical immunosuppression status. Pre-patent periods were 11 days and 9.7 days in average in Dex treated and untreated mice, respectively. Parasite infection was restricted to the stomach, and had a clear preferential colonization for fundic area in both groups. Among C. parvum-infected mice, Dex-treated SCID mice became chronic shedders with a prepatent period of 6.2 days in average. C. parvum-inoculated mice treated with Dex developed glandular cystic polyps with areas of intraepithelial neoplasia, and also with the presence of intramusosal adenocarcinoma.

Conclusion: For the first time C. parvum is associated with the formation of polyps and adenocarcinoma lesions in the gut of Dex-treated SCID mice. Additionally, we have developed a model to compare chronic muris and parvum cryptosporidiosis using SCID mice treated with corticoids. This reproducible model has facilitated the evaluation of clinical signs, oocyst shedding, location of the infection, pathogenicity, and histopathological changes in the gastrointestinal tract, indicating divergent effects of Dex according to Cryptosporidium species causing infection.
Background
Cryptosporidiosis represents a major public health problem. This infection, caused by protozoa of the genus *Cryptosporidium*, has been reported worldwide as a frequent cause of diarrhoea, and its prevalence varies according to different regions [1]. In developed countries, massive *Cryptosporidium* foodborne and waterborne outbreaks have been reported. In developing countries, *Cryptosporidium* affects mostly children under five [2]. Furthermore, cryptosporidiosis remains a clinically significant opportunistic infection in immunocompromised patients, causing potentially life-threatening diarrhoea, especially in those HIV-infected without access to highly active antiretroviral therapy (HAART) [3]. Additionally, these parasites not only infect humans, but also cause morbidity in farm animals, leading to economic losses [4]. Most *Cryptosporidium* species infect the epithelium of the gut but in severe infections, dissemination can occur to extra-intestinal sites [5]. Infection of the intestinal cells can result in blunting of the intestinal villi, crypt hyperplasia and inflammation. Epithelial cell apoptosis due to this parasite has also been described [6,7].

Molecular techniques have been developed to differentiate this parasite at the species and genotype levels, showing that there are at least 16 different species [1], and several methods have been used to study and characterize different parasite strains. However, the understanding about invasion, transmission, pathogenesis and epidemiology is limited, and no effective drug against this infection has been reported. In developed countries, massive *Cryptosporidium* foodborne and waterborne outbreaks have been reported. In developing countries, *Cryptosporidium* affects mostly children under five [2]. Furthermore, cryptosporidiosis remains a clinically significant opportunistic infection in immunocompromised patients, causing potentially life-threatening diarrhoea, especially in those HIV-infected without access to highly active antiretroviral therapy (HAART) [3]. Additionally, these parasites not only infect humans, but also cause morbidity in farm animals, leading to economic losses [4]. Most *Cryptosporidium* species infect the epithelium of the gut but in severe infections, dissemination can occur to extra-intestinal sites [5]. Infection of the intestinal cells can result in blunting of the intestinal villi, crypt hyperplasia and inflammation. Epithelial cell apoptosis due to this parasite has also been described [6,7].

We have developed a model to compare chronic *muris* and *parvum* cryptosporidiosis using SCID mice treated with corticoids in order to evaluate clinical signs, location of the infection, pathogenicity, oocyst shedding and histopathological changes in the gastrointestinal tract. This model was chosen for the following reasons: i) Previous studies have shown that host cell immunity during cryptosporidiosis is mediated by both Th1 and Th2 response [18,19], thus SCID mice are more susceptible to the infection and to develop a chronic disease due to their defect in T and B lymphocytes [20]. ii) It has been found that during cryptosporidiosis there is an IFNγ mucosal response with increased levels of IL15 [21]. IFNγ-induced enterocyte resistance against *C. parvum* has been reported [22]. Furthermore, glucocorticoids are known to have an effect on the priming of the innate immune response [23], and could suppress IFNγ-regulated gene expression [24]. Consequently, dexamethasone, a synthetic glucocorticoid, could be used to alter this innate immune response. iii) This model of SCID mice treated with steroids has been proven to be successful for the development of *Pneumocystis*, another opportunistic agent [25].

Results
The pathological damage due to *C. muris* or *C. parvum* infection was studied in SCID mice treated or not with Dex. Main data related to infected mice included in the study are shown in Table 1.

Dex induced a significant body weight loss in both *Cryptosporidium* infected and uninfected SCID mice (*P* < 0.001). In contrast, no significant body weight change was associated with parasite infection. Once infected with *C. muris*, mice from groups M and MDex became chronic shedders and produced high numbers of oocysts without marked differences between the groups. On the other hand, in animals inoculated with *C. parvum*, the oocyst excretion was sporadic and limited in P mice, and high and chronic in PDex mice, with a statistically significant difference (*P* = 0.002). Figure 1 shows the average of oocyst excretion in different groups of mice. Analysis of variance showed that the administration of Dex, *Cryptosporidium* species, and the interaction of these factors significantly influenced oocyst excretion (all the *P* < 0.001). The level of oocyst excretion was higher in mice infected with *C. muris* than in those infected with *C. parvum*.

Pre-patent period ranged from 6 to 11 days (9.7 days in average) in M mice and it was 11 days for all MDex mice. Geometric means of oocyst excretion before sacrifice were 1839 and 1159 oocysts/mg faeces respectively (Table 1). At histological level, no difference between mice of groups M or MDex was observed. *C. muris* localization was restricted to the stomach, with no extra-gastric dissemination. Different stages of the parasite life cycle were present.
### Table 1: Experimental infection of Dexamethasone-treated or untreated SCID mice infected with *C. parvum* or *C. muris*: Main clinical and histopathological data

| Group | Mouse N | Day of euthanasia (post-infection) | Oocysts/mg faeces at euthanasia | Clinical manifestations | Main histological changes |
|-------|---------|------------------------------------|---------------------------------|------------------------|--------------------------|
| P     | 1       | 20                                 | 0                               | Occasional diarrhoea   | Undetected               |
|       | 2       | 28                                 | 0                               | Lethargy               | Undetected               |
|       | 3       | 28                                 | 1                               | None                   | N.D.                     |
|       | 4       | 84                                 | 1                               | Occasional diarrhoea   | Undetected               |
|       | 5       | 84                                 | 4                               | Occasional diarrhoea   | Undetected               |
|       | 6       | 84                                 | 0                               | Occasional diarrhoea   | N.D.                     |
| Pdex  | 7       | 20                                 | 29                              | Occasional diarrhoea   | Undetected               |
|       | 8       | 28                                 | 102                             | Lethargy, ruffled coat | Undetected               |
|       | 9       | 28                                 | 95                              | Lethargy, ruffled coat | N.D.                     |
|       | 10      | 46                                 | 1182                            | Lethargy, ruffled coat | Polyps with areas of low-grade and high-grade intraepithelial neoplasia, and intramucosal adenocarcinoma at the ileocaecal region |
|       | 11      | 62                                 | 240                             | Lethargy, ruffled coat | Polyps with areas of low-grade and high-grade intraepithelial neoplasia, and intramucosal adenocarcinoma at the ileocaecal region |
|       | 12      | 84                                 | 695                             | Lethargy, ruffled coat | Polyps with areas of low-grade and high-grade intraepithelial neoplasia, and intramucosal adenocarcinoma at the ileocaecal region |
| M     | 13      | 20                                 | 1078                            | None                   | Stomach heavily infected mainly at the fundic region; dilated glands; hyperplasia |
|       | 14      | 20                                 | 407                             | None                   | N.D.                     |
|       | 15      | 28                                 | 742                             | None                   | Stomach heavily infected mainly at the fundic glands; hyperplasia |
|       | 16      | 84                                 | 4143                            | None                   | Stomach heavily infected mainly at the fundic region; dilated glands; hyperplasia |
|       | 17      | 84                                 | 4920                            | None                   | Stomach heavily infected |
|       | 18      | 84                                 | 5828                            | None                   | None                     |
| Mdex  | 19      | 20                                 | 1538                            | Occasional diarrhoea   | Stomach heavily infected; dilated glands |
|       | 20      | 20                                 | 166                             | Occasional diarrhoea   | N.D.                     |
|       | 21      | 27                                 | 1241                            | Occasional diarrhoea   | N.D.                     |
|       | 22      | 32                                 | 4155                            | Frequent diarrhoea, lethargy, ruffled coat | Stomach heavily infected mainly at the fundic glands; hyperplasia |
|       | 23      | 46                                 | 900                             | Lethargy, ruffled coat | Stomach heavily infected mainly at the fundic glands; hyperplasia |
|       | 24      | 84                                 | 2051                            | None                   | Stomach heavily infected mainly at the fundic glands; hyperplasia; a little inflammation |
| C     | 25      | 20                                 | 0                               | None                   | Undetected               |
|       | 26      | 67                                 | 0                               | Lethargy, ruffled coat | Undetected               |
|       | 27      | 84                                 | 0                               | None                   | Undetected               |
| Cdex  | 28      | 20                                 | 0                               | None                   | Undetected               |
|       | 29      | 43                                 | 0                               | None                   | Undetected               |
|       | 30      | 84                                 | 0                               | None                   | Undetected               |
| Cdex2 | 31      | 54                                 | 0                               | None                   | Undetected               |
|       | 32      | 54                                 | 0                               | None                   | Undetected               |
|       | 33      | 54                                 | 0                               | None                   | Undetected               |
|       | 34      | 54                                 | 0                               | None                   | Undetected               |
|       | 35      | 54                                 | 0                               | None                   | Undetected               |
|       | 36      | 54                                 | 0                               | None                   | Undetected               |

*Experimental groups were: P: *C. parvum*-infected SCID mice; Pdex: *C. parvum*-infected Dex-treated SCID mice; M: *C. muris*-infected SCID mice; Mdex: *C. muris*-infected Dex-treated SCID mice; C: Not infected SCID mice of control group inoculated with PBS; Cdex: Not infected Dex-treated SCID mice of control group inoculated with PBS; Cdex2: Not infected Dex-treated SCID mice that received an inoculum from which oocysts were previously removed by filtration; N.D.: Not done (These mice were not included in the histological examination).*
Stomachs of all mice euthanatized at different dates, from day 20 to day 84 post-infection, were heavily infected mainly in fundic mucosae (Figure 2). Fundic gastric glands were dilated and covered by a flattened epithelium. Oxyntic cells were less numerous than in control mice and preferentially visible in the deeper area. Fundic mucosa was twice thinner than in control mice while pyloric mucosa was similar to that in uninfected control mice. A moderate inflammatory infiltrate, made of mononuclear cells and neutrophile polynuclears, was observed in some cases.

For *C. parvum*-infected SCID mice from group P, geometric mean of oocyst excretion before euthanasia was 1 oocyst/mg faeces. SCID mice from P Dex group had a pre-patent period ranging from 4 to 11 days (6.2 days in average) and developed a chronic infection. Geometric mean of oocyst excretion before sacrifice was 195 oocysts/mg of faeces for mice from group P Dex. Two out of six P Dex SCID mice were severely ill and required euthanasia. In mice infected with *C. parvum*, histopathological differences were revealed between Dex-treated and untreated animals. Untreated SCID mice infected with *C. parvum* have neither detectable parasites nor lesions at the histological level (Figure 3) at any time during the course of the study.

In Dex-treated SCID mice infected with *C. parvum*, parasite localization was restricted to the gastrointestinal tract, mainly at the caecal region (Figure 3). Three out of five (60%) histologically examined P Dex SCID mice presented in the ileocaecal region polypoid, sessile, adenomatous masses, measuring approximately 2.5 mm in diameter. They appeared as closely packed, branching sometimes dilated tubular structures, separated by normal or inflammatory lamina propria. Focal cystic dilation was observed. Noticeably some tubules were covered by a low grade or high grade dysplastic epithelium, which showed mucin depletion and nuclear stratification. In some areas, architectural distortion was associated with marked cellular atypias (Figure 4). Epithelial cells presented a loss of their normal polarity. In addition, abnormal nuclear changes consisting of prominent nucleoli and irregularly scattered chromatin were observed (Figure 4). These mucosal changes were suggestive of intraepithelial neoplasia of low or high grade. In some areas, major cellular atypias, with foci of merged glands, typical of intramucosal adenocarcinoma, invasive into lamina propria, were found (Figures 4A and 4B).
These major histological changes were earliest observed in one mouse euthanatized at day 46 post-infection and were found in all PDex mice necropsied after (Table 1). They were always associated with the presence of *C. parvum* organisms (Figures 4 and 5). In contrast, no parasite was detected in the stomach, duodenum, jejunum, hepatic biliary system or pancreas. The possibility that biotic (e.g. virus) or abiotic factors present in the inoculum could be responsible for these lesions was discarded by administering Dex-treated SCID mice with a filtered inoculum (i.e. without *Cryptosporidium* oocysts) (group Cdex2). Neither parasites in the faeces or tissues nor lesions were detected in these mice at any time of the experience.

**Discussion**

We developed a new animal model that allows a good propagation of two different species of *Cryptosporidium*. In this study, adult SCID mice treated with Dex became chronically infected with $10^5$ *C. parvum* or *C. muris* oocysts, and had a significant oocyst shedding during all the course of the experiments. Furthermore, this model was useful to compare *C. muris* and *C. parvum* infections at clinical, histopathological and parasitological levels.

In this study, variations in the expression of the disease in terms of either *Cryptosporidium* species or Dex administration were shown. Amounts of oocysts discharged seemed slightly lower in MDex than in M mice but this difference was not statistically significant. However, it appeared that the aggravation of the immunosuppression status did not lead to an increase in the severity of *C. muris* infection. Miller *et al.* previously reported that immunosuppressed mice were as or less susceptible to *C. muris* than immunocompetent mice [26]. However, this conclusion can hardly be extrapolated to all *Cryptosporidium* species, as long as in our experiments, the oocyst shedding was markedly and significantly higher in PDex than in P group. Further studies are required to determine minimal infectious dose, ID$_{50}$ (infectious dose to 50 percent of exposed individuals) and other data for each species and according to the immunosuppression degree. Interestingly, data from very recent experiments with *C. parvum* confirmed the results of the present work (data not shown). The Dex-treated SCID mouse model revealed therefore to be reproducible.

*C. muris* infection caused damage to the gastric mucosa of both mice from M and MDex groups. But though M mice were less ill than MDex mice, there was no marked histological difference between them. Dilated, hypertrophied and highly parasitized gastric glands without an extra gastric dissemination of parasites were the main histopathological changes. Additionally, a little inflammatory response was observed. Other authors have reported similar findings in relation with *C. muris* gastric infection [12,27,28]. However, to our knowledge, this is the first report of a clear preferential localization of the parasite colonization at the fundic level of the stomach, where acid secreting glands are numerous [29]. This could suggest a favourable influence of lower gastric pH on the growth of *C. muris*.
On the other hand, several studies using animal models have described histological changes in the intestinal epithelium due to *C. parvum* infection, such as villous atrophy and crypt hyperplasia in the lower small intestine [13] or in the caecum and the colon [12], cryptic hyperplasia with abscessation of crypts in the large intestine [13], stunting and fusion of villi, replacement of enterocytes by immature cells and eosinophilia of lamina propria [30], small and large intestine mucosa severely damaged with villous contraction and little or absent epithelial layer [31]. Another report described an association between *Cryptosporidium* sp. and aural-pharyngeal polyps in iguanas. These polyps were pedunculated masses composed of glandular cystic structures lined by hyperplastic cuboidal to columnar epithelium, containing numerous parasites along the apical surfaces of the epithelial cells [32]. Nevertheless, none of these studies have described the presence of carcinoma lesions associated to cryptosporidiosis. These lesions were first observed unexpectedly after 46 days post-infection, when the infection had become chronic, and were found only in *C. parvum*-infected Dex-treated SCID mice. These findings were recorded using an inoculum relatively low (10⁵ oocysts) in comparison with the higher infectious doses, between 10⁶ and 10⁷, used by others [12,33].

Several observations in our study suggest that combination of *C. parvum* with Dex administration is involved in the generation of these significant histological changes. Indeed, Dex seemed to be a critical factor to the develop-
ment of intraepithelial adenocarcinoma in *C. parvum*-infected SCID mice. Dex potentially alters the innate immunity in *C. parvum* infected animals [23,24]. The higher oocyst shedding found in mice presenting these neoplastic lesions seems to confirm this assumption. Additionally, this kind of lesion was found neither in Dex-untreated *C parvum*-infected animals with a low oocyst excretion during the course of the study, nor in the control Dex-treated non-infected group. A possible contamination of the inoculum with a virus or other agent potentially responsible for these neoplastic lesions was also discarded. Interestingly, *C. muris* was not able to induce this type of epithelial changes and the reasons of this different expression of the disease are unclear. It has been reported previously a variability in pathogenicity for different hosts between different *Cryptosporidium* species and types, suggesting the existence of specific virulence factors among species and isolates [34]. Further studies should be done to clarify this difference in pathogenicity. It is important to mention that these colonic neoplastic lesions are not listed among the typical background diseases due to the SCID mice genetic defect [35].

To our knowledge, this is the first time that *C. parvum* is associated with cancerogenesis. There is one case report that described cryptosporidiosis of the biliary tract clinically mimicking a pancreatic cancer in an AIDS patient [36]. However, biopsy of the gallbladder revealed crypt-
osporidiosis causing inflammation of the biliary tract and ruled out the diagnosis of neoplasia [36]. Furthermore, a recent epidemiological study in Poland reported a high frequency of cryptosporidiosis in patients with colorectal cancer [37]. These data strengthen the interest of our experimental observations.

Several infectious agents, including parasites, have been linked to oncogenesis in humans. Some of these associations are strong. Particularly, *Schistosoma hematobium* has been described as a definitive cause of urinary bladder cancer by the International Agency for Research in Cancer. Also, a proportion of cholangiocarcinoma of the liver worldwide is attributable to Opisthorchidae liver flukes [38]. Other associations still speculative with some protozoa were suggested on the basis of epidemiological observations, such as *Trichomonas vaginalis* and cervical cancer, or *Toxoplasma gondii* and tumors [39]. As well, a microbial pathogen such as *Helicobacter pylori* has been classified as oncogenic for humans due to its strong epidemiological association with carcinoma of stomach and gastric lymphoma [38,40].

Because *C. parvum* is an opportunistic agent that causes significant morbidity and mortality in immunocompromised patients, it is possible that individuals infected with this parasite may have a higher risk of developing colorectal malignancies, especially when immunosuppression is more severe. In a retrospective study that shows the incidence and clinical course of colorectal malignancies in HIV/AIDS patients, adenocarcinoma was the type of cancer most frequently found among them. These patients

**Figure 5**

*Experimental Cryptosporidium parvum infection of Dex-treated SCID mice.* Low (A, D) and high magnification (B, C) of the caecal region showing the presence of abundant parasites (arrow), and high degree dysplasia (arrow). Hematoxylin & Eosin staining.
did not have familial antecedents of intestinal malignancies or other risk factors, and developed tumors at earlier ages in comparison to immunocompetent persons [41]. Unfortunately, no data about gastro-intestinal parasites in these patients were given in the study [41]. More studies have to be done in humans to evaluate cryptosporidiosis as a possible risk factor of colorectal cancer.

Epidemiological studies have shown that environmental factors may play a role in colon cancer susceptibility [42]. The association between chronic inflammation and cancer is also well known [43]. Particularly, colon cancer has also been associated to inflammatory bowel disease. In the latter case, cancer develops after sustained gut inflammation. However, in the present work Cryptosporidium infection was associated with only mild inflammation of the intestine as reported also by others [12].

Taking into consideration that the observations described herein were replicated in a second experiment (unpublished data), it can be concluded that Cryptosporidium is able to induce the formation of polyps and carcinogenic lesions in the gut of Dex-treated SCID mice. However, more work has to be done to elucidate this interesting association and to further understand cryptosporidiosis pathogenesis.

Conclusion
In summary, for the first time Cryptosporidium was associated with the generation of polyps and in situ adenocarcinoma in the gut of Dex-treated SCID mice. Additionally, we have developed a model to compare chronic muris and parvum cryptosporidiosis using SCID mice treated with corticoids. This reproducible model has allowed the evaluation of clinical impact, oocyst shedding, location of the infections, pathogenic power, and histopathological changes indicating divergent effects of Dex according to Cryptosporidium species.

Methods
Cryptosporidium oocysts
Cryptosporidium IOWA and C. muris RN66 oocysts were purchased from Waterborne™, Inc. (New Orleans, Louisiana) and stored in shipping medium (Phosphate-buffered saline (PBS) with penicillin, streptomycin, gentamycin, amphotericin B and 0.01% Tween 20) at 4°C until use. Oocyst viability before inoculation was determined by a trypsin-taurocholate excystation test [44] and absence of germs was assured by testing the oocyst suspensions on Plate Count Agar and on Sabouraud plates.

Animals
Nine week-old male and female CB17-SCID mice were obtained from a colony bred at the Pasteur Institute of Lille (France), and maintained under aseptic conditions in an isolator, with standard laboratory food for experimental mice and water ad libitum. All cages, food, water and bedding were sterilized before use. Faecal pellets were collected from all mice before inoculation to ascertain the absence of pre-existing Cryptosporidium infection. The conditions for the care of laboratory animals stipulated in European guidelines (Council directives on the protection of animals for experimental and other scientific purposes. J. Off. Communautés Européennes, 86/609/EEC, 1986, December 18th, L358) were followed.

Immunosuppression
When needed (see the next section), SCID mice were chemically immunosuppressed by administering 4 mg/L of dexamethasone sodium phosphate (Dex) (Merck, Lyon, France) via the drinking water. Immunosuppression was started two weeks prior to inoculation and was maintained during the whole experimentation. Dex-added water was replaced three times a week.

Experimental design
Twenty-four SCID mice, housed individually (one-mouse-cage) in capped cages, were randomly divided into four groups: P, M, PDex and MDex, described hereinafter. Six SCID mice, housed by three, constituted two control groups, C and CDex. Oral inoculation was done with 10⁵ oocysts in 200 μl of PBS. Six mice were infected with Cryptosporidium oocysts (group P) and six others with Cryptosporidium oocysts (group M). Under the effect of Dex immunosuppression, six mice were infected with Cryptosporidium oocysts (group PDex) and six others with Cryptosporidium oocysts (group MDex). Mice of control groups were inoculated only with PBS, without (group C) or with (group CDex) Dex administration. A further control group was constituted by six SCID mice that received an inoculum from which oocysts were previously removed by filtration with a Nanosep MF tube with a 0.45 μm pore size membrane (group CDex2).

Quantification of the oocyst shedding
To evaluate the intensity of oocyst shedding over the course of Cryptosporidium infection, freshly excreted faecal pellets were collected three times a week from each mouse and suspended in MilliQ water. Oocysts were detected and numbered after staining faecal smears with modified Ziehl-Neelsen stain [45].

Statistical analysis
Analyses of data were performed with the statistical software S-PLUS 2000 (MathSoft, Seattle, WA, USA). For statistical analyse purposes, logarithmic transformations of oocyst excretion values were used. Wilcoxon rank sum tests were used to compare body weights or oocyst excretion. An analysis of variance was conducted to account for the effects of relevant factors and their interactions on average daily excretion of oocysts. An average amount of oocysts excreted per day was estimated for each mouse.
from all the unitary values, and was represented as a function of the mouse group in a Box & Whiskers Plot.

**Histological examination**

Periodically or when signs of imminent death appeared, mice were euthanatized by a sodium pentobarbital (Ceva, Libourne, France) intra-cardiac injection. The liver, stomach and pancreas, a section of the duodenum, 3 sections of the jejunum, the caecum, and 3 sections of the colon were removed. Tissues were fixed in 10% neutral formalin and embedded in paraffin. Histological sections were stained with hematoxylin and eosin, and examined microscopically for the detection of *Cryptosporidium* organisms and/or histological modifications of the host tissue. Pathological changes found in the mouse caecum were classified according to the Vienna classification of tumors of the digestive system [46,47].

**Competing interests**

The author(s) declare that they have no competing interests.

**Authors' contributions**

GC & TN have equally contributed to this work. They participated in the conception and design of the study, carried out the experiments and drafted the manuscript. KG participated in the design of the experiments. NG, TC, AM participated in the performance of animal experiments. LF prepared the histological cuts. AP carried out the statistical analysis. ICC participated in the design of the study. ED participated in the design and coordination of the study and helped to draft the manuscript. CC carried out the pathological study and helped to draft the manuscript. All authors read and approved the final manuscript.

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