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RESEARCH ARTICLE

Bacillus subtilis SF106 and *Bacillus clausii* SF174 spores reduce the inflammation and modulate the gut microbiota in a colitis model

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Abstract

Chronic intestinal inflammation is associated with strong alterations of the microbial composition of the gut. Probiotic treatments and microbiota-targeting approaches have been considered to reduce the inflammation, improve both gut barrier function as well as overall gastrointestinal health. Here, a murine model of experimental colitis was used to assess the beneficial health effects of *Bacillus subtilis* SF106 and *Bacillus clausii* (recently renamed *Shouchella clausii*) SF174, two spore-forming strains previously characterised *in vitro* as potential probiotics. Experimental colitis was induced in BALB/c mice by the oral administration of dextran sodium sulphate (DSS) and groups of animals treated with spores of either strain. Spores of both strains reduced the DSS-induced inflammation with spores of *B. clausii* SF174 more effective than *B. subtilis* SF106. Spores of both strains remodelled the mouse gut microbiota favouring the presence of beneficial microbes such as members of the *Bacteroidetes* and *Akkermansia* genera.

Keywords

probiotics – spores – intestinal inflammation – microbiota

1 Introduction

The intestinal microbiota and its host form a symbiotic relationship that strongly contributes to the host metabolic health (Fan *et al.*, 2021). Alterations of the gut microbial composition (dysbiosis) are associated in the pathogenesis of inflammatory bowel diseases (IBD), characterised by an abnormal production of inflammatory cytokines by host cells and disruption of the epithelial integrity, as observed in Ulcerative Colitis (UC) and Crohn's Disease (CD) (Fan *et al.*, 2021; Piazzesi *et al.*, 2022). As a consequence, a series of microbiota-directed intervention strategies have been proposed to restore a healthy gut microbiota, including the oral administration of: (1) prebiotics, that once fermented by the intestinal microbiota, improve gut barrier functions; (2) probiotics, live bacteria that exert a beneficial health effect; (3) symbiotic, a combination of prebiotics and various probiotic strains; and (4) postbiotics, heat-inactivated probiotics or molecules secreted by probiotic strains (Ewaschuk *et al.*, 2008; Laval *et al.*, 2015; Sanders *et al.*, 2019; Yan *et al.*, 2007).

The oral use of probiotics is widely accepted and is based on the capacity of some bacteria to regu-

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late cytokine expression, reduce inflammation and protect gut barrier integrity (Fan *et al.*, 2021; Laval *et al.*, 2015; Piazzesi *et al.*, 2022; Sanders *et al.*, 2019). Several strains of Lactobacilli, Bifidobacteria and Bacilli have long been used as commercial probiotics (Da Silva *et al.*, in press; Saggese *et al.*, 2021), while various members of the *Bacteroidetes* genus and the *Faecalibacterium prausnitzii* (Martin *et al.*, 2017) and *Akkermansia muciniphila* (Depommier *et al.*, 2019) species have recently been proposed as next-generation probiotics.

Of all bacteria commercially used as probiotics the case of members of the *Bacillus* genus is peculiar. These are spore-forming bacteria and all *Bacillus*-based commercial probiotic preparations contain spores, metabolically quiescent and extremely stable cells produced when the environmental conditions no longer allow cell growth (McKenney *et al.*, 2013 Saggese *et al.*, 2021). The quiescent spore responds to the presence of nutrients by germinating, thereby allowing metabolically active cells to grow and eventually to sporulate again (Christie *et al.*, 2020). Spore formers of the *Bacillus* genus are ubiquitous in nature and are also common inhabitant of the animal gut (Egan *et al.*, 2021; Fakhry *et al.*, 2008; Hong *et al.*, 2009). It has been shown that *Bacillus* spores can conduct their entire life cycle in the animal gut, entering as spores through the oral route, safely passing the gastric barrier (Spinosa *et al.*, 2000) and germinating in the intestine (Casula *et al.*, 2002). In the small intestine, the germination-derived cells proliferate and temporarily colonise the upper intestinal tract, then re-sporulate in the colon and exit the body as spores (Hoa *et al.*, 2001; Tam *et al.*, 2006).

Both spores and germination-derived cells of various *Bacillus* species interact with intestinal and immune cells through complex mechanisms poorly understood. *In vitro* studies have shown that *Bacillus subtilis* spores are taken up and rapidly eliminated by both murine (Duc *et al.*, 2004) or human (Ceragioli *et al.*, 2009) macrophages. A different *in vitro* study has shown that spores protect human epithelial cells from oxidative stress by inducing the nuclear translocation of the transcriptional factor Nrf-2, that activates stress-response genes (Petruk *et al.*, 2018). Also vegetative cells of various *Bacillus* species have been shown to interact with model intestinal cells. Examples include: (1) the contribution exerted by a combination of *B. subtilis* and *Bacteroides fragilis* cells to the maturation of the gut associated lymphoid tissue (GALT) in rabbits (Rhee *et al.*, 2004); (2) the induction of the synthesis of the heat-shock proteins (HSPs) in intestinal epithelial cells stimulated by the competence and sporulation factor (CSF), a pentapeptide produced and secreted by *B. subtilis* cells (Fujita *et al.*, 2007). CSF-induced synthesis of HSPs contributes to the prevention of intestinal cell injury, loss of intestinal barrier function and it has been shown to have immunomodulatory and cytoprotective activities (Okamoto *et al.*, 2012). Although CSF is a *B. subtilis*-specific molecule, other *Bacillus* species, such as *Bacillus megaterium*, *Bacillus pumilus* and *Bacillus clausii*, produce and secrete peptides similarly able to induce HS proteins (CSF-like) (Di Luccia *et al.*, 2016; Vittoria *et al.*, 2023).

In addition, spores have been shown able to exert indirect beneficial effects by modulating the microbial composition of the gut and favouring the prevalence of potentially beneficial bacteria such as *Faecalibacterium prausnitzii* (Ji *et al.*, 2022), *Akkermansia muciniphila*, and *Bifidobacterium* spp. (Marzorati *et al.*, 2021).

A recent human trial has highlighted the safety, tolerance and positive health impact of the co-administration of *B. clausii*, *B. megaterium* and of a cocktail of probiotics containing *B. subtilis*, *B. megaterium*, *Bacillus coagulans* and *B. clausii* (Rea *et al.*, 2023). In addition, a double-blind, placebo-controlled trial indicated that a *B. subtilis*-based probiotic alleviates gastrointestinal symptoms (Garvey *et al.*, 2022).

Here, we used a dextran sodium sulphate (DSS)-induced colitis model (Chassaing *et al.*, 2014; Li *et al.*, 2019), to analyse the probiotic potential of spores from the strains SF106 and SF174, respectively, belonging to the *B. subtilis* and *B. clausii* species. The two strains have been previously characterised at the genomic and physiological level and shown to secrete vitamins, antimicrobial and antibiofilm molecules and to efficiently bind mucin and intestinal epithelial cells *in vitro*, without posing safety concerns (Saggese *et al.*, 2022).

2 Materials and methods

Spores production and purification

B. subtilis SF106 and *B. clausii* SF174 were isolated from ileal biopsies of healthy human volunteers and characterised in a previous work (Saggese *et al.*, 2022). For spores production, both strains were grown in Difco sporulation (DS) medium (for 1 l: 8 g Nutrient Broth, 1 g KCl, 1 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 10 μM $MnCl_2$, 1 μM $FeSO_4$, Sigma-Aldrich, Taufkirchen, Germany) at 37 °C with vigorous shaking (150 rpm), for 30 h, to allow complete sporulation (Saggese *et al.*, 2016). Spores were then harvested (10,000×*g* for 10 min) and purified on a step gradient of 20 to 50% of Gastrografin (Bayer S.p.A,

Leverkusen, Germany), as previously reported (Maia *et al.*, 2020). Spore preparations were routinely checked for purity under the light microscope and by colony forming units (cfu) determination of heat-treated and untreated samples. Spores were considered pure when less than 1% of vegetative cells were present in the sample. For animal trial, the spore-pellet stock was washed three times with sterilised phosphate buffer solution (PBS, pH 7.4) and diluted prior mice gavage.

Animal trial and induction of acute colitis in mice

The animal trial was approved by the Animal Welfare Ethical Review Body (AWERB) of the Royal Holloway University of London, and project license (PB9FA6ABB) was granted by the Home Office (UK). Twenty 'pathogen-free' BALB/c mice (age 8-10 weeks) were purchased from Charles River, UK. Mice were housed in HEPA-filtered individually ventilated cages and all the animals were kept in the Experimental Animal Center of Royal Holloway University of London under standard conditions (constant temperature of 18 ± 2 °C, humidity of 50 ± 5%, and 12-h light-dark cycle) and given free access to sterile standard mouse chow (5LF5, LabDiet) and water. Balb/c mice were randomly divided into four experimental groups (n = 5) and acclimatised to their social group and environment for one week before the study start. Two groups (106 and 174) received by oral gavage either 5×10^8 spores of SF106 or 5×10^8 spores of SF174 suspended in 200 µl of PBS buffer while the other two groups (Control and DSS) received the same volume of PBS buffer. After 7 days in all groups, except for the Control group (naïve mice), colitis was induced by daily treatment for 7 days with 4% (w/v) of DSS, added to the drinking water. Fresh DSS was replaced every 2-3 days. On day 14 all the mice were euthanised, colorectum and anus were excised and colon tissues were isolated for further analysis.

Assessment of colitis severity and histological analysis

During the DSS challenge, body weight, stool consistency and rectal bleeding were monitored daily, starting one day before the addition of 4% DSS to the water (day 6). The presence of occult blood in the faeces was also analysed using a faecal occult blood test kit (Hema-Screen, Immunostics Inc, Eatontown, NJ, USA). The scores assigned according to symptom severity were used to obtain the disease activity index (DAI), calculated as previously reported (Hidalgo-Cantabrana *et al.*, 2016). On the day mice were euthanised, the distal colon was collected and fixed overnight at 4 °C in a 10% (v/v) formalin solution. The samples were processed and stained with haematoxylin and eosin and for histological analysis, several slides were analysed under the microscope and scores were assigned according to the severity of the inflammatory state, as previously reported (Koelink *et al.*, 2018); two investigators evaluated each blinded sample separately and the mean of the two was used to obtain the mouse colitis histological index (MCHI) for each sample.

RNA extraction, retro-transcription and qPCR analysis

About 100 mg of rectosigmoid colon for each sample was placed in 1 ml of TRIzol™ reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) and homogenised for 30 s for RNA extraction. To avoid contamination of DSS in colonic tissue, which has been reported to inhibit the reverse transcription reaction and amplification of mRNA (Viennois *et al.*, 2013), the extracted total RNA was purified with lithium chloride using the RNA purification protocol described previously (Oldak *et al.*, 2018). Residual gDNA was removed and reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). The Real-Time was performed according to manufacturer's protocol using the QuantiNova RT-PCR kit (Qiagen) in a Corbett Rotor-Gene 6000 thermocycler. The primer sequences used in this study are listed in the Supplementary Table S1 and their amplification efficiency was validated before performing the qPCR analyses. The $2^{-\Delta\Delta Ct}$ method was used to determine the fold change, which was normalised to the β-actin expression levels of each gene. The relative quantification of each analysed gene expression was normalised to that of the *naïve* control mice (considered zero).

Bioinformatic analyses

Faeces was collected from each mouse on day 14. DNA was extracted using the Quick-DNA Fecal/Soil MicroPrep Kit (Zymo Research, Tustin, CA, USA), as per the manufacturer's guidelines. Blank extractions were systematically included to check for potential cross-contaminations. DNA quality and quantity were checked as described previously (Buglione *et al.*, 2022).

16S rDNA was amplified in the V3-V4 region, and sequenced with Ilumina's TruSeq DNA library (Novogene Co., Ltd., Beijing, China) for bioinformatic analysis. All analyses were performed using the R software (R-Cran project, http://cran.r-project.org/) within a jupyter notebook instance (Loizides *et al.*, 2016). Sequences were analysed using the DADA2 package (Callahan *et al.*, 2016), after removing primers. All sequences with an average call quality for each base between 20 and 40

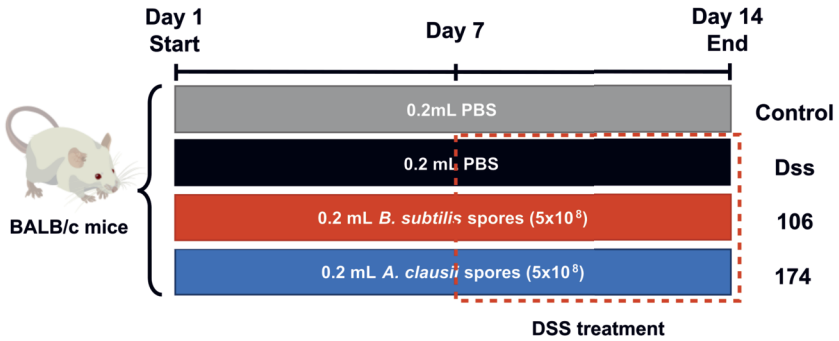

FIGURE 1 Experimental design. Grey rectangle: control (naïve mice); black rectangle: mice treated with DSS only; red rectangle: mice treated with SF106 spores; blue rectangle: mice treated with SF174 spores; red dotted line: 4% DSS-treatment.

were retained for downstream processing. DADA2's end product was an amplicon sequence variant (ASV) table, and the Silva database release 138 was used as a comparison to assign the taxonomy to the sequence variants (https://www.arb-silva.de/). The end product of DADA2 was used to investigate the prokaryotic diversity using the phyloseq package (McMurdie *et al.*, 2013), as previously reported (Cordone *et al.*, 2023). The alpha diversity was calculated using the Shannon diversity index while the beta diversity was analysed using weighted Jaccard dissimilarity index, and related to different variables using non-metric multidimensional scaling (nMDS) with the vegan package (Oksaen *et al.*, 2009).

Statistical analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Prism, La Jolla, CA, USA; version 9.1.0 for Windows). All graphs are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to detect differences between the groups compared to the DSS group, followed by Dunnett's multiple comparison test. A two-way ANOVA was used to determine the effect of different treatment groups over time on weight change and DAI, followed by Tukey's multiple comparison test with individual variances computed for each comparison. A value of $P \leq 0.05$ was considered statistically significant.

3 Results

SF106 and SF174 spores protect mice from the DSS-induced inflammation

Balb/c mice were randomly subdivided into four experimental groups (n = 5) as schematically reported in Figure 1. Two groups, 106 and 174, received, respectively, 5×10^8 spores of strain SF106 or SF174 every day for fourteen days by oral gavage. In addition, from day 7 to day 14, 4% DSS was added to their drinking water to induce symptoms of ulcerative colitis (UC). In parallel, two control groups received an oral gavage of PBS for fourteen days with the positive control having 4% DSS (via drinking water) from day 7 to day 14 (DSS) (Figure 1). All animals were monitored daily from the day before the beginning of the DSS treatment (day 6) to the end of the treatment (day 14) to assess the weight variations and the Disease Activity Index (DAI). As reported in Figure 2A, a weight gain was recorded in mice of the Control group (grey line, Figure 2A) while the treatment with DSS (black line, Figure 2A) caused a drastic weight loss. Mice of both spore-treated groups (106 in red and 174 in blue in Figure 2A) showed a slight initial weight loss (day 7) followed by a significant weight recovery from day 7 to day 10 and by a final weight reduction that was, however, significantly lower than that observed in animals of the DSS group ($P < 0.0001$) (Supplementary Table S2).

To assess disease progression typical symptoms of UC, including stool consistency and rectal bleeding (see Materials and methods) were monitored. Scores were assigned based on the severity of symptoms (see Materials and methods) and used to generate a DAI graph (Figure 2B). At day 11 mice of the DSS group (black line, in Figure 2B) showed severe symptoms (DAI = 4.0 ± 1.1). In contrast, animals of both spore-treated groups (red and blue line, Figure 2B) did not show significant symptoms until day 13 of the treatment, indicating a delay in disease progression. On day 14, 106 mice reached a DAI score of 7.0 ± 1.0, while 174 mice maintained a score of 5.0 ± 1.5, significantly lower than the value recorded for mice of the DSS group ($P < 0.0001$) (Supplementary Table S2).

SF106 and SF174 spores protect the intestinal integrity

On day 14 all mice were euthanised and distal colon samples collected, fixed and stained with H&E for histological analysis (see Materials and methods). Histo-

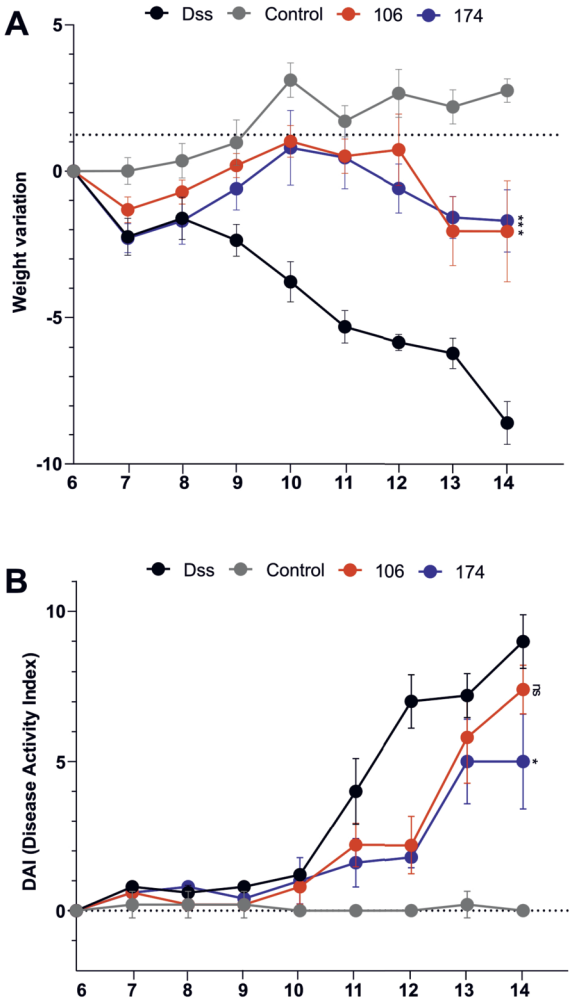

FIGURE 2 Effect of SF106 and SF174 spores on disease progression. Weight change (A) and disease activity index (B) were compared from the day before the DSS treatment (day 6) to day 14. Grey line: control (naïve mice); black line: mice treated with DSS only; red line: DSS-treated mice receiving SF106 spores; blue line: DSS-treated mice receiving SF174 spores. Data are reported as mean ± standard error of the mean (n = 5 per group). Two-way ANOVA was used to assess statistically significant differences between groups ($P < 0.0001$ for both analyses), followed by Dunnett's multiple comparison test. Only the significance of the SF106 and SF174 groups compared to the DSS group on the last day of treatment are reported. **** $P < 0.0001$; *** $P < 0.001$; ns = not significant.

logical samples of the Control group (top left panel of Figure 3A) showed an intact epithelial tissue, with intact crypts without inflammatory cell infiltration, numerous goblet cells (red arrows in Figure 3A) and a normal muscularis propria (black arrows in Figure 3A). In contrast, severe histological damages were observed in the colon of mice of the DSS group (lower left panel of Figure 3A), with erosion and destruction of the intestinal epithelium, ulceration of the mucosa (black star in Figure 3A), destruction of the intestinal crypts and villi, depletion of the goblet cells and abundant infiltration of inflammatory cells in the mucosa and submucosa (blue arrows in Figure 3A). In spore-treated mice (right panels in Figure 3A), there was minimal tissue damage, with visible crypts and goblet cells and reduced muscular hypertrophy (black arrows in Figure 3A). Furthermore, leucocyte inflammatory infiltrates (blue arrows in Figure 3A) were not observed in animals of the 174 group and were minimal in mice of the 106 group. To better evaluate the severity of the colitis, an objective histological scoring system was employed. The un-treated *naïve* mice showed no evidence of histological abnormality (not shown), whereas the histology from the DSS group resulted in a cumulative score of 13.0 ± 0.3 (Figure 3B). In mice treated with either spore type, a statistically significant reduction in the severity of histological inflammation was seen, with scores being of 11.6 ± 0.5 and 8.2 ± 0.6 for 106 and 174 groups, respectively. Although the protective effect was observed with spores of both strains, it was, however, stronger with SF174 than with SF106 spores (Figure 3B).

A qPCR approach was used to analyse the expression levels of the *muc-2* (a secretory mucin whose expression has been associated with several intestinal diseases (Liu *et al.*, 2020)) and *claud-1* (a tight junction protein altered in several IBDs (Pope *et al.*, 2014)) genes. Considering the level of expression in *naïve* mice as zero, the treatment with DSS drastically reduced the expression of the *muc-2* and *claud-1* genes (black boxes in Figure 3C,D), while the treatment with either spore type (red and blue boxes in Figure 3C,D) restored the expression of the two genes at levels similar to those observed in control animals. These results, consistent with histological analyses, indicate the beneficial effect of the treatment with either type of spores on maintaining and improving intestinal barrier function.

SF106 and SF174 spores reduce DSS-induced inflammation markers

To evaluate the effect of the spore treatment on the DSS-induced inflammatory response, the expression levels of genes coding for some of the major cytokines were analysed by qPCR. Normalising for the values of the Control group (equal to 0 in the graphs), clear variations were observed in animals of the DSS group with an increased expression of the pro-inflammatory cytokine tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β genes and a decrease of the anti-inflammatory cytokine IL-10 (Figure 4A-D). The TNF-α increase and IL-10 decrease were partially reverted by SF174 spores

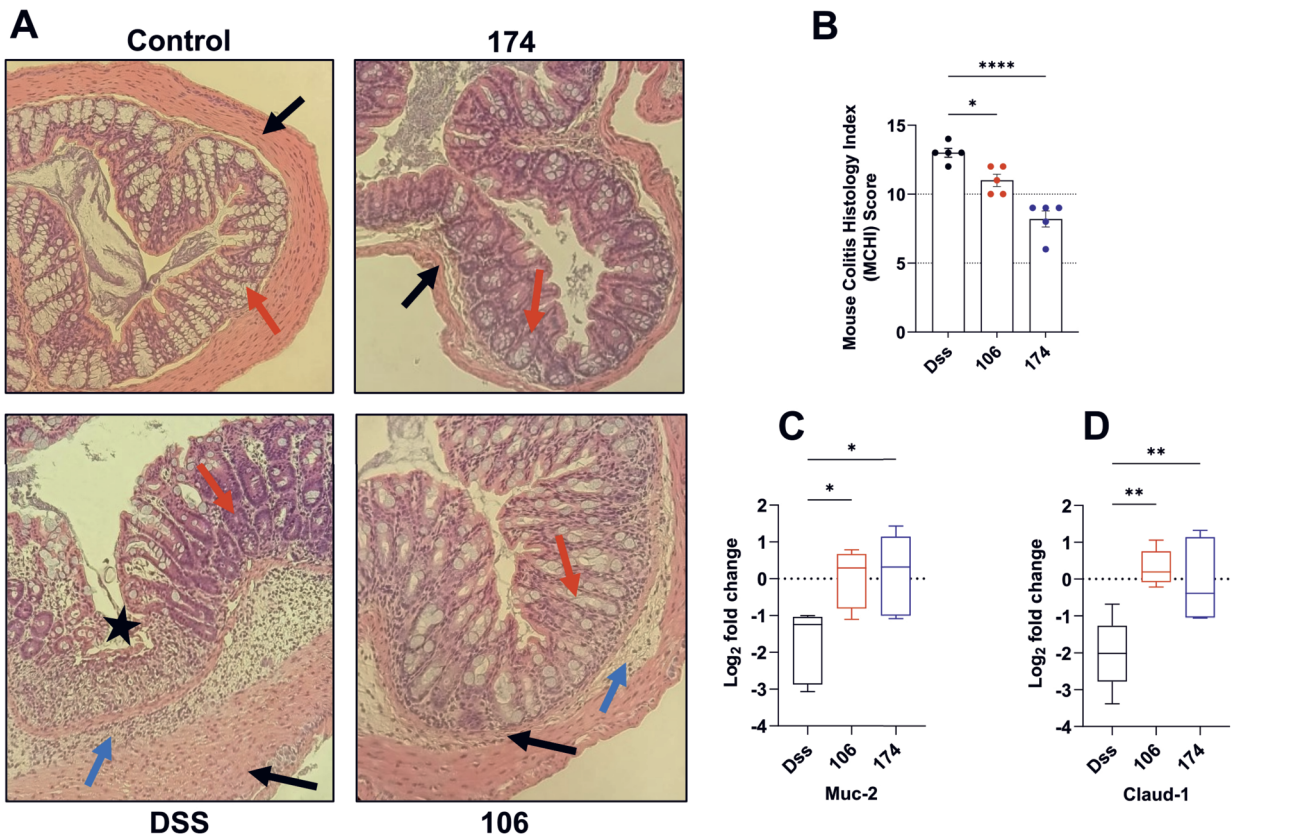

FIGURE 3 Effect of SF106 and SF174 spores on DSS-induced colonic injuries and gut integrity. (A) Microscopic pictures of proximal colon tissue stained with H&E. Red arrows: goblet cells; black arrows: muscularis propria; blue arrows: submucosal inflammatory infiltrate; black star: mucosa ulceration. (B) Mouse Colitis Histology Index scores assigned after microscopic analysis of colon sections (Methods). qPCR analysis of *muc-2* (C) and *claud-1* (D) genes. Data are shown as mean ± SEM (n = 5). One-way ANOVA was performed to determine the statistically significant differences between groups followed by Dunnett's multiple comparisons test against the DSS-only group. **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

(blue boxes in Figure 4A and 4D), while the effects of SF106 spores (red boxes in Figure 4A-D) were statistically significant only on the TNF-α levels (Figure 4A). The increased expression levels of the IL-6 (Figure 4B) or IL-1β (Figure 4C) genes were not affected by either spore treatment.

Expression levels of the genes coding for the Toll-like receptor 2 and 4 (TLR-2 and TLR-4), recognising, respectively, Gram-positive or Gram-negative bacteria, were also analysed by qPCR. As shown in Figure 4E and 4F, the DSS treatment down-regulated TLR-2 and did not affect TLR-4. While the treatment with SF106 spores tended towards the value of naive animals that with SF174 spores completely subverted ($P < 0.01$) the DSS-induced down-regulation (red and blue boxes, respectively, Figure 4E). No statistically significant differences were observed for the expression levels of the TLR-4 gene (Figure 4F).

Gut microbial composition

The effects of SF106 and SF174 spores on the composition of the mice gut microbiota was analysed by 16S rRNA gene sequencing of faecal samples collected on day 14. With the exception of the DSS group (n = 5), analyses for the other groups (Control, 106 and 174) were performed on four samples (n = 4) due to lack of material at the time of sampling. The alpha-diversity by the Shannon Index showed that the diversity was overall similar between the four experimental groups with a slightly higher and lower diversity for the 106 and Control group, respectively (Figure 5A). The DSS treatment strongly reduced the heterogeneity of the microbial community observed between the various animals of the group that was instead higher in the 106 group (Figure 5A). In the beta diversity analysis using non-metric multidimensional scaling (nMDS) based on weighted and unweighted Jaccard dissimilarity index, all groups formed clear clusters. The 106 and 174 clusters (respectively, red and blue symbols in Figure 5B) were well separated from Control and DSS clusters (Figure 5B). Consistently with the results of Figure 5A, all animals of the DSS group formed a small cluster close to that of the Control group (Figure 5B).

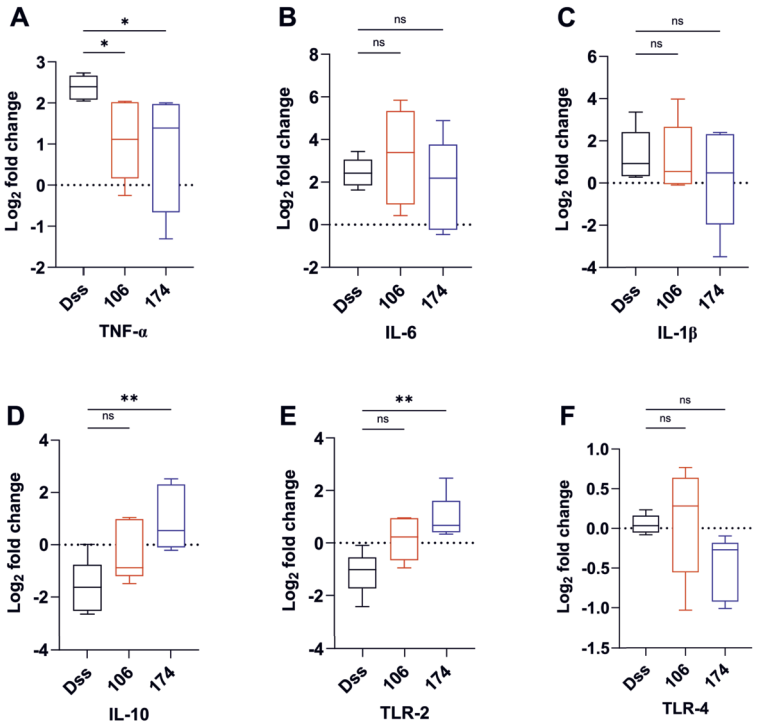

FIGURE 4 Effect of SF106 and SF174 spores on the expression of inflammatory and anti-inflammatory markers: (A) tumour necrosis factor (TNF)-α, (B) interleukin (IL)-6, (C) IL-1β, (D) IL-10, (E) Toll-like receptor (TLR)-2 and (F) TLR-4. Data are shown as mean ± standard error of the mean (n = 5). Black boxes: mice treated with DSS only; red boxes: DSS-treated mice receiving SF106 spores; blue boxes: DSS-treated mice receiving SF174 spores. One-way ANOVA was performed to determine the statistically significant differences between groups followed by Dunnett's multiple comparisons test against the DSS-only group. ** $P < 0.01$; * $P < 0.05$; ns = not significant.

The taxonomy analysis of the ten most represented phyla and genera is reported in Figure 6A and Figure 6B, respectively. In all four groups *Firmicutes* and *Bacteroidota* were the most abundant phyla but while they represented over 90% of the total phyla in the control and DSS groups, in the spore-treated groups were less abundantly represented (64.3 and 81.5% of the total, in SF106 and SF174, respectively) and replaced by members of the *Verrucomicrobiota*, *Proteobacteria* and *Actinobacteria* phyla (Table 1).

At the genus level the abundance of several genera was significantly altered by the spore treatment. In comparison with the microbiota of the DSS animals, the abundance of thirteen genera was reduced by both SF106 and SF174 spores in a statistically significant way (Table 2). Of those thirteen genera only one, the *Lactobacillus* genus, was reduced by the DSS treatment with respect to the control group and further reduced by both spore treatments (Table 2). All other twelve genera were increased by the DSS treatment with respect to the control group and rescued by the SF106 or SF174 spore treatment at the level observed in the control group or even at lower levels (Table 2).

The abundance of some other genera was, instead, increased by the spore treatment. With respect to mice of the DSS group, SF174 spores caused a statistically significant increase in the abundance of members of the *Bacteroides* and *Akkermansia* genera, while SF106 had a similar but not statistically significant effect (Figure 7). Only SF106 spores positively effected in a statistically significant way the abundance of the low represented genera *Hyphomicrobium*, *Devosia*, *Terrimonas* and *LWQ8* (uncultured family of *Saccharimonadales*) (Table 3).

4 Discussion

The main result of this study is that a preventive treatment with spores of *B. subtilis* SF106 or *B. clausii* SF174 has a beneficial health effect by reducing the DSS-induced inflammation in a murine model. Spores of the two strain behaved similarly but those of strain SF174 were more effective and SF174 pre-treated animals showed a lower disease activity and histology indices than those observed with mice pre-treated with SF106 spores. In addition, SF174 spores induced the expression of the anti-inflammatory cytokine IL-10 at levels statistically higher than those observed in the DSS-treated group while spores of the SF106 showed a slight, not statistically significant, increase of IL-10 expression.

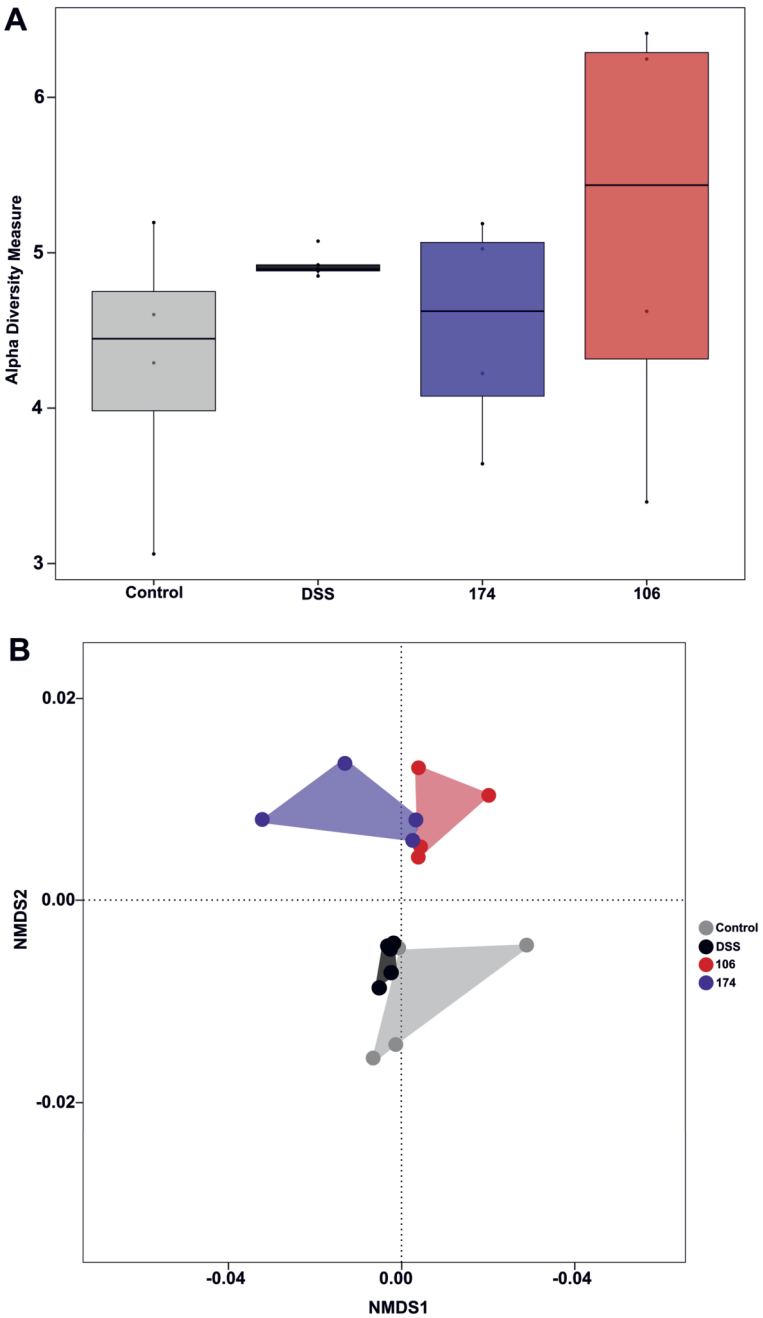

FIGURE 5 Gut microbial community diversity. Alpha diversity measure across the different groups by the Shannon index (A) and non-metric multidimensional scale (nMDS) plot based on weighted Jaccard dissimilarity index (B). Grey symbols: control (naïve mice); black symbols: mice treated with DSS only; red symbols: DSS-treated mice receiving SF106 spores; blue symbols: DSS-treated mice receiving SF174 spores.

The analysis of the expression of cytokines and Toll-like receptors 2 and 4 (TLR-2 and TLR-4) suggested that both spore-based treatments have an immunomodulatory trend in mice with DSS-induced colitis. Both spores reduced the expression of the pro-inflammatory cytokine TNF-α while only SF174 spores induced a statistically significant increase of the expression of the anti-inflammatory cytokine IL-10. Such increase was at levels higher than those observed in the Control (naïve) animals indicating a general anti-inflammatory activity independent of the DSS-induced inflammation.

TLR-2 and TLR-4 recognise commensal and pathogenic bacteria, with TLR-2 mainly active on Gram-positives and TLR-4 on Gram-negatives. Such recognition is essential for the release of pro- and anti-inflammatory cytokines and, therefore, for the maintenance of the intestinal homeostasis (Latorre *et al.*, 2018). The balance of TLR-2 and TLR-4 receptors can

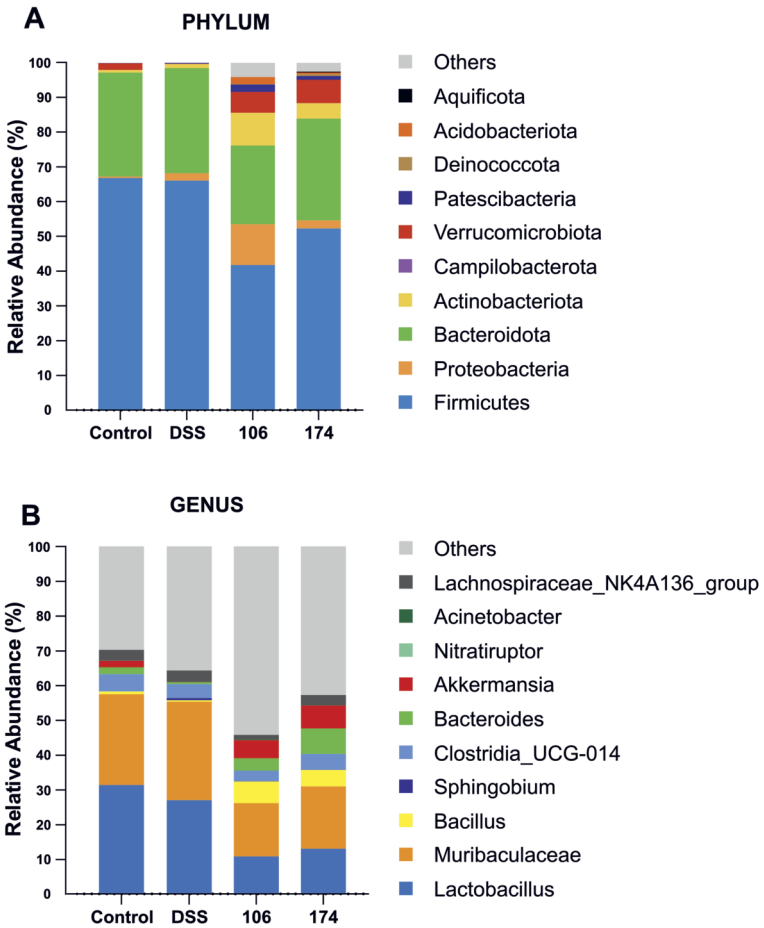

FIGURE 6 Taxonomic analysis. Bar plot of the relative abundance of the ten most abundant phyla (A) and genera (B) in the different groups.

TABLE 1 Relative abundance (%) of the ten most abundant Phyla in the four experimental groups

Phylum	Control	DSS	SF106	SF174
Firmicutes	66.79	66.03	41.71	52.24
Bacteroidota	29.84	30.22	22.59	29.26
Verrucomicrobiota	1.92	0.06	5.99	6.75
Proteobacteria	0.43	2.17	11.80	2.38
Actinobacteriota	0.82	1.17	9.43	4.40
Patescibacteria	0.08	0.29	2.13	1.09
Campilobacterota	0.00	0.00	0.03	0.01
Deinococcota	0.01	0.00	0.11	0.50
Acidobacteriota	0.00	0.00	2.01	0.51
Aquificota	0.00	0.00	0.03	0.21
Others	0.12	0.06	4.17	2.64

trigger intracellular signalling cascades and modulate the expression of cytokines (Jia *et al.*, 2020; Latorre *et al.*, 2018). In the present work the analysis of the expression of TLR-2 and TLR-4 indicated a down-regulation of TLR-2 and a slight up-regulation of TLR-4 in the DSS group, probably contributing to the increased production of pro-inflammatory cytokines and to the decreased production of the anti-inflammatory cytokine IL-10 observed as consequences of the DSS treatment. Such effects were reverted in a statistically significant way (TLR-2) or close to statistically significance (P = 0.08) (TLR-4) by SF174 spores. SF106 spores induced a similar but weaker effect on TLR-2 expression while the effect on TLR-4 was unclear due to the high variability of the expression observed in the mice of the SF106 group. The different balance of expression of TLR-2 (up-regulated) and TLR-4 (down-regulated) caused by the spore pre-treatment is a likely cause of the observed IL-10 increase and indicates that the oral administration of SF174 spores promotes an anti-inflammatory response in mice.

The analysis of the microbial composition of the mice gut indicated that both spore treatments modified the gut microbiota. With respect to animals treated only with DSS, both spores reduced the abundance of the same thirteen genera. In twelve of those thirteen genera the relative abundance was increased by the DSS treatment with respect to *naïve* animals and

TABLE 2 Relative abundance (%) of genera negatively affected by SF106 or SF174 spores

Genus	Control	DSS	SF106	SF174
Lactobacillus	31.34 ± 7.25	26.95 ± 7.21	10.80 ± 4.2[b]	13.00 ± 4.20[a]
Lachnospiraceae UCG-001 group	0.40 ± 0.34	1.32 ± 0.57	0.19 ± 0.14[b]	0.08 ± 0.09[b]
Enterorhabdus	0.56 ± 0.44	1.04 ± 0.30	0.25 ± 0.17[b]	0.17 ± 0.13[b]
Clostridioides	0.08 ± 0.10	0.80 ± 0.45	0.00 ± 0.00[b]	0.00 ± 0.00[b]
Roseburia	0.26 ± 0.25	0.71 ± 0.26	0.06 ± 0.05[c]	0.01 ± 0.02[c]
Tyzzerella	0.12 ± 0.04	0.37 ± 0.11	0.13 ± 0.04[c]	0.12 ± 0.05[c]
Eubacterium xylanophilum group	0.09 ± 0.10	0.35 ± 0.15	0.02 ± 0.02[b]	0.00 ± 0.01[c]
Incertae sedis	0.12 ± 0.03	0.25 ± 0.04	0.07 ± 0.01[d]	0.07 ± 0.04[d]
Gemella	0.12 ± 0.07	0.21 ± 0.05	0.09 ± 0.03[a]	0.08 ± 0.04[a]
Eubacterium nodatum group	0.13 ± 0.05	0.18 ± 0.04	0.05 ± 0.03[b]	0.07 ± 0.04[b]
Intestinimonas	0.07 ± 0.02	0.11 ± 0.03	0.05 ± 0.02[a]	0.07 ± 0.03
Chryseomicrobium	0.02 ± 0.03	0.04 ± 0.00	0.00 ± 0.00[b]	0.00 ± 0.00[b]
Eubacterium brachy group	0.03 ± 0.02	0.03 ± 0.01	0.01 ± 0.01	0.00 ± 0.00[a]

[a] $P \leq 0.05$.
[b] $P \leq 0.01$.
[c] $P \leq 0.001$.
[d] $P \leq 0.0001$ by a one-way ANOVA analysis performed comparing groups SF106 or SF174 vs DSS.

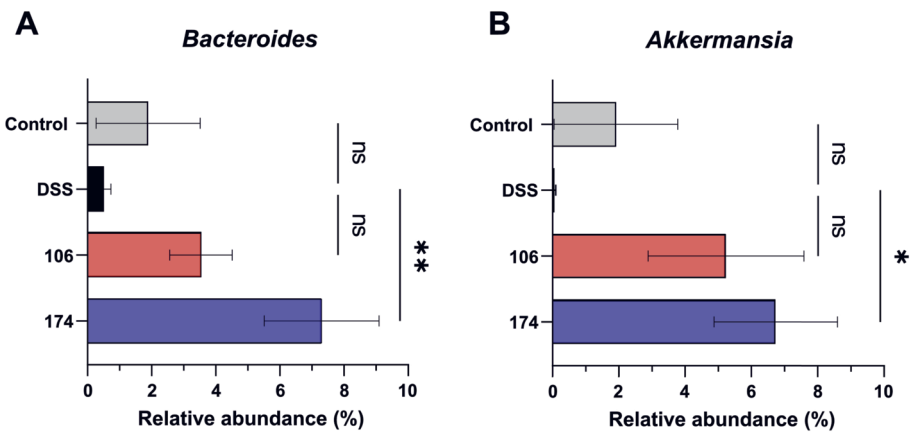

FIGURE 7 Effect of SF106 and SF174 spores on the abundance of *Bacteroides* and *Akkermansia* genera. Data are shown as mean ± standard error of the mean. One-way ANOVA was performed to determine the statistically significant differences between groups followed by Tukey's multiple comparison test. ** $P < 0.01$; * $P < 0.05$; ns = not significant.

rescued by both spore types. Only the abundance of members of the *Lactobacillus* genus was decreased by DSS and further decreased by both spore types. Both spore types had a positive effect on the abundance of members of the *Bacteroidetes* and *Akkermansia* genera, although the increase was statistically significant only with SF174. Similar effects have been previously reported with spores of other *Bacillus* strains able to increase the abundance of those bacteria *in vitro* (Ji *et al.*, 2022) or *in vivo* (Marzorati *et al.*, 2021). *Bacteroidetes* and *Akkermansia* are beneficial microbes, considered as 'next-generation probiotics' (Depommier *et al.*, 2019). Indeed, *Akkermansia mucinophilia* has been shown to increase colonic mucin production, providing a thicker mucosal barrier to the underlying epithelial layer, perhaps explaining the upregulation of MUC-2 seen in this study and, in part, the protective effects of SF174.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.25913347

Table S1. Sequence list of primers used for qPCR reactions.

Table S2. Statistics weight and DAI.

TABLE 3 Relative abundance (%) of genera positively affected by SF106 spores

Genus	Control	DSS	SF106	*P*-value[1]
Hyphomicrobium	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.07	0.049
Devosia	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.03	0.040
Terrimonas	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	0.002
LWQ8	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.01	0.038

[1] One-way ANOVA analysis performed comparing group 106 vs DSS.

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Authors' contribution

MV performed most of the experiments; EH contributed to the animal experiments; DB contributed to the bioinformatic analysis; AS, LB and SMC contributed to the analysis of the experimental results and to the text writing; ER supervised the work and wrote the manuscript.

Conflict of interest

E.R. acts as a consultant for Gruppo Savio that has the rights for the commercialization of the strains SF106 and SF174.

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