**Enterococcus faecium** CMUL1216 an Immunobiotic Strain with a Potential Application in Animal Sector

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Antibiotic misuse in the animal sector is the first cause of the emergence and spreading of MDR bacteria. Prevention of infectious diseases and enhancement of animal growth are the main effects of antibiotics that push farmers and veterinarians to use this molecule in animal farms. Thus, the use of alternative solutions such as natural antimicrobial substances as well as probiotic strains is a crucial need in this sector. *Enterococcus faecium* CMUL1216 was isolated from healthy human baby’s feces. This strain was assessed *in vitro* for probiotic properties including activity against many pathogens isolated from animal, human, and soil samples. CMUL1216 strain exhibits good antimicrobial activity against indicator pathogens in both planktonic and biofilm forms. In addition, CMUL1216 strain showed a strong biofilm formation. Furthermore, CMUL1216 exhibits a good anti-inflammatory effect by inducing the secretion of IL-10 *in vitro*. Moreover, this strain did not show any pathogenic characteristics such as hemolytic effect, presence of virulence genes as well as susceptibility to the majority of antibiotic families. *E. faecium* CMUL1216 could be a good candidate to be used as a probiotic strain in the animal sector in order to maintain animal health and therefore reduce antibiotic resistance caused by the excessive use in this sector.

**Key words**: Biofilm / Enterococcus / Immunomodulation / Probiotic / Antimicrobial activity.

**INTRODUCTION**

Antibiotic resistance is a major public health threat worldwide. Even if the increasing availability of life-saving antibiotics in developing countries is appreciated, the misuse and overuse of these drugs have created resistant strains of deadly bacteria, particularly in poorer nations (Ventola, 2015). The exceptional spread of multidrug-resistant (MDR) bacteria is highly worrisome, as it could lead to serious infections leaving patients and animals with very limited or no therapeutic options (Exner et al., 2017; Tartari et al., 2017). Unfortunately, poor antibiotic stewardship, inadequate antibiotic prescription, extensive agriculture use, low antibiotic awareness and knowledge, and promotion of antibiotic self-medication are key drivers of antibiotic resistance in low-income countries.

The emergence and spread of MDR bacteria from animals, food, and the environment assume that the dissemination of MDR bacteria in the community may have a zoonotic origin since the same clones were found in clinical specimens (Andremont, 2003). Thus, antibiotic resistance can be reduced by using other natural substances in the animal sector. Since antibiotics are used as preventive agents and growth enhancers in this sector, especially in fowl farms, the use of other agents will be not accepted easily. Thus, developing a combination of alternative agent(s) for infection treatment as well as an immunity booster seems to be more accepted in the
animal sector especially if these agents could be less expensive than antibiotics. Recently, beneficial bacteria, as well as their metabolites, are more studied to replace some chemical molecules already used in food, animal, and clinical sectors (Yang et al., 2014). Bacteriocins, lipoproteins, and Non-Ribosomal Peptide (NRP) are very interesting bioactive metabolites secreted by several types of micro-organisms including lactic acid bacteria (LAB) (Arias Argüelles et al., 2011). Bacteriocins are small peptides produced by several bacteria especially LAB (Al Kassaa et al., 2014). Some bacteriocins have a large antimicrobial spectrum reaching Gram positive, Gram negative, and fungi (Al Kassaa et al., 2018; Lim et al., 2016; Shelburne et al., 2007). These antimicrobial metabolites are stable under almost all processing conditions such as heating, pressure, and drying. Furthermore, probiotics are considered also as agents that can control infection as well as immunity leading to the reduction of drug use. Probiotics are broadly defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO and WHO, 2001). Probiotic strains can fight pathogens via several mechanisms. Strong colonization and formation of biofilm, co-aggregation and pathogen exclusion via nutritional competition, secretion of bacteriocins as well as other antimicrobial substances, and enhancement of local and systemic immunity are the main anti-pathogen mechanisms (Ashraf and Shah, 2014; Gomez et al., 2016; Kizerwetter-Swida and Binek, 2016; Lee and Puong, 2002; Lomax and Calder, 2009).

This study aimed to characterize a new LAB strain with probiotic properties for a potential application in the animal sector to reduce antibiotic use in Lebanese farms. Antimicrobial activity, biofilm formation, immunomodulation effects, and safety properties of the selected LAB strain were evaluated.

**MATERIAL AND METHODS**

**Isolation and Identification of LAB strain from baby’s feces**

In order to isolate (LAB) strains, stool samples, from less than 4 months old babies, were collected. Stool samples were serially diluted, and 1 ml of each dilution was spread onto MRS agar plates (Man, Rogosa and Sharpe, Bio-Rad, France), and incubated at 37 °C for 48 h under anaerobic atmosphere. Morphological and biochemical preliminary tests permitted to select Gram positive bacteria, with a cocci shape, and without catalase activity. The isolated strain was then subjected to Mass spectrometry identification technique by using VITEK® MS (Biomérieux-France).

*Enteropathogenic E. coli* CMUL616 (EPEC CMUL616) was delivered by the Lebanese university strain bank “Collection microbiologique-Univerté Libanaise” (CMUL). This strain was isolated previously from beef meat and conserved as freeze-dried powder. In addition, *Campylobacter jejuni* (C. jejuni) ATCC® BAA-1062™, *Salmonella typhimurium* (S. typhimurium) ATCC® 14028™ are commercial strains isolated also from animal sources. The other indicator strains used were: *Listeria monocytogenes* (L. monocytogenes) ATCC® 19115™ *Escherichia coli* (E. coli) ATCC® 8739™ and *Staphylococcus aureus* (S. aureus) ATCC® 25923™ isolated from human samples. *Pseudomonas aeruginosa* (P. aeruginosa) ATCC® 39327™ were isolated from soil samples.

**Inhibition of pathogenic bacteria by intestinal LAB isolates**

**Well diffusion method**

Antibacterial activity of the isolated LAB strains was evaluated using the well diffusion method on Brain Heart infusion (BHI) 1% agar. The supernatant of LAB strains was prepared as follows: A concentration of 1 McF of each LAB strains was prepared in a medium containing sterile MRS broth and incubated for 24 h at 35 °C, then culture broth was centrifuged, the supernatant was exposed to heat (90 °C / 5min) in the water bath and pH was measured. 500 μl of 10^4 CFU / ml of each indicator strain was incorporated in 20 ml BH (1% agar) cooled at 45 °C. Wells (diameter=6mm) were filled with 50 μl of LAB supernatant and then incubated at low temperature for 2 h. The petri dish was then incubated at 37°C for 24 h. The inhibition zone was reported in millimeters (mm). After the incubation period, the diameter of the growth inhibition zones was measured. The sterile MRS was used as negative control while bacteriocinogenic strain *Enterococcus faecium* CMUL20-2 (AL Kassaa et al., 2018) was used as a positive control. The test was performed in duplicate.

**Kinetic method**

To prepare the supernatant of the CMUL1216 (strain which showed good activity against indicator strains), 1 McF of this strain was prepared in a medium containing sterile BHI broth (Bio-Rad, France) and incubated for 48 h at 35°C. The supernatant obtained after centrifugation of the culture (4000 g / 15 min) was filtered by using 0.45 μm filter (Millipore, USA), exposed to heat (90°C/15min) in the water bath. Then 50μl of BHI broth containing 10^5 CFU/ml of each pathogen (EPEC CMUL616 and *L. monocytogenes* ATCC® 19115™) was added with 50μl of the supernatant of the isolated strain in a 96-well microplate. The microplate was incubated aerobically at 35°C. The Optical Density (OD)
of the different wells was measured by ELISA reader (Biotek, USA) every 2 h at 630 nm for 24 h. BHi was used as negative control; colistin was used as a positive control for EPEC CMUL16 whereas E. faecium CMUL20-2 (AL Kassaa et al., 2018) was used as a positive control for L. monocytogenes ATCC® 19115™.

**Evaluation of probiotic properties of CMUL1216 strain**

**Detection of the capacity to form a biofilm by the isolated strain**

The capacity of forming a biofilm by the isolated strain was evaluated using the Tissue culture plate method (TCP): 1 McF of the strain was prepared in a medium containing sterile MRS broth and incubated for 24 h at 35 °C, then 200 μl of this bacterial culture were added in sterile 12 well flat-bottomed polystyrene microplate containing 2 ml of MRS broth. The plate was incubated at 35°C for 24 h under CO2 condition. The content of the wells was poured off and washed 3 times with 2 ml of sterile distilled water. The bacteria adhering to the wells were fixed with 2 ml of methanol for 15 min. Then the wells were washed with sterile distilled water, followed by staining with 2 ml of 1% crystal violet solution for 5 min. Excess stain was removed by washing and air drying. The remaining dye that binds to the wells was extracted with 2 ml of 33% glacial acetic acid for 10 min. Then 1.5 ml of acetic acid was removed from each well, and the optical density was measured at 595 nm using a spectrophotometer (Thermo Fisher Scientific, USA). The strain was tested seven times (7 independent wells). Sterile MRS was used as a negative control whereas a commercial probiotic strain (Lactobacillus paracasei, Bion3-France) was used as a positive control.

**Exclusion method**

A concentration of 1 McF of the CMUL1216 strain was prepared in a medium containing BHi broth and incubated for 24 h at 35°C. In order to form a biofilm, 200 μl of CMUL1216 culture were added to 12 well sterile flat-bottomed polystyrene microplate containing 2 ml of BHi broth. After 24 h of incubation, 1 ml of BHE solution was removed (solution which potentially contains the antimicrobial substance and will be used in section “CMUL1216 strain can inhibit pathogen’s biofilm”) and 1 ml of fresh BHi was added to the microplate and reincubated for 24 h. After that, the well contents were poured out, washed with PBS (Gibco, Scotland) and 2 ml of BHi broth contain 10^4 CFU/ml of each indicator strains were added in the wells (3 wells per pathogen). Using the serial dilution method to count the maximum number of colony-forming units (CFU) of each pathogen was used after 48 h of incubation. 100 μl of each well contain the pathogen was diluted from 10^1 to 10^9 CFU/ml in a medium containing sterile BHi broth. Then 100 μl of each dilution of the pathogen were spread onto specific agar

for enumeration of the growing colonies. Mannitol salt agar (Bio-Rad, France) aerobically incubated was used for S. aureus ATCC® 25923™, Salmonella-Shigella agar (Sharlau, Spain) aerobically incubated was used for S. typhimurium ATCC® 14028™, Cetrimeide agar (Bio-Rad, France) aerobically incubated was used for P. aeruginosa ATCC® 39327™, PALCAM agar (Bio-rad, France) under CO2 incubation condition was used for L. monocytogenes ATCC® 19115™, MacConkey (BioMérieux, France) for E. coli ATCC® 8739™, and Blood agar base (Bio-Rad, France) supplemented with sheep blood (5%) incubated under micro-aerophilic condition was used for C. jejuni ATCC® 29429™.

**Assessment of CMUL1216 supernatant in the inhibition of the biofilm formation by indicator strains**

1 McF of each aforementioned indicator strain was prepared in a medium containing BHi broth. After that, 200 μl of this bacterial culture were added to 12 well sterile flat-bottomed polystyrene microplate containing 2 ml of BHi. After 8 h of incubation, 1 ml of BHi was removed from each well and 1 ml of the BHE solution already taken from the previous microplate (section “Exclusion method”), was added to this microplate. After 24 h of incubation, 100 μl were removed from each well, and the OD was measured at 630 nm using an ELISA reader (Biotek, USA). 2 ml of sterile BHi broth with 1 McF of each pathogen incubated 48 h at 35°C with the renewal of 50 % of the BHi after 24 h of incubation, were used as control of biofilm formation by the indicator strains. The difference of OD between control and treated wells was calculated and the percentage of this difference was given by using the following formula: (ODc/ODt)×100, where ODc is the OD of control well and ODt is the OD of treated well.

**CMUL1216 strain can alter the biofilm of indicator strains**

The formation of indicator strains’ biofilm was realized using the Tissue culture plate method (TCP): 1 McF of each indicator strain was prepared in a medium containing sterile BHi broth and incubated for 24 h at 35°C. After that, 200 μl of each bacterial culture was added in sterile 12 well flat-bottomed polystyrene microplate and incubated for 48 h under the same culture. After incubation, the medium was removed from each well and three piles of washing were realized using PBS solution followed by adding 2 ml of BHE. The plate was incubated at 35°C for 8 hours. The content of the wells was poured off and washed 3 times with 2 ml of sterile distilled water. The dispersion of biofilms was revealed with the same method mentioned in section “Detection of the capacity to form a biofilm by the isolated strain” and compared to the wells which contain biofilms with 2 ml of BHi instead of BHE as positive control and wells containing sterile BHi and BHE was used separately as a negative control.
This test was repeated three times.

**Detection of the presence of gene coding for a bacteriocin**

The presence of bacteriocin’s gene in the isolated strain was detected using the conventional PCR analysis according to previous studies (du Toit et al., 2000; Henning et al., 2015). The target genes (entA, entB, entC and entD) were detected after extraction of the total DNA by using QIAamp DNA minikit (Qiagen, Germany) according to the manufacturer’s instructions. The primers sequences used are shown in Table 1. The total DNA extraction was done according to the manufacturer’s instructions.

**In vitro immunomodulatory properties of the isolated strain**

PBMCs were isolated from human blood obtained from five healthy informed donors upon approved agreement (signed consents) by authorized staff, as previously described (Foligne et al., 2007). Briefly, the blood was placed on a Ficoll gradient (Pharmacia, Stockholm, Sweden) and PBMCs were recovered at the interface after centrifugation, washed with PBS, and adjusted to $2 \times 10^8$ cells/ml in RPMI 1640 (Roswell Park Memorial Institute, Gibco, Scotland) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Scotland), 1mM glutamine and 150 μg/ml gentamycin. PBMCs were plated in 24-well cell culture plates and stimulated with the isolated strain at a bacteria/cells ratio of 10:1 (20 μl of a thawed bacterial suspension at $10^9$ CFU/ml in well). PBS containing 20% glycerol was used as a negative control, *Lactobacillus gasseri* CMUL 057 was used as a positive control for interleukin 12p70 (IL-12p70), and *Bifidobacterium longum* CMUL CXL001 was used as positive control for interleukin 10 (IL-10) (Zaylaa et al., 2018). After 24h of stimulation at 37°C under 5% CO2, supernatants were collected, clarified by centrifugation, and stored at −20°C for cytokine assay. The cytokines IL-10 and IL-12p70 were measured by ELISA using ELISA MAX® Deluxe kits (BioLegend, San Diego, USA) according to the manufacturer’s instructions.

**Strain safety assessment**

**Hemolysis test**

The strain was cultured in MRS broth and incubated overnight, then a loopful was streak on Columbia base agar amended with sheep blood, then incubated anaerobically at 37°C for 16 h. *S. aureus* ATCC® 25923™, a hemolytic strain, was used as a positive control.

**Antibiotic susceptibility testing**

Disc diffusion method was used to evaluate the antibiotic susceptibility of isolated strain by using 21 antibiotics (Bio-Rad, France). Tests were done according to the criteria of the National Committee of Clinical Laboratory Standards (NCCLS) using Müller-Hinton agar amended with blood and nicotinamide adenine dinucleotide (NAD). Diameters of inhibition zones were measured after incubation at 37°C for 24 h under CO2.

**Screening for the presence of virulence genes**

Conventional PCR was used to detect the presence of virulence genes to evaluate the safety of the isolated strain, according to Vankerckhoven et al. (Vankerckhoven et al., 2004). The total DNA extraction was carried out by using QIAamp DNA minikit (Qiagen, Germany) according to the manufacturer’s instructions. Four target genes were tested: *gelE* (gelatinase), *esp* (enterococcal surface of protein), *asai* (aggregation substance) and *hyl* (hyaluronidase). A clinical strain from “CMUL” bank strain” was used as a positive control for the presence of virulence genes.

**Statistical analysis**

Experimental results were analyzed for statistical significance using GraphPad Prism 6.0. Bonferroni’s and sidak’s multiple comparison test analysis was performed. The statistical significance level was defined as P < 0.05. All the results were expressed as the mean ± SEM.

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### Table 1. Primer sequences of three enterocin genes

| Genes            | Primer sequences                          | References                  |
|------------------|-------------------------------------------|-----------------------------|
| Enterocin A      | F: AAATATTATGGAAATGGAGTGAT T<br>R: GCACCTCCCTGGAAATTGCTC | (du Toit et al., 2000)     |
| Enterocin B      | F: GAAAATGATCAGAATGCGCTA<br>R: GTTGATTAGAATGATAGTATTG | (du Toit et al., 2000)     |
| Enterocin C      | F: AGGTCAGCTGCTATTG GA<br>R: CCATTAGAATGATACTGAAGAAAA | (Henning et al., 2015)     |
| Enterocin D      | F: ATGGGAGCACAATCGCAAATTA<br>R: TAGCCATTTCATATTGATC | (De Vuyst et al., 2003)    |
| Structural gene  |                                           |                             |
RESULTS

Strain identification

The strain CMUL1216 was Gram positive cocci and didn’t exhibit a catalase activity. The MALDI-TOF technique (VITEK® MS method (Biomerieux, France)) identified this strain as *E. faecium*.

Detection of antimicrobial activity

*E. faecium* CMUL1216 was active against *L. monocytogenes* ATCC® 19115™ and EPEC CMUL616. Based on the well diffusion method, the inhibition zone diameter was 29 and 23 mm respectively (data not shown). These two indicator strains were used in the kinetic method.

Kinetic method

The detection of antimicrobial activity by the kinetic method shows that the supernatant of the *E. faecium* CMUL1216 strain was active against *L. monocytogenes* ATCC® 19115™ “P1” as well as against the EPEC CMUL616 “P2”. The Fig. 1 shows the growth curves (GC) of indicator strains P1 and P2 in the absence of the supernatant of *E. faecium* CMUL1216 strains (GCP1, GCP2), as well as in the presence of this supernatant (GCP1/CMUL1216/GCP2/CMUL1216). The effect of antibacterial activity of this strain was observed by the large shifting between GCP1 and GCP2 and GCP1/CMUL1216 and GCP2/CMUL1216 respectively. In addition, compared to the control strain, GCP1/CMUL20-2 and GCP1/CMUL1216 are almost superposed; and GCP2/CO1ISTIN and GCP2/CMUL1216 are close.

Evaluation of probiotic properties of the CMUL1216 strain

The capacity to form a biofilm by *E. faecium* CMUL1216

In order to evaluate the capacity of forming a biofilm by the *E. faecium* CMUL1216, the following referred formulas have been used: If OD.C < OD.E < 2*OD.C: the biofilm is considered as weak biofilm, If 2*OD.C < OD.E < 4*OD.C: the biofilm is considered as moderate biofilm, If 4*OD.C < OD.E: the biofilm is considered as strong biofilm (Borges et al., 2012). Biofilm production for this strain was strong according to this formula: 4*OD.C=0.268 < OD.E=1.08. OD.C: Optical density for control was 0.067, OD.E: Optical density for evaluated strain was 1.08.

Exclusion method

The exclusion method was used to detect the antibacterial activity of *E. faecium* CMUL1216 against different indicator strains. The surface colony count method shows that *E. faecium* CMUL1216 is capable of reducing pathogen growth to more than 5 logs compared to the pathogen control test (Fig. 2).

Inhibition of biofilm formation by CMUL1216 supernatant (BHE)

On the other hand, the formation of biofilm by different pathogens was inhibited by *E. faecium* CMUL1216 supernatant (BHE) at a rate of more than 30% for *P. aeruginosa* ATCC® 39327™, more than 40% for *L.
monocytogenes ATCC® 19115™ and S. aureus ATCC® 25923™, more than 55% for S. typhimurium ATCC® 14028™ and more than 75% for E. coli ATCC® 8739™ compared with the indicator strains’ control wells (Fig. 3).

Alteration of biofilm-forming indicator strains by CMUL1216 supernatant (BHE)

The biofilm alteration was measured after the incubation of the 48 hours-aged biofilms of the indicator strains treated with BHE. After 8 hours of contact time under culture conditions, the results showed that the BHE could alter the biofilm of all indicator strains in a different dispersion percentage. As shown in Fig. 4, the percentage of biofilm dispersion was 80%, 57%, 50% and 33% of E. coli, S. typhimurium, S. aureus, and P. aeruginosa biofilms respectively.

Detection of bacteriocin genes

The molecular detection of bacteriocin genes in E. faecium CMUL1216 strain showed positive amplification results for two enterocin genes entA and entB.

In vitro immunomodulatory properties of the isolated strain E. faecium CMUL1216

To determine the immunomodulatory effect of the selected strain, PBMCs were stimulated and cytokines’ release was measured. Two control strains were used: Bifidobacterium longum CMUL CXL 001 and Lactobacillus gasseri CMUL 057 that respectively exhibit anti-inflammatory (990 pg/ml of IL-10) and pro-Th1 effects (190 pg/ml of IL-12). As shown in Fig. 5, E. faecium CMUL1216 was able to stimulate the secretion of the anti-inflammatory cytokine IL-10 with a value 901 pg/ml. On the other hand, this strain induced the secretion of a very low level of the pro-Th1 IL-12 cytokine with a value of 93 pg/ml.

Strain safety assessment

Hemolysis activity, resistance to antibiotics, and detection of virulence genes showed safety properties of E. faecium CMUL1216. This strain didn’t exhibit a hemolytic activity. In addition, as shown in Table 2, E. faecium CMUL1216 was resistant to Penicillin 1, Cefotaxim, Cefalexin, Clindamycin, Tetracyclin, Minocyclin, Oxacillin, Moxifloxacin and Meropenem. The PCR detection of virulence genes revealed that E. faecium CMUL1216 didn’t harbor any of the virulence genes tested.

**DISCUSSION**

The massive use of antibiotics for animal growth and the prevention of infectious diseases in the animal sector is the main cause of the emergence of resistant bacteria. For this reason, the use of natural antimicrobial agents such as probiotics and their metabolites as alternatives to ATB is one of the solutions to reduce the emergence of MDR bacteria. In this context, the purpose of our study was to select a probiotic strain for a potential application in the animal sector. In order to select this probiotic strain, several tests, such as biofilm formation, antimicrobial effect, safety assessment, and immunomodulatory effect should be evaluated. The new strategy of identification of the isolated strain was based on a molecular method of identification. The first indicator strains used in this study was an EPEC strain isolated...
from an animal source to evaluate the anti-Gram-negative bacteria activity of CMUL1216 strain whereas *L. monocytogenes* was used in order to investigate a potential secretion of bacteriocins or bacteriocin-like inhibitory substances (BLIS) by CMUL1216 strain.

As mentioned before, our team has isolated CMUL20-2 strain which was used in this study as control to the anti-listerial activity. The difference between the CMUL20-2 and CMUL1216 strains was the anti-Gram-activity showed by the CMUL1216. For this reason, we decided to conduct this study with the CMUL1216 strain.

Commensal microorganisms will prevent pathogenic colonization phenomena through competitive processes: nutrient metabolism, pH modification, secretion of antimicrobial peptides, effects on cell signaling pathways (limitation of virulence factors). The antibacterial effects induced by the microbiota improve the host's response to pathogens. The effect studied in our case is the production of antimicrobial peptides which are potential bacteriocins to control pathogens. The heat-treated supernatant from a 24 h culture of *E. faecium* CMUL1216 strain was added in the presence of the pathogens already mentioned, and then the growth of each pathogen in the presence of this supernatant was evaluated by measuring the OD every 2 h for 18 h. Supernatants have been heat-treated to degrade all other thermolabile antibacterial substances. The *E. faecium* CMUL20-2 strain was used as a positive control for *L. monocytogenes* ATCC® 19115™, and colistin was used as a positive control for EPEC CMUL616. The supernatant of *E. faecium* CMUL1216 was able to inhibit pathogen growth as shown in Fig. 1, from where this strain was considered a strain that produces a heat-resistant antibacterial substance.

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In another hand, the exclusion method was used to confirm the antibacterial activity of our strain against more indicator strains isolated from different sources and that could be transmitted by animals. The surface

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**TABLE 2.** Antibiotic susceptibility test of strain CMUL 1216.

| Antibiotics | Profile | Antibiotics | Profile |
|-------------|---------|-------------|---------|
| PEN: Penicillin 1 | R | LIN: Linozeid | S |
| AMP: Ampicillin | S | TET: Tetacyclin | R |
| CTX: Cefotaxim | R | MIN: Minocyclin | R |
| CN: Cefalexin | R | RIF: Rifampicin | S |
| CLN: Clindamyacin | R | COT: Cotrimoxazol | S |
| ERY: Erythromycin | I | GEN: Gentamicin | S |
| PST: Pristinamycin | S | OXA: Oxacillin | R |
| TEC: Teicoplanin | S | MOXI: Moxifloxacin | R |
| VAN: Vancomycin | S | MER: Meropenem | R |
| SPI: Spiramycin | S | CHL: Chloramphenicol | R |

R : resistant, I : intermediate, S : susceptible

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**FIG. 5.** In vitro immunomodulatory profiles of the strain CMUL1216. Cytokine production was evaluated in the supernatants of PBMCs (n=5) different donors stimulated for 24h by the tested strains and two control strains (*B. longum* CMUL CX 001 and *Lactobacillus gasseri* CMUL 057), in comparison to non-treated cells (NS). Results indicate levels of (A) IL-10 and (B) IL-12p70. Data represent means ± SEM of 5 independent donors. *refers to the comparison of bacteria stimulated PBMCs versus untreated cells; *p < 0.05, ***p < 0.001.
colony count method shows that *E. faecium* CMUL1216 is capable of reducing pathogen colony growth to more than 5 logs compared to the pathogen control test as shown in Fig. 2. Regarding the incubation time, our results showed that after 48 h of incubation, the strain showed higher antibacterial activity in comparison to 24 h of incubation (data not shown), which is similar to another study on *E. faecium* strain (Nascimento et al., 2010) that demonstrates the high inhibition of Gram positive bacteria co-cultured with *E. faecium* after 48 h of incubation due to the high production of bacteriocin among the time.

In addition, the capacity to make a biofilm by pathogens themselves was evaluated in presence and absence of the supernatant of *E. faecium* CMUL1216. In this experiment, the indicator strains were incubated in the 12-well microplate for 8 hours, and then control and treated plates were treated with 1 ml of sterile BHi and 1 ml of BHE respectively. The OD of the well’s supernatant was measured to assess the percentage of bacterial adhesion to the well. The results showed that in the control wells the OD was less than in the treated wells due to the bacterial cell adhesion and biofilm formation in control wells. Although the antibacterial activity of BHE, the OD of the control well remained lowest than the BHE treated cells. Thus, these results could be related to the inhibition of bacterial adhesion by the BHE. The biofilm formation of all pathogens was inhibited by *E. faecium* CMUL1216 supernatants at a different rate for each pathogen as shown in Fig. 3. In this Figure, the difference of OD between control and treated wells was measured and the percentage was calculated to present the biofilm formation inhibition by CMUL1216.

In order to evaluate the anti-biofilm capacity of CMUL1216 supernatant (BHE), 48 hours-aged biofilms of each indicator strain were dispersed by BHE after 8 hours of contact as shown in Fig. 4. The Crystal violet technique was used to compare the control biofilm with biofilm treated with BHE. These results are preliminary data on the capacity of CMUL1216 metabolites to destroy a 48 hours-aged biofilm of Gram-positive and Gram-negative bacteria. However, as results showed, *P. aeruginosa* biofilm was the most resistant to BHE effect in comparison with biofilms of the other indicator strains.

All these results confirm that the CMUL1216 strain was able to inhibit Gram-positive and Gram-negative bacteria in planktonic form and after the formation of biofilm. Therefore, the hypothesis that the antimicrobial activity of *E. faecium* CMUL1216 is due to the bacteriocin produced can be established. Enterocins (bacteriocins produced by enterococci strains) are antimicrobial peptides that showed a broad spectrum of activity such as antibacterial and antiviral activities (Belguesmia et al., 2010). To confirm the hypothesis that the antimicrobial activity of *E. faecium* CMUL1216 is due to the enterocin production, conventional PCR was used to detect the presence of genes coding to enterocins such as *entA*, *entB*, *entC* and *entD* (*entL50A/B*). The results revealed that *E. faecium* CMUL1216 carries genes encoding enterocin A and B that confirm this hypothesis. In general, the wide distribution of enterocin genes in the enterococcal strain may be due to the high ability of enterococci to disseminate and receive genetic material between strains as well as between genera (e.g. between staphylococcus and enterococci) (Strompfova et al., 2008). This combination detected in our strain of the 2 genes encoding enterocin A and B has already been observed in other strain of *E. faecium* by Casaus et al (Casaus et al., 1997) who have demonstrated that enterocin B and enterocin A can work synergistically to kill a wide range of pathogens. These two enterocins belong to the pediocin family (class II bacteriocin). Enterocin A exhibits the YNGNgxx motif and thus it belongs to sub class IIA (Cotter et al., 2005). However, enterocin B exempts of this motif and therefore it belongs to class II because of the sequence similarity with members of this family.

The intestinal microbiota plays an essential role in the development and maturation of the immune system, and therefore regulates its functions. Some bacteria particularly stimulate pro-Th1 intestinal cell populations which induce the production of pro-inflammatory cytokines such as IL-12, while other microbial strains stimulate Treg lymphocytes by promoting the production of anti-inflammatory cytokines such as IL-10. The composition of the microbiota, therefore, plays a major role in the balance between Th1 and Treg, which is essential for maintaining intestinal homeostasis (CDU-HE, 2014). In this study, the immunomodulatory capacity of the isolated strain was studied after stimulation of human peripheral blood mononuclear cells (PBMC) using heparinized blood from five healthy donors. The cell culture supernatant was analyzed for the concentration of IL-10 and IL-12p70 secreted by PBMCs in the presence of *E. faecium* CMUL1216. The results showed that *E. faecium* CMUL1216 induced a high level of IL-10 (901 pg/ml) almost equal to that induced by *B. longum* CMUL CXL 001 the reference strain (990 pg/ml) as shown in Fig. 5. Hence, this strain presented an in vitro anti-inflammatory profile characterized by a high IL-10/IL-12 ratio (IL-10/IL-12=9.6 > 1) with a value of 901 pg/ml for anti-inflammatory effect and 93 pg/ml for pro-Th1 effect (Zaylaa et al., 2018). Indeed, previous studies have shown that probiotic effects, in particular, are mediated through immunoregulation, that mediates the suppression of lymphocyte proliferation and cytokine production.
by T cells (Isolauri et al., 2001) and downregulates the expression of proinflammatory cytokines such as tumor necrosis factor-α, IL-12, and IFN-γ (Stewart et al., 1996). It is well known that anti-inflammatory cytokines interact with intestinal epithelium and attenuate the synthesis of inflammatory effector molecules elicited by diverse proinflammatory stimuli (Bai et al., 2004; Otte and Podolsky, 2004). Thus, probiotics can be responsible for the unique tolerance of the gastrointestinal mucosa to proinflammatory stimuli, and thus, undesirable effects of such inflammation will be reduced. In addition, the human and animal gut inflammation enhances pathogen colonization and decreases intestinal permeability. Therefore, the use of anti-inflammatory probiotics can increase intestinal barrier as well as prevent pathogen invasion (Penha Filho et al., 2015). In this context, our strain can be used in the animal sector as anti-inflammatory probiotic able to modulate the intestinal immune response through the stimulation of certain cytokine secretion by epithelial cells. This immunomodulatory effect is related to the beneficial effects of probiotics in this sector.

Finally, to obtain a strain with probiotic properties, safety concerns, and potential application in the animal sector, a safety assessment, such as hemolytic activity, antibiotic resistance, and virulence gene detection (WHO/FAO, 2002) was analyzed for E. faecium CMUL1216. The results showed that E. faecium CMUL1216 had no hemolytic activity and did not contain any of the virulence genes tested. Furthermore, CMUL1216 strain exhibits antibiotic resistance against antibiotics used already in the animal sector especially in the poultry sector such as the cyclines (Mehdi et al., 2018).

In conclusion, E. faecium CMUL1216 exhibits good properties as a potential antimicrobial probiotic. Moreover, this strain can reduce inflammation by the stimulation of an anti-inflammatory response in vitro. E. faecium CMUL1216 could represent a good strain for a potential application in the animal sector. Further in vivo tests should be realized to ensure the viability, stability, and activity of this strain.

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