Beneficial Microbes, 2021; 12(3): 295-305

Wageningen Academic Publishers

Gut microbiota, body weight and histopathological examinations in experimental infection by methicillin-resistant *Staphylococcus aureus*: antibiotic versus bacteriocin

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Received: 2 August 2020 / Accepted: 23 November 2020

RESEARCH ARTICLE

Abstract

Bacteriocins have been steadily reported as potential agents that may contribute, in different ways, to overcome antimicrobial drug resistance. Here, holoxenic NMRI-F mice microbiota, their body weight recovery and histopathological alterations of organs like colon, spleen and liver were examined in mice intraperitoneally infected with 10^8 cfu of a clinical methicillin-resistant *Staphylococcus aureus* (MRSA-1), and treated with enterocin DD14 alone (165 mg/kg), erythromycin alone (100 mg/kg) or their combination. Animals that received both antimicrobials presented a better body weight recovery than other groups. Less pronounced histopathological alterations were observed in mice MRSA-infected and treated with bacteriocin than in those MRSA-infected but untreated or MRSA-infected and treated with erythromycin. Noteworthy, these alterations were absent when mice were treated with MRSA-infected and treated with both antibacterial agents. Furthermore, the genus richness was significantly lower in mice infected and treated with erythromycin, compared to mice infected and treated with both antimicrobials. The beta-diversity analysis showed that non-infected mice and those infected and treated with both antimicrobials, stand apart from the other groups as supported in a NMDS model. This *in vivo* study shows the relevance of bacteriocin, or bacteriocin-antibiotic formulation in protecting colonic, liver and spleen soft tissues and controlling the mouse gut microbiota, following MRSA infection.

Keywords: methicillin-resistant *Staphylococcus aureus*, enterocin DD14, erythromycin, histological sections, microbiota modifications

1. Introduction

The antimicrobial resistance (AMR) crisis is attributable, among other things, to massive use of antibiotics for human and animal health, and also to the drying up of the antibiotic discovery pipeline. By 2050 more people will die in the world because of AMR than any other illness according to different official sources (O'Neill, 2016). AMR involving methicillin-resistant *Staphylococcus aureus* (MRSA) have been reported in the hospital environment as being responsible for a high number of deaths (Dumitrescu *et al.*, 2010; Köck *et al.*, 2010). MRSA is a successful modern pathogen, living as a commensal and transmitted in both health-care and community settings. This harmful pathogen is a leading cause of clinical conditions, such as bacteraemia, endocarditis, skin and soft tissue infections, bone and joint infections and various hospital-acquired infections. This pathogen is still posing serious clinical menace, with high morbidity and mortality rates (Turner *et al.*, 2019). Treatment options for MRSA are limited and several antimicrobials are under development, including vaccination as preventive measure (Lee *et al.*, 2018). Of note, MRSA has become a therapeutic challenge, even though antibiotics like teixobactin, with a distinct mode of action from methicillin, exist (Ling *et al.*, 2015). Discovery and development of new antibiotics has not been of high

priority during the last decades, which could be partially explained by lack of investment incentive due to continuing lack of success.

To help curb AMR, new antibiotics and other therapeutic options are needed. Antimicrobial peptides (AMPs) stand as key alternatives to fading antibiotics and could rekindle the drying pipeline. AMPs possess similar, or even better effective activity profiles than traditional antibiotics (Seo *et al.*, 2012), in spite their sensibility to proteolytic and gastric degradation (Renukuntla *et al.*, 2013). The World Health Organization is presently calling for a groundbreaking action to avert this AMR crisis. International agencies and experts are also calling for immediate and coordinated action to tackle AMR. In this perspective, the global action encourages not only research of new antibiotics, in spite of industry recalcitrance, but also fosters implementation of key alternatives like phage therapy, faecal transplantation and antimicrobial peptides.

AMPs are produced by all living cells, as part of their innate immune system (Mishra *et al.*, 2017), and do play a role in the protection of the host-cells against invading pathogens (Spencer *et al.*, 2014). AMPs are endowed with intrinsic antibiotic activities, and are foreseen to replace fading or help improving their activities through synergistic interactions (Lei *et al.*, 2019). Bacteriocins are ribosomally synthesised by both Gram-negative and Gram-positive bacteria (Drider and Rebuffat, 2011), as well as *Archea* (Kumar and Tiwari, 2017). Applications of bacteriocins, mainly those produced by lactic acid bacteria (LAB-bacteriocins), were limited to the food industry as food preservatives (Chen and Hoover, 2003; O'Connor *et al.*, 2020), and the lantibiotic nisin is presently the only LAB-bacteriocin to be qualified by the FDA (US Food, Drug and Administration) as food-preservative E234. The last decades has witnessed a steadily increasing number of studies dedicated to the potential use of LAB-bacteriocins as therapeutics. Indeed, these molecules are gaining more and more interest, and their momentum to treat multi-drug species, in humans and animals is increasing day by day (Cotter *et al.*, 2013; Meade *et al.*, 2020; Vieco-Saiz *et al.*, 2019).

Recently, we established the capabilities of the bacteriocins enterocin 28 (DD28), and enterocin 93 (DD93) to potentiate the *in vitro* activity of erythromycin against a MRSA-1 strain (Al Atya *et al.*, 2016). Interestingly, this synergistic interaction was also observed for enterocin 14 (DD14), when added to erythromycin. Of importance, the DNA sequences of EntDD14, EntDD28 and EntDD93 were identical (unpublished data). Of note, further studies were performed only on EntDD14, namely, genome sequencing and analysis (Belguesmia *et al.*, 2017), and also by identification of the peptide amino acid sequence of this bacteriocin (Caly *et al.*, 2017). These studies collectively enabled us to classify EntDD14, as a leaderless class IIb bacteriocin. To extend our knowledge on the potential use of this *in vitro* synergistic bacteriocin-antibiotic interaction against the MRSA-1 strain, we looked at the effects of EntDD14-erythromycin combination *in vivo* using mice models. To that purpose, the NMRI-F mice were examined for the body weight recovery; microbiota stability and histological alterations of the intestine; spleen and liver; after their MRSA-infection and treatment with antibiotic (erythromycin), bacteriocin (EntDD14) or their combination.

2. Materials and methods

EntDD14 preparation and antibiotic activity

The bacteriocinogenic *Enterococcus faecalis* 14 was grown overnight at 37 °C, in Brain Heart Infusion (BHI) broth (Fluka Analytical, Steinheim, Germany). Then, the culture was centrifuged (8,000×*g*, 4 °C, 10 min). 40 ml of the resulting cell-free supernatant (CFS) were passed, at room temperature (20-25 °C) with a flow rate of 1 ml/min, through a C_{18} solid phase extraction (SPE) cartridge containing octadecyl silica as a filler to retain non-polar compounds by strong hydrophobic interaction (Agilent, Santa Clara, CA, USA). Several washing steps with 40 ml of eluents containing successively 10, 20, 30 and 40% (v/v) of acetonitrile, mixed with deionised water, permitted the removal of contaminants. The semi-purified EntDD14 was eluted with 50% (v/v) of acetonitrile. This active fraction was dried using a SpeedVac and resuspended in ultrapure water. The DD14 concentration was determined by the Bradford protein assay (Bradford, 1976), whereas its total activity was assessed as described by Dabard *et al.* (2001). Then, and according to the protocol described by Al Atya *et al.* (2016), the minimal inhibitory concentrations (MICs) of EntDD14 or erythromycin (Ery) were determined as well as their fractional inhibitory concentration index (FICI) to assess their interaction.

Animal design, diet and housing conditions

Holoxenic NMRI-F (Naval Medical Research Institute) strain of mice were obtained from the Pasteur Institute of Algiers (Algeria). Thus, 35 female mice (NMRI-F), with a weight of 30 g were randomly assigned to 5 different groups (G1, G2, G3, G4 and G5). They were housed individually in metal cages under controlled room temperature (20±2 °C), humidity (50±5%), constant 12 h/12 h light/dark cycle and fed with mouse diet obtained from a local company (ONAB, Bejaia, Algeria), and water for 1 week *ad libitum* before the experimental analysis and monitoring.

Challenge tests

The antibacterial agents, namely erythromycin and EntDD14, were dissolved in 0.9% (v/v) saline sterile water. The animals were divided in five groups (G1 to G5). G1 was used as control, the other four groups were challenged intraperitoneally (right side) with 100 µl of MRSA-1 strain at 10^9 cfu/ml. Then, 1 h later, all groups, except G2, received a single dose of a solution of 100 µl of erythromycin at 100 mg/kg (G3), or EntDD14 at 165 mg/kg (G5) or of both of them (G4) (left-side). Following these challenge tests and administration of the antibacterial agents above-cited, animals were regularly examined for adverse effects and morbidity during 96 h. Their body weights were daily registered. At the end of the 96 h of experimental monitoring, 5 different samples of faeces from each group were randomly taken for DNA extraction and microbiota analyses.

Ethical procedure

Animals were sacrificed and one animal per group was used for histological examinations following eosin-staining of organs, such as colon, liver and spleen. All international and national applicable guidelines were scrupulously respected for animal care and welfare.

Histological sections examinations

Following animal euthanasia and dissection, liver, spleen and colon were removed and immediately put into 10% (v/v) of neutral-buffered formalin solution. The histological sections were prepared at a private laboratory of anatomy, cytology and pathology (Bejaia, Algeria). The inclusion of the sections was carried out as previously reported (Lussier, 1989), and the staining of the slides was performed using the haematoxylin-eosin procedure (Sobotta and Welsch, 2000).

DNA extraction and purification

Total bacterial DNA was extracted from 25 mg of faeces using a QIAamp PowerFecal extraction kit (Qiagen, Venlo, the Netherlands), according to the manufacturer's recommendations. A first mechanical lysis step of 5 min using lysing matrix E tubes (MP Biomedicals, Illkirch, France) and extraction kit lysis buffer was applied. The DNA was then diluted with DNAse and RNAse-free water and its concentration and purity were evaluated by optical density at 260/280 nm using a Nano Drop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). Then, DNA samples were stored at -20 °C until they were used for the sequencing analysis.

16S rDNA high throughput amplicon sequencing

PCR-amplification of the V1-V3 region of the 16S rDNA, and library preparation were performed with the following primers (with Illumina overhand adapters), forward (5'-GAGAGTTTGATYMTGGCTCAG-3'), and reverse (5'-ACCGCGGCTGCTGGCAC-3'). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter, Pasadena, CA, USA) and submitted to a second PCR round for indexing, using the Nextera XT index primers 1 and 2. After purification, PCR products were quantified using the Quant-IT PicoGreen (ThermoFisher Scientific, Waltham, MA, USA) and diluted to 10 ng/µl. A final quantification, by qPCR, of each sample in the library was performed using the KAPA SYBR® FAST qPCR Kit (KapaBiosystems, Wilmington, MA, USA) before normalisation, pooling and sequencing on a MiSeq sequencer using v3 reagents (Illumina, San Diego, CA, USA). Positive control using DNA from 20 defined bacterial species and a negative control (from the PCR step) were included in the sequencing run. Raw amplicon sequencing libraries were submitted to the NCBI database under bioproject number PRJNA623490.

Sequence analysis and 16S rDNA profiling

Sequence reads processing was performed as previously described using MOTHUR software package v141.1 (Schloss *et al.*, 2009) and VSEARCH algorithm for chimera detection (Rognes *et al.*, 2016). A clustering distance of 0.03 was used for operational taxonomic unit (OTU) generation. 16S rDNA reference alignment and taxonomical assignment were based upon the SILVA database (v1.32) of full-length 16S rDNA sequences (Quast *et al.*, 2013).

Data analysis

Subsample datasets were obtained and used to evaluate ecological indicators (Goods Coverage, Chao richness index and reciprocal Simpson microbial diversity of the samples) and beta-diversity (using a distance Bray-Curtis dissimilarity matrix) using MOTHUR. Beta-diversity was visualised with a Bray-Curtis dissimilarity matrix based non-parametric dimensional Scaling (NMDS) model using vegan (Oksanen *et al.*, 2019) and vegan3d (Oksanen *et al.*, 2018) packages on R. Sample clustering and beta-dispersion were respectively assessed on Bray-Curtis dissimilarity matrix with AMOVA and HOMOVA tests using MOTHUR (using 10,000 iterations on the rarefied table).

Statistical analysis

All results were expressed as mean ± standard deviation. Statistical analysis, except for microbiota and mouse bodyweight analyses, was performed using the one-way analysis of variance (ANOVA) procedure of Statistica

5.5 (1999 edition) software (Statsoft, Tulsa, OK, USA). Differences among means were detected by paired Student's test. Values of $P<0.05$ were considered statistically significant. Statistical analysis of mouse body weights was conducted in GraphPad prism v6.1 (La Jolla, CA, USA), one way ANOVA was applied with Tukey's test for post comparison ($P<0.05$). Microbial population structure and microbial population abundance difference between groups were assessed with the Kruskal-Wallis non parametric test followed by paired-tests corrected by Benjamini, Krieger, Yekutieli False Discovery Rate using PRISM 7 (Graphpad Software), differences were considered significant for a q-value <0.01.

3. Results

EntDD14 and erythromycin enabled synergistic interaction *in vitro* against MRSA-1

MRSA-1 strain was resistant to erythromycin (MIC 16 µg/ml) according to the CA-SFM guideline (CA-SFM, 2019). Of note, the MIC of erythromycin decreased from 16 to 1 µg/ml, in the presence of EntDD14, arguing a potential synergistic interaction. Indeed, this interaction was confirmed by the FIC value 0.313. Antibacterial activities of EntDD14, erythromycin and their combination are given in Table 1. Interpretation of these data were performed as reported by Petersen *et al.* (2006).

Animals infected with MRSA-1 and treated with erythromycin + DD14 show a better body weight recovery than groups G2, G3 and G5

Results showed that the mouse body weights were not significantly different between groups during the first three days of the experiment but at day 4 all the treatments led to a significant body weight decrease when compared to G1 (Supplementary Figure S1). However, the mice for which EntDD14 and erythromycin were administrated, alone (G5) or in association (G4), presented a significantly higher body weight than mice from G2. Moreover, the mice treated by both EntDD14 + erythromycin (G4) appeared to be less affected than other mice treated with either drug alone. The *P*-values obtained were 0.0688 (G4 versus G5), and 0.0375 (G4 versus G5).

Histopathological examination revealed severe alterations in G2 and G3 mice

The histological sections were analysed for the main organs, namely colon, spleen and liver. These examinations revealed G1 presented a preserved colic architecture with normal height crypts and devoid of any inflammatory and/or ulcerated signs (Figure 1 – IA). Nevertheless, intraperitoneal administration of MRSA-1 strain to mice caused villous atrophy, and other adverse effects like infectious and inflammatory symptoms, outlined by a mesenteric lymphadenopathy and ileal intestinal mucosa, respectively (Figure 1 – IB). These adverse effects were also observed for animal infected with MRSA-1 strain and treated with erythromycin (Figure 1 – IC). Architecture of crypts was mostly impaired, and inflammatory signs with lymphocytic infiltration were observed (Figure 1 – IC). Of note animals challenged with MRSA-1 strain and treated with EntDD14 exhibited less pronouncedly these adverse effects, as indicated on Figure 1 – IE. Importantly, when mice were challenged with MRSA-1 and concomitantly treated with both antibacterial agents, EntDD14+Erythromycin, a normal anatomy was observed. This normality, as seen on Figure 1 – ID, is associated with normal intestinal mucosa and intact villosities. This anatomy and cellular architecture are fairly equivalent to those from the animal group used as positive control. Despite that the MRSA-1 strain is resistant to erythromycin, and EntDD14 weakly

Table 1. Minimum inhibitory concentrations (MICs) of antimicrobials and their association against methicillin-resistant *Staphylococcus aureus* (MRSA)-S1.[1,2,3]

Strain	DD14 (µg/ml)	Ery (µg/ml)	DD14-Ery (µg/ml)	FIC_{DD14}[4]	FIC_{Ery}[5]	FICI[6]
MRSA-S1	154	16	38.5/1	0.25	0.063	0.313

[1] Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.
[2] FIC index = (MIC of DD14 in association/MIC of DD14 alone) + (MIC of erythromycin in association/MIC of erythromycin alone). In antimicrobial association, Petersen *et al.* (2006) defined synergy as $\sum FIC \leq 0.5$, additivity as $0.5 < \sum FIC \leq 1$, indifference as $1 < \sum FIC \leq 4$ and antagonism as $\sum FIC > 4$.
[3] DD14 = enterocin DD14; Ery = erythromycin; FIC = fractional inhibitory concentration; FICI = fractional inhibitory concentration index.
[4] FIC_{DD14} = MIC of DD14 in association/MIC of DD14 alone.
[5] FIC_{Ery} = MIC of Ery in association/MIC of Ery alone.
[6] $FICI = FIC_{DD14} + FIC_{Ery}$.

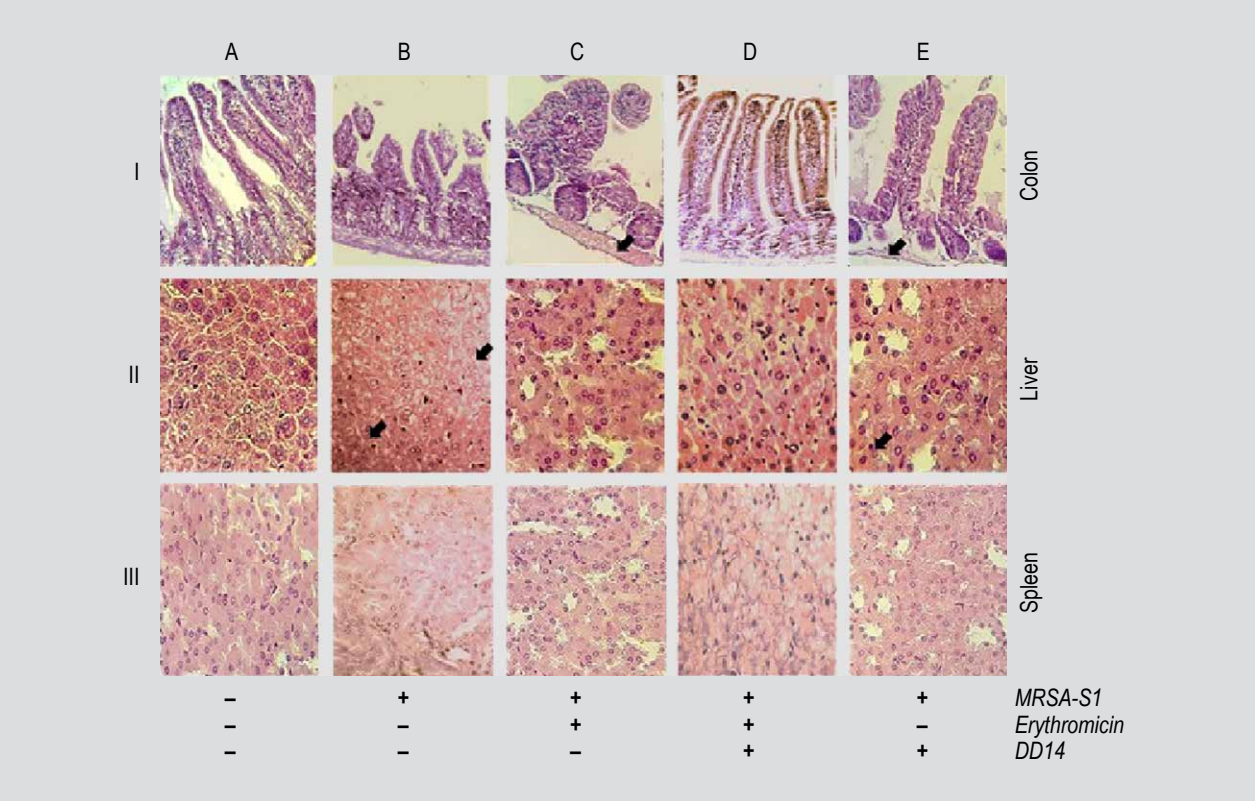

Figure 1. Histological examination and eosin-stained sections of organs: colon (I), liver (II) and spleen (III). Tissues were from mice G1 (A) (–, –, –), G2 (B) (+, –, –), G3 (C) (+, +, –), G4, (D) (+, +, +) and G5 (E) (+, –, +). Organs are indicated on the right side of the picture. Arrows indicate lymphocytes infiltrating, binucleation and granulo-vitreous degeneration.

active, their association enabled synergistic interaction *in vitro* (Table 1). These results convey the potency and utility of such combination in fighting staphylococcal infections.

The histological sections of liver were studied similarly. As depicted on Figure 1 – IIA, the animal from the control group presented a well conserved cellular architecture, which is typically present in intact organ. Nevertheless, this architecture was affected when mice were intraperitoneally challenged with the MRSA-1 strain. Indeed, symptoms of liver injury were marked by several adverse events such as haemorrhage, increase in both cytoplasm density, and chromatin content, binucleation process, centrolobular hepatic necrosis and inflammatory reactions (Figure 1 – IIB). Of note, centrolobular hepatic necrosis was markedly associated with congested veins, while inflammatory reaction was, in turns, associated with hepatocyte degeneration (Figure 1 – IIB). The histological analysis revealed overall similar adverse effects in mice infected with the MRSA-1 strain and treated with erythromycin (Figure 1 – IIC). Accordingly, necrosis was noticeably represented by the disappearance of caniculi and loss of the zone of drainage, whereas cellular intoxication was marked by loss of polygonal and radial hepatic cell shapes, cell junctions/ends as well as bi-nucleation process in the nucleus, and increased chromatin concentration (Figure 1 – IIC). These lesions were less pronounced in mice infected with MRSA-1 strain and treated with EntDD14 alone (Figure 1 – IIE). Nevertheless, mice infected with MRSA-1 strain and treated by EntDD14+erythromycin were less afflicted since signs of necrosis were insignificant, as noted with a faint vascular congestion, and increase in the nuclear chromatin concentration (Figure 1 – IID). Mice from group 1 were used as baseline control, since they had received no antibacterial agent and/or MRSA-1 strain. Histological sections of spleen from an animal taken from this group were normal, with well-organised cellular architecture and typically healthy spleen cells (Figure 1 – IIIA). Similar to other organs, challenge and treatment caused modifications, which were treatment-dependent. Signs of severe adverse effects, supporting a vascular affection, were observed in mouse of G2. These signs included manifest venous congestion of sinusoids, and haemorrhagic suffusion. In addition, infection and inflammatory reactions were observed. They showed on one hand a myeloid metaplasia and abundance of megakaryocytes, and on the other hand granulo-vitreous degeneration (Figure 1 – IIIB). However, when mice came from groups challenged with MRSA-1 and receiving erythromycin or EntDD14, only signs of bacterial infection characterised by a splenomegaly were noticeably observed (Figure 1 – IIIC and IIID). The histological analyses did not show, at the cellular level, clear

differences with that of the group I (Figure 1 – IIIA, IIIC and IIIE). Nonetheless, the spleen tissues of mice infected with MRSA-1 strain and treated with both antibacterial agents, EntDD14+Erythromycin, displayed similar organisation to those of the animal from group 1, that was used as control baseline (Figure 1 – IIID).

Analysis of faecal microbiota

From the 25 libraries, we obtained 3,204,042 raw reads and 1,690,250 reads after cleaning and chimera detection (median length of 503 bp). We retained a subsampled pool of 10,000 reads per sample to proceed with OTU binning (0.03 cut-off) for a total of 5,945 OTUs. Mean sampling Good's coverage was 99.87%, with no statistical difference between groups. Notably, the faecal microbial population structure was assessed at the genus level. Intrinsic diversity analysis revealed that genus richness was significantly lower in G2, compared to mice from G1 and G4 (Supplementary Figure S2A). Beta-diversity analysis showed that G1, and G4, stand apart from the other groups as illustrated in a NMDS model (K=3, stress 0.04) (Supplementary Figure S2B). This clustering, however, is only significant for G1 versus G2 (AMOVA test, P=0.00396). Moreover G4 versus G2 does not reach significance but is close (P=0.00689).

The alpha biodiversity index Shannon is a continuous variable with a lower bound of zero and no upper bound. Higher is the value, greater is the diversity. As seen in Supplementary Figure S2A, the level of diversity declined following the infection with MRSA-1 strain, and the Shannon's index declined significantly compared to the control. However, the diversity was partly recovered after administration of any drug alone, raising the biodiversity at the same level. However, the noticeable effect was obtained when these drugs were administered concomitantly. The Shannon index increased, reflecting an enhancement of the richness at the genus level.

Supplementary Figure S3 indicates that *Bacteroidetes* and *Firmicutes* are the prevailing phyla as expected for faecal microbiota. At the genus level, the main genera in G1, G2, G3 and G5 belong to the *Firmicutes* with *Lactobacillus*, *Enterococcus* and *Turicibacter* being present. However, in G1 and G4, the most abundant population belong to the *Bacteroidetes* with *Alistipes* genus and population belonging to the *Prevotellaceae* and *Muribaculaceae* families. Moreover, Kruskal-Wallis H test evaluation for the population abundance in the different groups was performed and highlighted several populations of interest (Supplementary Table S1). Mainly we confirmed the shift *Firmicutes*/*Bacteroidetes* between G1 and G2 (q<0.0001) but also between G4 and the other infected groups G2, G3 and G5 (q<0.0001). These differences can also be found at the genus level with *Lactobacillus* being significantly more abundant in G2, G3 and G5 compared to G1 and G4. The *Enterococcus* population is also more abundant in G3 and G5 compared to the others.

4. Discussion

To bolster antibiotic stewardship initiatives, the LAB-bacteriocins are gaining more and more interest for therapeutic applications (Dischinger *et al.*, 2014; Meade *et al.*, 2020; Ołdak and Zielińska, 2017). With respect to this objective, we provide in the present study original data on the *in vivo* cytotoxicity of class IIb enterocin DD14 (Belguesmia *et al.*, 2017; Caly *et al.*, 2017), and studied its impact on different markers as discussed below. Overall, studies dedicated to cytotoxicity and immunotoxicity of bacteriocins are very limited, in spite of the relevance of this aspect to food or medical applications. Nisin, which is the only authorised and marketed bacteriocin used as a food additive (E234), is the most studied model for cytotoxicity aspects.

The effects of EntDD14, erythromycin, or their association were examined *in vivo* using the NMRI-F mice, as murine model. These effects concerned markers such as body weight change, gut microbiota stability and histopathological lesions in the NMRI-F mice, challenged with a clinical MRSA-1 strain, and treated with EntDD14, erythromycin, or both. To clarify the use of these molecules, we suggest referring to our recently reported *in vitro* data, in which we showed that DD28 and DD93 were able to potentiate erythromycin against the target MRSA-1 strain (Al Atya *et al.*, 2016). Importantly, the DNA sequences of EntDD14, EntDD28 and EntDD93 are fully identical (unpublished data), despite producing strains having been isolated from different samples (Al Atya *et al.*, 2016; Caly *et al.*, 2017). Of note, the EnDD14 was the first one which was fully characterised (Caly *et al.*, 2017). In addition, all these bacteriocins are weakly active against the MRSA-1 strain. Nevertheless, when added to erythromycin, any of these bacteriocins provides *in vitro* synergistic interactions against the MRSA-1 strain (Al Atya *et al.*, 2016) (Table 1). Here, we examined the effects of EntDD14, erythromycin, or both, when administered intraperitoneally to NMRI-F mice concomitantly infected with the MRSA-1 strain. The route of administration of bacteriocins is a key element. Oral administration can be counterproductive because of conditions met during the gastrointestinal transit, such as low pH, proteases, etc. These conditions are unfavourable to bacteriocins, and can impair their activity and stability (Fernandez *et al.*, 2013). For example, Kheadr *et al.* (2010) outlined the instability of pediocin PA-1/AcH in the dynamic *in vitro* TIM model, which mimics the upper human gastrointestinal tract.

Nevertheless, the oral administration of bacteriocins can gain insight into their safety aspects. Thus, Frazer *et al.* (1962) administered orally nisin to rats and proclaimed the

safety of this bacteriocin. However, de Almeida Vaucher *et al.* (2011) reported histopathological alterations of spleen, skin and liver following oral nisaplin administration to mice. On the other hand, Sahoo *et al.* (2017) showed that mice receiving orally and daily 0.5 mg/kg of bacteriocin TSU4 for 21 days, showed no deaths, and were devoid of any immunogenicity or toxic effect.

Furthermore, the class IIc enterocin AS 48 was intraperitoneally administered to BALB/c mice and the authors reported (Baños *et al.*, 2019) moderate vacuolar degeneration in the hepatocytes, conversely to mice fed with nisin. However, Ketaren, *et al.* (2016) showed that mice receiving intraperitoneally high amounts of pediocin N6 (up to 20,000 mg/kg) did not show any sign of acute toxicity.

In the present study, EntDD14 was administrated intraperitoneally to NMRI-F mice, to avoid proteolytic and gastric degradation of EntDD14. Additionally, this route was also used for administration of erythromycin, and challenge with the MRSA-1 strain to allow its rapid spread into the bloodstream. As reviewed by Turner *et al.* (2019), *Staphylococcus aureus* expresses different virulence factors, such as toxins, immune-evasive surface factors and enzymes that promote its tissue invasion. Other harmful toxins like exfoliative toxins, adhesins and haemolysins were reported (Malachowa and DeLeo, 2010). These authors revealed that some MRSA strains can utilise bacteriocins as MGE (mobile-genetic-elements) to inhibit competing or commensal bacteria (Malachowa and DeLeo, 2010). In the present study, no death was registered during 4 days of experimental analysis and monitoring, in spite of a dose of pathogen administrated to the mice. This can be explained by different factors, such as strain adaptation, genetic reorganisation and finally loss of the virulence traits (Mizobuchi *et al.*, 1994).

In terms of the body weight incremental, we noticed at the end of 4 days experimental analysis that the mice from G4 were less affected than those from other infected groups G2, G3 and G5. Examination of histological sections revealed severe histopathological damage in the colon, spleen and liver from the mice of G2 and G3. These symptoms and signs of necrosis, cellular toxicity and inflammation observed in G2 and G3 can be attributed to many factors including toxins secreted locally by MRSA-1 and their dissemination via the blood circulation. In MRSA-infected mice treated with erythromycin alone (G3), these adverse effects were expected because this strain is resistant to this antibiotic (Al Atya *et al.*, 2016) (Table 1). Erythromycin is used for treatment of nosocomial infections mostly caused by *S. aureus*, but because surviving bacteria are frequently found most patients are recommended to receive combination therapy of erythromycin with other agents active against *S. aureus*. Erythromycin is suitable for treating Gram-positive infections in humans. By increasing the concentration of erythromycin to 600 mg/g of faeces, its spectrum has been shown to be extended and became active against some intestinal Gram-negative bacteria such as those from *Enterobactericeae* family (Hartley *et al.*, 1978). The histopathological alterations reported in mice from G2 and G3 persisted, but to a lesser extent to the animals in G5, which had been infected and EntDD14 treated. Finally, these clinical signs were cleared following treatment of infected mice with erythromycin+ EntDD14. The cationic nature of LAB-bacteriocins can prejudice their effectiveness, because they can bind to blood components (Ghobrial *et al.*, 2010). Data gathered here indicate the absence of toxicity of EntDD14, and revealed that erythromycin+EntDD14 is a suitable association that provides protection from deleterious effects of MRSA.

With respect to the microbiota aspects, it should be remembered that although antibiotics are invaluable weapons for eliminating malevolent pathogens, they can also induce long-lasting deleterious effects on the host such as the destabilisation of the microbiota, leading to dysbiosis (Lagier *et al.*, 2012; Lange *et al.*, 2016). The impact of antibiotics on the gut microbiota depends on its route of administration, dose and time of administration (Ruppé *et al.*, 2018). Thus, we looked at the impacts of erythromycin, EntDD14, or their association on the NMRI-F mouse gut microbiota under different experimental conditions. Firstly, we looked at the phyla composition in each mouse group. The *Firmicutes*/*Bacteroidetes* ratio (F/B) is a key factor in mouse obesity (Ley *et al.*, 2005), and this is also consistently confirmed for humans (Barlow *et al.*, 2015; Sweeney and Morton, 2013).

Metagenomic analyses of the faecal samples from G1 revealed, amongst other phyla, *Epsilonbacteteraeota*, *Lentisphaerae* and *Proteobacteria. Epsilonbacteteraeota* phylum has been introduced into the bacterial taxonomy, following re-classification of class *Epsilonproteobacteria* (Waite *et al.*, 2017), whereas the *Lentisphaerae* phylum, has been included in the PVC super-phylum, which initially contained three phyla *Planctomycetes, Verrucomicrobia* and *Chlamydiae* (Fuerst, 2013). In the present study, a shift in the gut faecal composition occurred following infection of mice with MRSA-1strain (G2), and their subsequent treatment with erythromycin (G3), or EntDD14 (G5).

The *Proteobacteria* phylum has been reported to be associated with obesity complex disease (Bai *et al.*, 2019; Rizzatti *et al.*, 2017). *Proteobacteria*, qualified as key dysbiosis players, are not only present in the gut and stools but also in others human body sites, like skin, oral cavity, tongue or vaginal tract (Huttenhower *et al.*, 2012). This phylum also contains pathogens such as *Brucella, Rickettsia, Bordetella, Neisseria, Escherichia*, *Shigella*, *Salmonella*, *Yersinia,* and *Helicobacter,* which are associated with both intestinal and extra-intestinal diseases (Langgartner *et al.*,

2017; Maharshak *et al.*, 2013). The metagenomic analyses performed here showed that faecal samples from mice of G4 contained more *Bacteriodetes* than *Firmicutes*. This is an important point because mice from G4 exhibited greater body weight recovery profiles (Supplementary Figure S1), and earlier studies reported a predominance of *Bacteroidetes* in overweight and obese individuals (Schwiertz *et al.*, 2010), and a positive correlation between the faecal concentrations of *Bacteroides* and the body mass index (Ignacio *et al.*, 2016). Deeper analyses of these metagenomic data revealed a relative abundance of *Helicobacter*, *Lachnospiraceae*, Bacteroidales/Bacteroides, *Prevotellaceae* and *Muribaculaceae*. Interestingly, *Muribacaculaceae* is known to be the dominant bacterial group in the mouse gut (Seedorf *et al.*, 2014). Nevertheless, this profile has been modified in other groups (G2, G3, G4 and G5), being noteworthy the relative abundance of probiotic genera, such as *Lactobacillus* and *Enterococcus* in G3 and G5.

5. Conclusions

During this study we established that Holoxenic NMRI-F mice infected with the MRSA-1 strain, and treated with erythromycin, EntDD14 or both of them, responded differently based on the clinical parameters used. Interestingly the MRSA-infected mice concomitantly treated with erythromycin and EntDD14 showed the best body weight recovery. Of note, this bacteriocin-antibiotic combination enabled protection of the soft tissue of organs such as colon, liver and spleen, against the MRSA-1 strain. Moreover the faecal microbiota composition, and more particularly the beta-diversity analysis revealed a convergence between the uninfected mice and those treated with MRSA-infected and treated concomitantly with bacteriocin-antibiotic. These new advances constitute a new step in the valorisation of LAB-bacteriocins as therapeutic agents. This unique study carried out *in vivo* on the Holoxenic mice models permitted to show the clinical and metagenomic alterations following mice infection with MRS-1 strain and their treatment with erythromycin, EntDD14 and both of them. To strengthen this approach, further clinical investigations using other molecules and infection models will be tested in the near future.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2020.0155.

Table S1. Abundance of bacterial taxa expressed in percentage that are statistically impacted by the MRSA challenge or the post-challenge treatment, as determined by 16S amplicon sequencing.

Figure S1. Body weight evolution during animal experimentation and monitoring.

Figure S2. Intrinsic bacterial genus diversity, genus richness and evenness deduced respectively from reciprocal Simpson Index, Chao1 index and Simpson index (A). Non-metric dimensional scaling model with three axis of the five mouse groups (B).

Figure S3. Changes in microbial populations in the faecal content, assessed by 16S profiling.

Acknowledgements

The authors would like to thank la Région des Hauts-de-France for supporting part of this work, through CPER/FEDER Alibiotech (2016/2021). The authors are indebted to Dr. Steve W Elson, Dr. Bruce Seal (Oregon State University) and Prof. Luis A Nero (Universidade Federal de Viçosa, Brazil) for critical reading of the manuscript.

Conflict of interest

The authors declare no competing interest

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