The Biotherapeutic Potential of *Lactobacillus reuteri* Characterized Using a Target-Specific Selection Process

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A growing body of clinical and experimental data supports the view that the efficacy of probiotics is strain-specific and restricted to particular pathological conditions, which means that newly isolated probiotic strains need to be targeted to a specific disease. Following national and international guidelines, we used a conventional *in vitro* experimental approach to characterize a novel strain of *Lactobacillus reuteri*, LMG P-27481, for safety (sensitivity to antibiotics and genome analysis) and putative efficacy (resistance to gastro-intestinal transit, adhesiveness, induction of cytokines, and release of antimicrobial metabolites). *In vitro* assays, which were carried out to examine the probiotic’s effect on diarrhea (lactose utilization, inhibition of pathogens such as bacteria and Rotavirus), showed that it was more efficacious with respect to well-known reference strains in antagonizing *Clostridioides difficile* (CD). Data confirming that the probiotic can effectively treat CD colitis was gained from *in vivo* trials involving mice conditioned with large spectrum antibiotics before they were subjected to CD challenge. Two out of the three antibiotic-treated groups received daily LMG P-27481 for different time durations in order to simulate a preventive approach (LMG P-27481 administered prior to CD challenge) or an antagonistic one (LMG P-27481 administered after CD challenge). Both approaches significantly reduced, with respect to the untreated controls, CD DNA concentrations in caecum and *C. difficile* toxin titers in the gut lumen. In addition, LMG P-27481 supplementation significantly mitigated body weight loss and the extent of inflammatory infiltrate and tissue damage. The study results, which need to be confirmed by *in vivo* clinical trials, have demonstrated that the *L. reuteri* LMG P-27481 strain is a promising probiotic candidate for the treatment of CD infection.

**Keywords:** probiotic, lactobacilli, pathogen, intestine, gut, diarrhea

**INTRODUCTION**

A variety of studies have evaluated the efficacy of probiotics in treating diarrhea, which represents a potentially serious health problem with more than 4 billion cases reported world-wide every year. McFarland et al. (2018) carried out an extensive systematic meta-analysis of the results of randomized controlled trials examining the use of probiotics to treat different types of diseases.
In accordance with Ouwehand (2017), the investigators confirmed the strain- and disease-specificity of probiotics in treating diarrhea. Despite a wide heterogeneity across clinical studies, some probiotics have been found to be effective in treating antibiotic-associated adult diarrhea, Clostridium difficile diarrhea (CDAD), acute Rotavirus diarrhea, radiation therapy-related diarrhea, and enteral nutrition-related diarrhea. Their efficacy in treating traveler’s diarrhea (TD) has not, however, been supported by clinical data (Williams and Adcock, 2018). Hypothetical mechanisms explaining probiotics’ effects include competitive inhibition of pathogens, stabilization of the resident microbiota, and attenuation of increased gut barrier permeability. Their beneficial effects in terms of reducing the duration and intensity of fever has been demonstrated in children although they did not seem to affect the number of daily evacuations and accompanying symptoms such as vomiting (Salarí et al., 2012).

The efficacy of probiotics in adults for the different types of diarrhea listed above has not yet been convincingly established and still requires further investigation (Ritchie and Romanuk, 2012; Salarí et al., 2012). When Szajewska et al. (2019) examined in a systematic review 15 randomized controlled trials investigating the efficacy of Lactobacillus rhamnosus GG (LGG) in children affected by acute gastroenteritis, they concluded that the probiotic reduced the duration of diarrhea and hospitalization. Conflicting findings, which may be explained by the heterogeneity of the patients studied and methodological limitations, have nevertheless been reported. Capurso (2019) likewise reviewed data examining the use of L. rhamnosus GG supplementation to treat gastrointestinal infections and diarrhea, antibiotic and C. difficile associated diarrhea as well as its use for other pathological conditions.

Belonging to the Lactobacillus genus, Lactobacillus reuteri represents a natural inhabitant of the human and animal gastro-intestinal tract. It is an obligatory heterofermentative Lactobacillus that in particular conditions leads to the formation of carbon dioxide, ethanol, acetic acid and lactic acid following sugar fermentation. L. reuteri can also produce 3-hydroxypropionaldehyde (reuterin), a bacteriocin with antimicrobial properties, as well as folate and cobalamin (Mu et al., 2018). Some L. reuteri strains, such as DSM 17938 and RC-14, are already present in many commercially available food supplements. Evidence confirming the efficacy of L. reuteri in treating gastrointestinal disorders such as infantile colic, regurgitation, functional constipation, abdominal pain, and necrotizing enterocolitis (Srinivasan et al., 2018) has been extensively outlined in the literature.

Using the FAO/WHO (2002) for the evaluation of probiotics in food, whose timeliness has been confirmed by an expert panel (Hill et al., 2014; Shewale et al., 2014), the current study set out to evaluate the efficacy of a new probiotic strain in treating C. difficile-associated diarrhea. The new strain, named LMG P-27481, meets the conventional safety criteria for probiotics which is based on their survival in the GI tract as well as their ability to adhere to gut mucosa, to counteract the activity of pathogens and to boost the immune system. Its safety profile for human use was also assessed following the most recent international guidelines (EFSA Scientific Opinion, 2018). Characterization to assess the strain’s efficacy was, moreover, performed using an in vivo murine model of CD-associated diarrhea to investigate its ability to counteract the pathogen.

MATERIALS AND METHODS

Identification of L. reuteri LMG P-27481

The LMG P-27481 L. reuteri strain was isolated in 2013 from a human fecal sample of a 9-month old vaginally delivered healthy infant. Written informed consent was obtained from the parents. The strain was identified at the genus level and then as a distinct L. reuteri species. LMG P-27481 was compared to other L. reuteri strains available on the Italian market to assess its originality using strain-specific REP-PCR amplification (Versalovic et al., 1994). Other relevant well-known commercial strains such as L. reuteri RC-14, L. reuteri DSM 17938, and L. harnmosus ATCC 53103 were used as references for the assays conducted during the study (Mogna et al., 2012).

Safety Assessment

Minimum inhibitory concentrations (MICs) of 15 different antibiotics were determined for the strain under investigation in accordance with ISO 10932:2010 (IDF 223:2010). MIC values were determined for the strain under investigation were measured by agar dilution according to the guidelines described in the EUCAST document (EUCAST, 2018). Antibiotics were determined for the strain under investigation were measured by agar dilution according to the guidelines described in the EUCAST document (EUCAST, 2018).

| Antibiotic                  | Concentration range (µg/ml) |
|-----------------------------|----------------------------|
| Gentamicin                  | 0.5–256                    |
| Kanamycin                   | 2–1024                     |
| Streptomycin                | 0.5–256                    |
| Neomycin                    | 0.5–256                    |
| Tetracycline                | 0.12–64                    |
| Erythromycin                | 0.016–8                    |
| Clindamycin                 | 0.03–16                    |
| Chloramphenicol             | 0.12–64                    |
| Ampicillin                  | 0.03–16                    |
| Vancomycin                  | 0.25–128                   |
| Virginiamycin (Quinupristin-Dalfopristin) | 0.016–8               |
| Linezolid                   | 0.03–16                    |
| Trimethoprim                | 0.12–64                    |
| Ciprofloxacin               | 0.25–128                   |
| Rifampin                    | 0.12–64                    |
Complete Genome Sequencing of L. reuteri LMG P-27481 Strain

Genomic DNA from an overnight culture of LMG P-27481 L. reuteri, which was extracted using the MasterPure Gram Positive DNA Purification kit (Epicenter, Cambio, United Kingdom) following the manufacturer’s instructions, was quantified utilizing NanoDrop™ spectrophotometer A280/260 (Thermo Fisher Scientific, Milan, Italy). Total DNA sequencing was determined by GenProbio Srl (Parma, Italy) using a MiSeq Illumina platform. Genomic libraries were constructed employing the TruSeq DNA PCR-Free LT Kit (Illumina) using 2.5 µg of genomic DNA, which was fragmented using a Bioruptor NGS ultrasonicator (Diagenode, United States), and size was assessed using Agilent 2200 Tape Station (Agilent Technologies). Library samples were loaded onto a Flow Cell V3 600 cycles (Illumina) following the technical support guide instructions. Five hundred sequencing cycles resulted in an average reading length of approximately 250 nucleotides for both paired-end sequences.

Bioinformatic Analysis of L. reuteri LMG P-27481 Genome

The version 4.0.2 MIRA program (Chevreux et al., 1999, Computer Science and Biology: Proceedings of the German Conference on Bioinformatics) was used for de-novo assembly of the LMG P-27481 L. reuteri genome sequence. Assembly resulted in 134 contigs with a total genome size of 2,016,419 bp using a MIRA v4.0.2 assembler (Chevreux et al., 1999), with a coverage of 70.1X, GC content of 38.8%, N50 29849. Open Reading Frames (ORFs) prediction and subsequent GeneBank file construction were performed using Prodigal v2.6 software (Hyatt et al., 2010). Automatic annotation of the ORFs was carried out using a custom script performing a BLASTp search against the NCBI database (Milani et al., 2014). Ribosomal RNA gene prediction was performed using RNAmer v1.2 (Lagesen et al., 2007), and transfer RNA gene prediction was carried out using tRNAscan-SE v1.21 (Lowe and Eddy, 1997).

Reordering of the final contigs comparable to NCBI template genomes L. reuteri DSM 20016 was performed using Mauve v2.3.1 (Darling et al., 2004). Burrows-Wheeler Alignment with SAMTools software (Li and Durbin, 2009) and VarScan v2.2.3 (Koboldt et al., 2013) were carried out to optimize the assembly to obtain final contigs. To obtain the predicted protein sequences, the antibody resistome of LMG P-27481 L. reuteri was identified using Rapshear (Zhao et al., 2012) against a custom database and CARD (McArthur et al., 2013) and Transporter Classification Database (TCDB) (Saier et al., 2014). Prediction of the mobilome was carried out using the PHAge Search Tool PHAST (Zhou et al., 2011) and the ISfinder database (Siguier, 2006). Prediction of the putative virulence genes was carried out using the Virulence Factors Database (VFDB) (Chen et al., 2005) and that of the bacteriocin encoding genes was performed using the Bagel3 software package (van Heel et al., 2013). Other relevant factors with a putative adhesion to probiotic functions, such as mucus and fibronectin-binding proteins and S-layer proteins, pilus encoding genes and those involved in Extracellular polysaccharide synthesis (EPS), were predicted using a custom database based on NCBI RefSeq (Pruitt et al., 2007).

Conventional Characterization of L. reuteri LMG P-27481

The procedures described by Charteris et al. (1998) and Gilliland et al. (1984) were followed to investigate the ability of the newly isolated and reference L. reuteri DSM 17938 and L. reuteri RC-14 strains to survive the hostile conditions of the gastrointestinal environment including the high pH levels of gastric and duodenal juices. Their ability to adhere to the human mucosa was verified and quantified using cultured monolayers of HT-29 cells (Jensen et al., 2012). All the strains were cultured in MRS medium at 37°C in anaerobiosis and collected by centrifugation. Bacteria were resuspended in sterile DMEM at 10^6 CFU/ml and added to HT-29 monolayers (MOI 1:50/cells to probiotic). The medium was incubated for 60 min at 37°C. Then, it was removed and discarded and sterile DMEM was added to the monolayers. The medium was checked three times to verify that all unbound bacteria were removed. One ml of sterile MRS was then added to each monolayer and the cells were collected using a cell scraper. The material was collected using a sterile syringe and passed three times through an 18-needle gauge, and following decimal dilutions, seeded on MRS agar to quantify the live bacteria. The adhesion was expressed as the percentage of viable bacterial cells adhering to HT-29. The experiments were performed in triplicate. L. paracasei ATCC 344 was used as the technical control.

The in vitro Effect on Human Dendritic Cells

Following international guidelines for the characterization of the properties of probiotics, the immune modulatory potential of the LMG P-27481 strain was verified on human-derived dendritic cells (DCs). The DCs were generated from monocytes obtained from the peripheral blood of healthy donors and cultured in complete RPMI medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF, 50 ng/mL; Peprotech) and interleukin-4 (20 ng/mL; Peprotech, Milan, Italy) for 6 days (Miletí et al., 2009). On the day of the experiment, the DCs were collected, washed by centrifugation, counted, and either incubated in medium alone or in medium without antibiotics containing the specified probiotic strain (MOI 1:10/DC to probiotic) obtained from a fresh culture in the logarithmic growth phase. The dendritic cells were incubated 1 h at 37°C, then collected, pelleted by centrifugation, resuspended in 1 ml of sterile RPMI and subjected to centrifugation (800 rpm × 10 min). The cells were then incubated in fresh complete medium containing gentamicin (100 µg/ml). After 24 h, the DCs were collected by centrifugation, IL-10 and IL-12p70 were quantified in the culture supernatants by ELISA (eBioscience), and the anti-inflammatory index was calculated (IL-10/IL-12p70 concentration ratio).
The Release of Hydrogen Peroxide

The ability to release H$_2$O$_2$ in the surrounding environment confers antimicrobial and preservative properties to some lactobacilli. Bacterial strains were grown in MRS broth to assess the LMG P-27481 strain’s ability to release H$_2$O$_2$. The cultures were then centrifuged, washed twice, and finally resuspended with sodium phosphate buffer containing glucose 5 mM. After being incubated in refrigerated conditions for a maximum of 24 h, the cultures were centrifuged, and the supernatants were assayed for H$_2$O$_2$. Hydrogen peroxide was quantified following the protocol of Yap and Gilliland (2000). The RC-14 and DSM 17938 strains were used as positive and negative controls, respectively.

Reuterin Secretion

Some strains of _L. reuteri_ are known to produce broad-spectrum antimicrobial substance, reuterin (Talarico et al., 1988), a product of the glycerol metabolism that is active against several intestinal pathogens. The ability to produce this bacteriocin was verified using the colorimetric test described by Cadieux et al. (2008) on a 24-h culture of the strain grown in MRS medium supplemented with glycerol (250 mM final concentration). The principle behind the test is that of transforming glycerol into 3-hydroxypropionaldehyde (reuterin) and to quantify it using a spectrophotometric method at 560 nm (Doleyres et al., 2005; Ortiz-Rivera et al., 2017).

Lactose Metabolism

The ability to use lactose as the growth substrate was tested by inoculating washed pure suspensions of _L. reuteri_ DSM 17938, _L. rhamnosus_ ATCC 53103, and _L. reuteri_ LMG P-2748 in MRS medium without dextrose, containing either 1% glucose or 1% lactose as the sole carbon source (Arnold et al., 2018). After being incubated for 18 h in microaerophilic conditions at 37°C, live bacteria were enumerated by seeding decimal dilution of the cultures on MRS agarose plates. T18 results were compared to the initial counts at T0 for the different carbohydrate sources.

The in vitro Effect on Intestinal Pathogen Growth

These experiments were designed to assess the ability of the LMG P-27481 strain to exert a direct inhibitory effect on some of the most common intestinal pathogens. The pathogen strains used for these tests were obtained from ATCC and from the Culture Collection of the University of Göteborg (Sweden). The two _L. reuteri_ strains (LMG P-27481 and DSM 17938) and _L. rhamnosus_ ATCC 53103 were cultured in MRS medium, _E. coli_ and _Salmonella enterica_ in Luria Bertani (LB) broth, and _C. difficile_ in Brain Heart Infusion (BHI) broth. To test the antimicrobial activity, the lactobacilli were separately inoculated at 10$^7$ CFU/ml and grown in MRS broth anaerobically at 37°C for 24 h. Once the bacteria had grown, the cultures were centrifuged, the supernatants were sterile filtered (0.2 μm), and the pH corrected to 7.0 before being inoculated with _E. coli_, _Salmonella_ or _C. difficile_ (10$^7$ CFU/ml) and incubated at 37°C. The aliquots of bacterial cultures were collected and plated 6, 12, and 24 h after inoculation onto appropriate agar media to quantify the viable pathogens. Each experiment was performed three times with triplicate determinations.

The in vitro Effect on the Rotavirus Infection

Rotavirus (ATCC VR-2018) was expanded and quantified on MA-104 (rhesus monkey kidney) cells. The efficacy of the _L. reuteri_ LMG P-27481 strain in treating the Rotavirus infection was assessed utilizing two _in vitro_ HT-29 cells protocols mimicking the human intestinal epithelium. In the pre-treatment _in vitro_ model the strains were placed in contact with HT-29 cells (MOI 1:50/cells to probiotic) prior to exposure to trypsin activated human rotavirus (Guerrero et al., 2000). Seventy-two hours later, the cells were collected, and total RNA was extracted. In the competition protocol, trypsin activated human rotavirus was pre-incubated with the different probiotic strains for 90 min. The bacteria + virus mixture was briefly centrifuged, and then the clear supernatant was used to infect confluent monolayers of human intestinal epithelial HT-29 cells. Seventy-two hours later, the cells were collected, total RNA was extracted using the SV Total RNA Isolation System (Promega), and reverse-transcribed into complementary DNA. Viral genome copies were quantified with SYBR Green PCR Master Mix in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using specific primers (van Maarseveen et al., 2010).

The in vivo Inhibitory Effect on _C. difficile_ Infection

LMG P-27481’s ability to inhibit _C. difficile_ infection was assessed _in vivo_ by verifying intestinal colonization and inflammation in mice treated with cefoperazone (Theriot et al., 2011). Twenty-four adult C57Bl/6 mice, purchased from Envigo Srl (Udine, Italy), were randomly allocated to one of four experimental groups. Experimental group 1 was not subjected to antibiotic treatment and did not receive LMG P-27481; experimental groups 2, 3, and 4 received a broad-spectrum antibiotic (cefoxperazone 0.5 mg/ml) in drinking water for 10 days. Two days after the antibiotic was discontinued mice received an intragastric suspension of _C. difficile_ VPI strain 10463 (10$^5$ CFUs) grown in BHI broth in anaerobic conditions. Experimental group 2 received only the _C. difficile_ challenge without LMG P-27481 treatment. Experimental group 3 was administered an intragastric suspension of _L. reuteri_ LMG P-27481 (10$^9$ CFU/day) 2 days before the antibiotic administration, which lasted for 9 days. Experimental group 4 received _L. reuteri_ LMG P-27481 (10$^9$ CFU/day) beginning on day 1 after _C. difficile_ challenge, that continued for 4 days (Figure 1).

Body weight was monitored daily until the animals were sacrificed 5 days after the _C. difficile_ challenge. The caecum content was collected to assess the presence of _C. difficile_ toxins, diluted 1:1 (vol/weight) with sterile PBS, and centrifuged at 10,000 × g for 5 min. The clear supernatant was then sterile filtered (0.2 μm) and serially diluted on Vero cell monolayers to determine the cytotoxin titer, which was defined as the highest dilution needed to cause > 50% rounding of all cells. _C. difficile_
FIGURE 1 | The experimental protocol of L. reuteri LMG P-27481 treatment of C. difficile colitis in mice.

FIGURE 2 | Lactobacillus reuteri LMG P-27481’s tolerance to in vitro gut transit gastric acid (A), pancreatic juice (B) and bile (C) exposure. (A) The probiotic’s growth, expressed in CFU/ml, following 0, 90, and 180 min of exposure to simulated gastric juice (n = 3 replicates for each strain). (B) The probiotic’s growth, expressed in CFU/ml, following 0 and 240 min of exposure to simulated pancreatic juice (n = 3 replicates for each strain). (C) Growth increase of probiotics, expressed as optical density OD 600 nm, following exposure for 6 h to bile salts. There are two curves for each strain tested: the reference growth standard on conventional laboratory medium MRS and the test curve reflecting the same medium supplemented with 0.3% vol/vol Oxgall.
FIGURE 3 | Lactobacillus reuteri LMG P-27481 hydrogen peroxide release and adherence to human HT-29 monolayers. (A) Production of H$_2$O$_2$ by probiotics expressed as µg/ml of metabolite released by $10^9$ viable CFUs after 24 h of incubation. (B) Antibiotic resistance image obtained by CARD showed the presence of nonperfect and strict and 111 loose hits. (C) Confluent HT-29 monolayers were washed and incubated with the probiotic strain (MOI 1:10) for 1 h at 37°C. To quantify adhering bacterial cells, the monolayers were extensively washed, lysed and aliquots seeded on MRS agar plates. Following 48 h of incubation in anaerobiosis, the colonies were enumerated and adhering bacteria expressed as the percentage of the total initial population of viable probiotic cells ($n=3$ different experiments in duplicate). Data are presented as mean ± SD.

colonization was evaluated by seeding aliquots of caecal content on C. difficile selective Agar (CDSA) plates (Becton Dickinson) incubated at 37°C in anaerobiosis. In addition, total DNA was extracted from 200 mg of caecal content using QIAmp DNA Stool mini extraction kit (Qiagen), and purified DNA was used as a template for qPCR using C. difficile 16S specific primers (Truong et al., 2017). Data were quantified using the ΔΔCt method and 16S rDNA as the reference gene. Tissue specimens from the colon of the mice were collected immediately after they were sacrificed and either fixed in 4% buffered formalin or snap frozen in liquid nitrogen. After the tissues had been kept in formalin for 24 h, they were paraffin embedded. At that time, 6 µm thick sections were cut, subjected to standard hematoxylin & eosin staining and scored for inflammatory damage by a pathologist (A.P.) (Castagliuolo et al., 1994). The frozen specimens were used to quantify myeloperoxidase activity (MPO) as a marker of neutrophil infiltration, as described elsewhere (Castagliuolo et al., 2005). All the experimental protocols were approved by the Animal Care and Use Ethics Committee of the University of Padua under license from the Italian Ministry of Health, and they were compliant with national and European guidelines for handling experimental animals and using them in experiments.

**Statistical Analysis**

Data are reported as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism Software 6.0 (GraphPad Software Inc., La Jolla, CA, United States). Student’s t-test was used to compare two independent groups; One-way ANOVA was used to compare more than two experimental group. $p < 0.05$ values were considered statistically significant.

**RESULTS**

**Identification and Probiotic Features of L. reuteri LMG P-27481**

The LMG P-27481 strain was selected from other human isolates belonging to the Lactobacillus reuteri species in view of its susceptibility to antibiotics according to the breakpoint values
### TABLE 2 | “Unique” genes of *L. reuteri* LMG P-27481.

| LMG_P-27481 ORFs | LMG_P-27481 annotation |
|------------------|------------------------|
| LMG_P-27481_0045 | Hypothetical protein   |
| LMG_P-27481_0048 | Hypothetical protein   |
| LMG_P-27481_0205 | Hypothetical protein   |
| LMG_P-27481_0214 | Type III restriction protein, res subunit |
| LMG_P-27481_0286 | YSIRK signal domain/LPXTG anchor domain surface protein |
| LMG_P-27481_0293 | Cytochrome C554         |
| LMG_P-27481_0387 | Transposase             |
| LMG_P-27481_0432 | Acetylornithine deacetylase |
| LMG_P-27481_0511 | Hypothetical protein   |
| LMG_P-27481_0514 | Hypothetical protein   |
| LMG_P-27481_0520 | Cro/C1-type HTH DNA-binding domain-containing protein |
| LMG_P-27481_0523,LMG_P-27481_0840 | Hypothetical protein |
| LMG_P-27481_0583 | Oxalyl-CoA decarboxylase |
| LMG_P-27481_0650 | Hypothetical protein   |
| LMG_P-27481_0666 | Competence protein CoIA |
| LMG_P-27481_0754 | Hypothetical protein   |
| LMG_P-27481_0846 | Integrase               |
| LMG_P-27481_0849 | Hypothetical protein   |
| LMG_P-27481_0850 | Hypothetical protein   |
| LMG_P-27481_0851 | Alpha-amylase           |
| LMG_P-27481_0852 | Hypothetical protein   |
| LMG_P-27481_0853 | Hypothetical protein   |
| LMG_P-27481_0854 | Hypothetical protein   |
| LMG_P-27481_0856 | Glycosyl hydrolase family 25 |
| LMG_P-27481_0869 | Peptidoglycan-binding protein LysM |
| LMG_P-27481_0908 | Hypothetical protein   |
| LMG_P-27481_0909 | Hypothetical protein   |
| LMG_P-27481_0910 | PHAGE protein, ATPase   |
| LMG_P-27481_0914 | Cro/CI family transcripational regulator |
| LMG_P-27481_0915 | Hypothetical protein   |
| LMG_P-27481_0919 | Hypothetical protein   |
| LMG_P-27481_0920 | Hypothetical protein   |
| LMG_P-27481_0921 | Hypothetical protein   |
| LMG_P-27481_0922 | Hypothetical protein   |
| LMG_P-27481_0923 | Hypothetical protein   |
| LMG_P-27481_0924 | Hypothetical protein   |
| LMG_P-27481_0927 | Hypothetical protein   |
| LMG_P-27481_0928 | Phage protein           |
| LMG_P-27481_0936 | Hypothetical protein   |
| LMG_P-27481_0937 | DNA replication protein |
| LMG_P-27481_0938 | Hypothetical protein   |
| LMG_P-27481_0942 | Hypothetical protein   |
| LMG_P-27481_0943 | Hypothetical protein   |
| LMG_P-27481_0944 | Hypothetical protein   |
| LMG_P-27481_0946,LMG_P-27481_1265 | Hypothetical protein |
| LMG_P-27481_0947 | Hypothetical protein   |
| LMG_P-27481_0948 | Hypothetical protein   |
| LMG_P-27481_0950 | Hypothetical protein   |
| LMG_P-27481_0952 | Hypothetical protein   |
| LMG_P-27481_0969 | NLP P60 protein         |
| LMG_P-27481_0970 | Hypothetical protein   |
| LMG_P-27481_0971 | Hypothetical protein   |
| LMG_P-27481_0972 | Tail protein            |

(Continued)
| LMG_P-27481 ORFs | LMG_P-27481 annotation |
|------------------|------------------------|
| LMG_P-27481_0973 | Hypothetical protein    |
| LMG_P-27481_0974 | Hypothetical protein    |
| LMG_P-27481_0975 | Hypothetical protein    |
| LMG_P-27481_0976 | Hypothetical protein    |
| LMG_P-27481_1014 | Hypothetical protein    |
| LMG_P-27481_1098 | Cell wall anchor        |
| LMG_P-27481_1185 | Hypothetical protein    |
| LMG_P-27481_1187 | N-acetylmuramoyl-L-alanine amidase |
| LMG_P-27481_1193 | Hypothetical protein    |
| LMG_P-27481_1194 | Polysaccharide biosynthesis family protein |
| LMG_P-27481_1195 | Exopolysaccharide biosynthesis protein |
| LMG_P-27481_1196 | Transposase             |
| LMG_P-27481_1197 | O-antigen polysaccharide polymerase Wzy |
| LMG_P-27481_1239 | PTS sugar transporter subunit IIA |
| LMG_P-27481_1249 | Chromosome segregation ATPase, partial |
| LMG_P-27481_1250 | NLP/P60 protein         |
| LMG_P-27481_1251 | Phage tail protein      |
| LMG_P-27481_1252 | Tape measure protein    |
| LMG_P-27481_1253 | Hypothetical protein    |
| LMG_P-27481_1254 | Small major structural protein |
| LMG_P-27481_1255 | Tail component protein  |
| LMG_P-27481_1256 | Tail component protein  |
| LMG_P-27481_1257 | Phage head-tail adaptor |
| LMG_P-27481_1297 | Hypothetical protein    |
| LMG_P-27481_1304 | Hypothetical protein    |
| LMG_P-27481_1455 | Hypothetical protein    |
| LMG_P-27481_1458 | Hypothetical protein    |
| LMG_P-27481_1459 | Hypothetical protein    |
| LMG_P-27481_1460 | Hypothetical protein    |
| LMG_P-27481_1461 | Hypothetical protein    |
| LMG_P-27481_1462 | Acyltransferase         |
| LMG_P-27481_1472 | CDP-diacylglycerol diphosphatase |
| LMG_P-27481_1479 | N-acetylmuramoyl-L-alanine amidase |
| LMG_P-27481_1491 | Amino acid permease     |
| LMG_P-27481_1525 | Hypothetical protein    |
| LMG_P-27481_1526 | Transposase             |
| LMG_P-27481_1527 | Uracil-DNA glycosylase  |
| LMG_P-27481_1541 | Hypothetical protein    |
| LMG_P-27481_1543 | Hypothetical protein    |
| LMG_P-27481_1651 | NAD-dependent malic enzyme |
| LMG_P-27481_1671 | ABC transporter related |
| LMG_P-27481_1735 | MFS transporter         |
| LMG_P-27481_1744 | Amino acid permease     |
| LMG_P-27481_1745 | Decarboxylase           |
| LMG_P-27481_1755 | Hypothetical protein    |
| LMG_P-27481_1762 | Hypothetical protein    |
| LMG_P-27481_1763 | Hypothetical protein    |
| LMG_P-27481_1764 | Hypothetical protein    |
| LMG_P-27481_1765 | Short-chain dehydrogenase |
| LMG_P-27481_1767 | Acetyltransferase       |
| LMG_P-27481_1772 | Hypothetical protein    |
| LMG_P-27481_1785 | CsB family protein      |
| LMG_P-27481_1846 | Hypothetical protein    |
### TABLE 2 | Continued

| LMG_P-27481 ORFs | LMG_P-27481 annotation |
|------------------|------------------------|
| LMG_P-27481_1847 | Hypothetical protein   |
| LMG_P-27481_1855, LMG_P-27481_2203, LMG_P-27481_1454 | Transposase |
| LMG_P-27481_1871 | Hemolysin              |
| LMG_P-27481_2037 | Transposase            |
| LMG_P-27481_2073, LMG_P-27481_1453 | Integrase, catalytic region |
| LMG_P-27481_2075 | hypothetical protein   |
| LMG_P-27481_2077 | integrase              |
| LMG_P-27481_2078 | Hypothetical protein   |
| LMG_P-27481_2079 | Hypothetical protein   |
| LMG_P-27481_2080 | Hypothetical protein   |
| LMG_P-27481_2081 | DNA polymerase III     |
| LMG_P-27481_2084 | Hypothetical protein   |
| LMG_P-27481_2085 | Hypothetical protein   |
| LMG_P-27481_2086 | Hypothetical protein   |
| LMG_P-27481_2087 | Hypothetical protein   |
| LMG_P-27481_2088 | Hypothetical protein   |
| LMG_P-27481_2089 | XRE family transcriptional regulator |
| LMG_P-27481_2096 | Hypothetical protein   |
| LMG_P-27481_2101 | Integrase              |
| LMG_P-27481_2102 | Transposase            |
| LMG_P-27481_2103 | LysR family transcriptional regulator |
| LMG_P-27481_2104 | 5-methyltetrahydropteroylglutamateg-homocysteine methyltransferase |
| LMG_P-27481_2105 | 5,10-methylenetetrahydrofolate reductase |
| LMG_P-27481_2107 | Cell wall anchor protein |
| LMG_P-27481_2108 | Mucus-binding protein  |
| LMG_P-27481_2109 | Hypothetical protein   |
| LMG_P-27481_2110 | DNA-binding protein    |
| LMG_P-27481_2111 | Restriction endonuclease |
| LMG_P-27481_2112 | DNA methylase N-4/N-6 family protein |
| LMG_P-27481_2113 | Hypothetical protein   |
| LMG_P-27481_2114 | Hypothetical protein   |
| LMG_P-27481_2115 | Hypothetical protein   |
| LMG_P-27481_2116 | ATPase para family protein |
| LMG_P-27481_2117 | Phage integrase        |
| LMG_P-27481_2118 | MFS transporter, partial |
| LMG_P-27481_2119 | K02314 replicative DNA helicase |
| LMG_P-27481_2120 | hypothetical protein   |
| LMG_P-27481_2121 | Guanine permease       |
| LMG_P-27481_2122 | Adenine phosphoribosyltransferase |
| LMG_P-27481_2123 | Hypothetical protein   |
| LMG_P-27481_2124 | Glycerol-3-phosphate transporter |
| LMG_P-27481_2125 | Hypothetical protein   |
| LMG_P-27481_2126 | Restriction endonuclease |
| LMG_P-27481_2127 | (Continued)            |
TABLE 2 | Continued

| LMG_P-27481 ORFs | LMG_P-27481 annotation |
|------------------|------------------------|
| 27481_1775, LMG_P-27481_1107, LMG_P-27481_2274, LMG_P-27481_1908, LMG_P-27481_1770, LMG_P-27481_0532, LMG_P-27481_2288, LMG_P-27481_1190, LMG_P-27481_1863, LMG_P-27481_1243, LMG_P-27481_2300, LMG_P-27481_1487, LMG_P-27481_1710, LMG_P-27481_0409, LMG_P-27481_2319, LMG_P-27481_0645, LMG_P-27481_0196 | Hypothetical protein |
| LMG_P-27481_2195 | 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase |
| LMG_P-27481_2198 | Bifunctional folylpolyglutamate synthase/dihydrofolate synthase |
| LMG_P-27481_2204 | Phage integrase |
| LMG_P-27481_2206 | Hypothetical protein |
| LMG_P-27481_2208 | Glycerol-3-phosphate transporter |
| LMG_P-27481_2212 | AI-2E family transporter |
| LMG_P-27481_2222 | 2,5-diketo-D-gluconic acid reductase |
| LMG_P-27481_2223 | Restriction endonuclease |
| LMG_P-27481_2225 | Restriction endonuclease |
| LMG_P-27481_2228 | Multidrug ABC transporter permease |
| LMG_P-27481_2229, LMG_P-27481_2183 | YSIRK signal domain/LPXTG anchor domain surface protein |
| LMG_P-27481_2240 | Acetylmethionine deacetylase |
| LMG_P-27481_2242 | Glycerol-3-phosphate transporter |
| LMG_P-27481_2244, LMG_P-27481_2162 | Hypothetical protein |
| LMG_P-27481_2245 | K02314 replicative DNA helicase |
| LMG_P-27481_2248 | Phospholipase |
| LMG_P-27481_2250 | MazF family toxin-antitoxin system |
| LMG_P-27481_2253 | 3-beta-hydroxysteroid dehydrogenase |
| LMG_P-27481_2257 | Glycerol-3-phosphate transporter |
| LMG_P-27481_2262 | YSIRK signal domain/LPXTG anchor domain surface protein |
| LMG_P-27481_2266 | Integrase |
| LMG_P-27481_2268 | Guanine permease |
| LMG_P-27481_2276 | Hypothetical protein |
| LMG_P-27481_2278 | DNA polymerase III subunit gamma/tau |
| LMG_P-27481_2286 | Two-component system sensor histidine kinase |
| LMG_P-27481_2287 | Farnesyl pyrophosphate synthetase |
| LMG_P-27481_2292 | Non-canonical purine NTP pyrophosphatase |
| LMG_P-27481_2296 | Glutamine amidotransferase |
| LMG_P-27481_2297 | 30S ribosomal protein S4 |
| LMG_P-27481_2299, LMG_P-27481_2305 | UDP-glucose 4-epimerase |
| LMG_P-27481_2307 | Asparagine-tRNA ligase |
| LMG_P-27481_2313 | Transposase |
| LMG_P-27481_2315 | Peptide ABC transporter substrate-binding protein |
| LMG_P-27481_2325 | Transposase |
| LMG_P-27481_2327 | |
| LMG_P-27481_2338 | |

for *L. reuteri* suggested by EFSA’s Scientific Opinion for 2018 (EFSA Scientific Opinion, 2018). *L. reuteri* species can be found, in fact, in the list of qualified presumption of safety (QPS) species, and our investigations using phenotypic and genotypic methods have confirmed the safety of LMG P-27481 strain. The strain was compared to other well-known, commercially available probiotic strains, such as *L. reuteri* DSM 17938, RC-14 and *L. rhamnosus* ATCC 53103 during our assessment and convincingly resulted a new potential probiotic product. We found that strain LMG P-27481 was able to survive exposure to the pH of the simulated gastric juice; it was comparable to DSM 17938 and higher than RC-14 strain (Figure 2A). Moreover, all the tested strains showed a marked resistance to exposure to simulated pancreatic juice (Figure 2B). After 6 h of incubation at 37°C, the presence of bile in the culture medium caused a comparable reduction in optical density in strains DSM 17938 and LMG P-27481; RC-14 strain showed instead a greater susceptibility (Figure 2C). LMG P-27481’s ability to produce oxygen peroxide placed it an intermediate level of the reference strains. In fact, 2.58 µg/10⁹ CFU was detected after 1-h of incubation and 3.96 µg/10⁹ CFU after 24 h (Figure 3A). The data obtained during the course of two different tests to
determine the presence of reuterin in the culture medium were in agreement with previous studies uncovering reuterin release by DSM 17938 (positive control) and RC-14 (negative control) strains and the absence of reuterin in LMG P-27481 culture medium (data not shown).

**Genome Sequencing: The Safety and Functional Profile of the LMG P-27481 Strain**

As reported by Saulnier et al. (2011) genome analysis of *L. reuteri* LMG P-27481 strain revealed features that are compatible with those of other *L. reuteri* strains. The average genome size for the *L. reuteri* species is approximately 2.0 Mbp with a total average number of 1.600 genes, and G+C content of 39%. Genome sequencing has been deposited and is available (WHOJ00000000 release public date 2020-02-03). The "unique" genes of *L. reuteri* LMG P-27481 are listed in Table 2. With regard to resistome detection, 15 putative antibiotic resistance genes with enzymatic function and 13 putative antibiotic resistance genes with efflux/transport function were identified. Graphical representation of antibiotic resistances obtained by CARD indicated the presence of 111 loose with the absence of perfect or strict hits underlying the safety of the *L. reuteri* LMG P-27481 (Figure 3B). Most of the sequences identified belonged to β-lactamase enzymes, but those gene(s) in *L. reuteri* were considered non-transferable by several authors because of their chromosomal location. No other antibiotic resistance mechanisms were identified. *L. reuteri* LMG P-27481 MIC values are listed in Table 3. During the investigation for possible virulence genes, 38 sequences were sorted out by bioinformatic tools as predictors of virulence factors. However, based on strict BLASTP standard (90% identity cutoff) none of the sequences raised from the VFDB analysis were considered as significant reporting identity at most of 70.4% (Li et al., 2019). The investigation confirmed that the LMG P-27481 strain was safe given the absence of significant sequence identity with any well-known virulence genes. No S-layer putative proteins were found in the *L. reuteri* LMG P-27481 genome. We performed comparative genomics between the LMG P-27481 strain and 18 other *L. reuteri* genomes available in public databases (Table 4). The analysis uncovered 188 unique sequences for strain LMG P-27481; 81 of these were classified as hypothetical proteins of unknown function. Most of the remaining putative genes, such as mucus-binding proteins, EPS biosynthesis and cell wall anchor domains, seemed to be involved in cell surface design and therefore in the interaction with the host.

**L. reuteri** LMG P-27481 Efficiently Adheres to Human Intestinal Epithelial Cells

In terms of adhesive capacity to the HT-29 cell line, a high percentage of viable cells of the reference strains adhered to human enterocytes although with a slightly different aptitude (Figure 3C). As expected, the *L. rhamnosus* ATCC 53103 and DSM 17938 reference strains showed the highest robust adhesion ability (79 and 78% on average, respectively); *L. paracasei* ATCC 334 was the least effective (adhesion ranging between 67 and 71%). Indeed, *L. reuteri* strain LMG P-27481 adhered efficiently to human intestinal epithelial cells since it was only slightly less adhesive (73%) with respect to the most efficient strains.

**L. reuteri** LMG P-27481 Induces a Distinctive Cytokine Profile in Human DC

We exposed human immature DCs obtained from peripheral blood monocytes to viable logarithmic-phase lactobacilli and quantified the cytokines in the culture medium to determine the effect of our strain on DC phenotype. After 1 h of incubation with either *L. reuteri* LMG P-27481, *L. reuteri* DSM 17938 or *L. rhamnosus* ATCC 53103, the cells were washed thoroughly, the medium was changed to one containing antibiotic and 23 h later the cytokine released was quantified by ELISA. The three lactobacilli tested induced IL-10 and IL-12p70 release, although with a different profile (Figures 4A,B). Both *L. reuteri* strains were more effective than *L. rhamnosus* ATCC 53103 in stimulating cytokine secretion. Moreover, *L. reuteri* LMG P-27481 appeared more efficient than *L. reuteri* DSM 17938 in inducing an anti-inflammatory response in the DCs (Figure 4C). The same experiments were also conducted after the DCs were challenged with *Salmonella*. Overall, all the probiotic strains were significantly less efficient than *Salmonella* in inducing cytokine release (data not shown).

**L. reuteri** LMG P-27481 Efficiently Metabolizes Lactose

The ability to counteract or at least mitigate the negative effects of various etiological agents of a chemical and/or microbial origin

| Antibiotic          | L. reuteri LMG P-27481 (µg/ml) | EFSA cut-off 2018 for L. reuteri (µg/ml) |
|---------------------|---------------------------------|----------------------------------------|
| Gentamycin          | 4                               | 16                                     |
| Kanamycin           | 64                              | 64                                     |
| Streptomycin        | 32                              | 64                                     |
| Tetracycline        | 16                              | 32                                     |
| Erythromycin        | 0,5                             | 1                                      |
| Clindamycin         | 0,063                           | 4                                      |
| Chloramphenicol     | 4                               | 4                                      |
| Ampicillin          | 1                               | 2                                      |
| Vancomycin          | >128                            | n.r.                                   |
| Neomycin            | 4                               | /                                      |
| Virginiamycin       | 1                               | /                                      |
| Ciprofloxacin       | 64                              | /                                      |
| Linezolid           | 4                               | /                                      |
| Rifampicin          | 0,125                           | /                                      |
| Trimethoprim        | 64                              | /                                      |

n.r.: not requested; / not reported.
TABLE 4 | Comparative genomics analyses performed with 18 L. reuteri publicly available strains. The percentages of identity with L. reuteri LMG P-27481 are reported.

| L. reuteri strain | Percentage of Identity |
|-------------------|-----------------------|
| ATCC53608         | 96.67                 |
| JCM1112           | 98.88                 |
| LTH5448           | 96.65                 |
| I5007             | 96.76                 |
| ZLR003            | 96.69                 |
| TMW1.656          | 96.20                 |
| TD1               | 96.53                 |
| MM2-3             | 96.88                 |
| DSM20016          | 96.88                 |
| mlc3              | 96.69                 |
| 100-23            | 95.68                 |
| TMW1.112          | 96.35                 |
| lpuph             | 96.14                 |
| IRT                | 96.53                 |
| LTH2584           | 96.12                 |
| SD2112            | 98.86                 |
| MM4-1A            | 98.86                 |
| CF48-3A           | 95.68                 |

FIGURE 4 | Lactobacillus reuteri LMG P-27481 induces an anti-inflammatory cytokine profile in human dendritic cells. The DCs obtained from differentiated peripheral blood monocytes were incubated (1:10 ratio) with the reported live bacterial strains for 1 h in medium without antibiotics. The cells were then collected, washed by centrifugation and incubated for an additional 23-h period in complete medium with antibiotics. Culture supernatants were collected. (A) IL-10 content quantified by ELISA. (B) IL-12p70 concentration assessed by ELISA. (C) Shows the anti-inflammatory index calculated as the ratio between IL-10 and IL-12p70 listed in Panels (A,B). Data are reported as mean ± SD on values obtained on 3 different donors. *P < 0.05 versus control and ºP < 0.05 versus LMG P-27481.

that are able to cause diarrhea is a highly desirable feature for a probiotic strain. Since lactose intolerance is one of the most common causes of diarrhea, probiotic strains were tested for their ability to hydrolyze lactose and thus, potentially, to limit the
symptoms associated with lactose intolerance. Two well-known probiotic strains, *L. reuteri* DSM 17938 and *L. rhamnosus* ATCC 53103, were selected as our reference of comparison with the LMG P-27481 strain tested.

Bacterial growth in the presence of glucose or lactose was compared. *L. reuteri* DSM 17938 seemed more active in the presence of lactose in the growth medium, indicating its ability to vigorously metabolize this carbon source. *L. rhamnosus* ATCC 53103 was instead clearly more active in presence of glucose with respect to lactose in the culture medium (Figure 5). The LMG P-27481 strain produced in the end the most significant growth in the presence of lactose with respect to the other strains (Figure 5). All strains tested showed a negligible growth in MRS broth without sugars (Figure 5).

**L. reuteri** LMG P-27481 Antagonizes Pathogen Growth in vitro

Intestinal pathogens, such as *E. coli*, *Salmonella* and *C. difficile*, were inoculated into supernatants of the probiotic strains tested and the inhibitory effects were verified. Figure 6 illustrates the growth patterns of the pathogens 6, 12, and 24 h following incubation in the probiotic supernatants. Although the probiotics tested were unable to significantly affect the growth of *Salmonella*, they all, however, slightly reduced pathogen growth as early as 6 h after incubation (0.2–0.3 log10 CFUs reduction compared to the control). *L. reuteri* DSM 17938 was the most effective in reducing *Salmonella* growth 24 h after incubation. Similarly, both *L. reuteri* strains hindered *E. coli* growth 6 h after incubation, and growth inhibition was maintained for the 24 h following co-incubation. *L. rhamnosus*, instead, did not modify the pathogen’s growth pattern. More interestingly, *L. reuteri* LMG P-27481 significantly inhibited *C. difficile* growth 24 h after incubation as the pathogen growth was reduced by more than 0.5 log10 CFUs. All the other strains produced a growth increase similar to that in the controls.
### TABLE 5 | The average number of copies of viral genome in the presence of the probiotics tested during the study (raw data expressed as DNA copies/5 × 10^3 cells ± SEM) in the two experimental designs.

| Probiotic               | DNA copies viral genome (DNA copies/5 × 10^3 cells ± SEM) |
|-------------------------|----------------------------------------------------------|
|                         | Pre-treatment | Competition |
| Rotavirus alone         | 4371 ± 660 × 10^3 | 4630 ± 720 × 10^3 |
| L. reuteri DSM 17938     | 2198 ± 450 × 10^3 | 531 ± 85 × 10^3 |
| L. reuteri DSM 17938     | 1528 ± 390 × 10^3 | 1012 ± 410 × 10^3 |
| L. reuteri DSM 17938     | 627 ± 90 × 10^3  | 421 ± 83 × 10^3  |
| L. rhamnosus ATCC 53103  |              |              |

*p < 0.05 versus Rotavirus alone.

### DISCUSSION

Lactobacilli are important members of the gut microbiota community that are able to influence host health through a variety of mechanisms (Oh et al., 2010). L. reuteri, a separate species within the Lactobacillus species, was chosen for isolation because it has been shown to exhibit probiotic efficacy and to confer broad-spectrum protection against disease in humans (Casas and Dobrogosz, 2000). Beyond its ability to survive the hostile conditions of the gastrointestinal environment including the high pH level of gastric juices and to tolerate bile salts, it shows resistance to antibiotics. It is, in addition, able to metabolize lactose, meaning that it can potentially alleviate the symptoms of intolerant subjects. Like some control strains (L. reuteri DSM 17938 and L. rhamnosus ATCC 53103), L. reuteri LMG 27481 has been shown to produce a marked inhibitory effect on several pathogens (E. coli, Salmonella and Rotavirus) in different in vitro models. But as opposed to the control strains, L. reuteri LMG 27481 has also been shown to diminish C. difficile growth in vitro and to significantly reduce C. difficile colonization and inflammatory colitis in mice.

Its safety, effectiveness, and genetic stability were investigated by means of genome sequencing, which uncovered the presence of two amino acid sequences similar to the mucus-binding MUB domains as well as a sequence coding for the typical N-terminal signal peptide (YSIRK motif) targeting the protein for secretion, and a C-terminal sortase recognition site (LPxTG), targeting the protein for covalent attachment to the peptidoglycan layer on the outside of the bacterial cell (Sengupta et al., 2013). As described by Etzold et al. (2014) and Bath et al. (2005), our experiments showed that the strain carried five sequences with a significant homology to mucus-binding proteins, cell wall anchor domains, and signal peptides characterizing the surface-exposed adhesins from L. reuteri. Moreover, the presence of putative EPS-coding sequences, which are known to interfere with the adhesion of enteropathogenic E. coli (EPEC) to pig erythrocytes, was also confirmed in the LMG P-27481 genome (Chen et al., 2014). L. reuteri is, in fact, one of the lactobacilli species known to produce EPS, whose chemical features seem to depend on the type of sugar substrate metabolized by the strain. Wang et al. (2010) demonstrated that EPS from L. reuteri was able to interfere with the in vitro adhesion ability of EPEC to pig erythrocytes (hemagglutination). Peptidoglycan hydrolases and EPS production could enhance the strain’s potential to exert antimicrobial activities against gut pathogens. Our experiments confirmed the safety of the LMG P-27481 strain because they demonstrated that sequences of well-known virulence genes were absent. The unforeseen finding regarding the anti-inflammatory potential of the LMG P-27481 strain that emerged here was the higher stimulation of IL-10 cytokine with respect to IL-12p70, as demonstrated by the resulting anti-inflammatory index (IL-10/IL-12 ratio). The
Lactobacillus reuteri LMG P-27481 reduces the severity of C. difficile colitis in a murine model. The mice were randomly divided in four experimental groups (Figure 1). Five days after intragastric (IG) challenge with C. difficile (10⁵ CFU) the animals were sacrificed, and the colon content collected. (A) Body weight was measured at baseline (IG challenge) and every day for the following 5 days. Weight change was calculated as the variation in weight at sacrifice compared with that at the baseline. (B) Full thickness colonic specimens were fixed in 4% formalin, paraffin embedded and 8 µm thick sections stained with H&E. Tissue edema, inflammatory infiltrate and mucosal ulcers were evaluated and indicated with * in the Group 2 image. (C) Thickness colonic specimens were homogenized and used to quantify MPO activity as a marker of neutrophils infiltration. Data are reported as mean ± SD on values obtained on six animals for each experimental group. *P < 0.01 versus group 1 and **P < 0.05 versus group 2.

CONCLUSION

Taken together, the results of our work characterizing a new probiotic lactobacillus lead to the conclusion that L. reuteri LMG P-27481 represents a promising strain by virtue of its ability to adhere to human enterocytes and to stimulate anti-inflammatory cytokine secretion. After an initial assessment of the probiotic’s unique properties carried out following conventional protocols and international guidelines, the strain was subjected to a battery of assays leading to target-selection and

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characterization. The probiotic not only showed conventional probiotic features, which are essential for its successful activity, but also the ability to counteract the growth and colonization in vitro and in vivo of *C. difficile*. Further studies are warranted given the strain’s efficacy even in those cases in which it is administrated in association with antibiotic therapy. Its clinical application to treat *C. difficile* infection and forms of diarrhea caused by other conditions (i.e. lactose intolerance) also warrant further investigation.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study can be found in the WHOJ00000000.

**ETHICS STATEMENT**

The strain involving human subjects was isolated following the written informed consent of the parents. Approval by an Ethical Committee was not required by local legislation at the time the strain was isolated. The animal study was carried out in accordance with the principles of the Basel Declaration and the recommendations of the Animal Care and Use Ethics Committee of the University of Padua under license from the Italian Ministry of Health; they were in compliance with national and European guidelines for handling and use of experimental animals. Experimental protocols were approved by the Animal Care and Use Ethics Committee of the University of Padua under license from the Italian Ministry of Health.

**AUTHOR CONTRIBUTIONS**

VS, FU, GB, AP, PB, IC, and ME performed the experiments. LB, LR, GM, PB, IC, and ME designed and interpreted the experiments. VS, FU, IC, and ME wrote the manuscript. LM critically reviewed the manuscript.

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Conflict of Interest: LB was employed by the Moviscom S.r.l. company that funded the study and owns the strain. GM and LR are employed by the Nòos S.r.l., the company that hold the license for the strain’s commercialization. ME, FU, and VS are employed by the AAT S.r.l. company, the contract laboratory that performed the assays.

The remaining 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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