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**Impact of colistin and colistin-loaded on alginate nanoparticles on pigs infected with a
colistin-resistant Enterotoxigenic *Escherichia coli* strain**

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Abstract: Colistin is frequently used for the control of post-weaning diarrhoea in pigs. Colistin resistance caused by plasmidic genes is a public health issue. We evaluated, in experimental animal facilities, whether free colistin or colistin-loaded on alginate nanoparticles (colistin/Alg NPs) could select a colistin-resistant Enterotoxigenic *Escherichia coli*. The Alg NPs were produced by a simple top-down approach through ball milling of sodium alginate polymer precursor, and colistin loading was achieved through physical adsorption. Colistin loading on Alg NPs was confirmed using various tools such Fourier transform infrared spectroscopy and dynamic light scattering measurements. Thirty-four piglets were orally inoculated or not with a *mcr-1*-positive, rifampicin-resistant enterotoxigenic *E. coli* strain, and the inoculated pigs were either treated or not during five days with commercial colistin (100,000 IU/kg) or colistin/Alg NPs (40,415 IU/kg). Clinical signs were recorded. Fecal and post-mortem samples were analyzed by culture. The result clearly indicated that colistin/Alg NPs had a slightly better therapeutic effect. Both treatments led to a transitory decrease of the total *E. coli* fecal population with a majority of colistin-resistant *E. coli* isolates during treatment, but the dominant *E. coli* population was found susceptible at the end of the trial. Further studies are needed to evaluate, in diverse experimental or field conditions, the therapeutic efficacy of colistin/Alg NPs for post-weaning diarrhoea.

Keywords: Post-weaning diarrhea; pig; colistin; Enterotoxigenic *Escherichia coli*; alginate; nanoparticles

1. Introduction

Post-weaning diarrhea (PWD) is one of the most frequent diseases encountered in pig production, leading to important economic losses. PWD is caused by Enterotoxigenic *Escherichia coli* (ETEC) strains, belonging to different serogroups (e.g. O138, O139, and O141), and possessing various adhesive factors (e.g. F4 (K88), F8, AIDA, and Eae) and virulence genes (e.g. enterotoxins LT, STa, STb, and EAST). The control of PWD must first rely on improvement of breeding management of the weaning piglets in order to avoid stressful growth conditions. Other prevention strategies including immunization, feed additives, breeding of resistant pigs, probiotics, phages... are also suggested or under development (Rhouma et al., 2017). However, when prevention measures result to be ineffective, then antibiotics may be used (Luppi, 2017). Although in 2012-2013, 70% of the French herds did not report any use of antimicrobials for their fattening pigs, a high antimicrobial treatment incidence of 320.1 per 1,000 pig/day was observed for weaned piglets in France (Anses, 2020b). The use of these antimicrobials enabled selection of antimicrobial resistant *E. coli* strains. Thus, according to the 2019 results published by the Resapath network, only 26.4% of *E. coli* from diseased pigs are susceptible to all of the following antimicrobials, tetracycline, trimethoprim-sulfonamide, gentamicin, ceftiofur, enrofloxacin and marbofloxacin (Anses, 2020a). Resistance to colistin, a critically important antimicrobial for human health (WHO, 2019) is also encountered for about 10% of *E. coli* from diseased piglets in 2019 (Anses, 2020a).

In line with national recommendations (<https://agriculture.gouv.fr/le-plan-ecoantibio-2-2017-2021>) as well as international agencies advice (EMA/CVMP/CHMP, 2016), a reduction of the antimicrobial use, and that of colistin, in particular, in animals is urgently needed. In France, the target objective was a reduction of 50% in 5 years (2017-2021) of cattle, pig and poultry exposure to colistin.

1 Nanotechnology, developed in the late 20th century, is considered as an emerging science
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3 offering possibilities of diverse applications. This approach is based on the use of ultrafine
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5 particles of materials, with a size ranging from 1 to 100 nm. Nanoparticles exhibit a high
6
7 surface-to-volume ratio and a small size, which are favorable for adsorption of diverse
8
9 biological molecules. Furthermore, nanoparticles are considered as efficient drug and antibody
10
11 carriers, allowing better accumulation and biodisponibility in the sites of infection (Kreuter,
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13 1996).
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17 Different nanoparticles-based materials can be used with the aim to provide new means for drug
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19 delivery, antibiotics rationale and overcome major problems related to infections encountered
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21 in animal health. In light of this, Liu et al. (Liu et al., 2021) reported beneficial and controversial
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23 effects of zinc nanoparticles (Cnz) when used as feed additive. Indeed, these authors showed
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25 that Cnz can permit enhancement of antimicrobial activity, in a swine model, but in meanwhile
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27 these nanoparticles led to alterations of the intestinal microbiota. Another study underpinned
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29 the positive effects of zinc phosphate nanoparticles (ZnA NPs) on the piglet growth
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31 performance (Kociova et al., 2020).
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35 Related to that, we conducted an experiment to evaluate the potential efficacy of an innovative
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37 formulation of colistin, based on alginate nanoparticles (Alg NPs), in comparison with free
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39 colistin. The alginate nanoparticles investigated in this work were produced by a simple top-
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41 down approach through ball milling of sodium alginate polymer precursor (Belguesmia et al.,
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43 2020). Colistin loading was achieved through physical adsorption. The benefits of this approach
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45 include the possibility of loading several active molecules on the same nanoparticle and the low
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47 cost associated with the nanoparticles' production (Zgheib et al., 2021). In this context, we
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49 compared the impact on the *E. coli* fecal population and the efficacy of free colistin and colistin-
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51 loaded on alginate nanoparticles on pigs infected with a colistin-resistant ETEC using a
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53 previously developed PWD model (Le Devendec et al., 2018).
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2. Materials and Methods

2.1. Preparation of strains, media and inocula

The previously described *E. coli* strain 12-269M (Le Devendec et al., 2018) was used. Briefly, this *mcr-1*-positive *E. coli* strain has been isolated from PWD-suffering piglets. It was made resistant to rifampicin. It is hemolytic, belongs to the phylogenetic group A, and contains the virulence markers O149-F4, *astA*, *capU*, *gad*, *hlyA*, *iha*, *ltcA*, *staI*, *stb*, *terE*, and *ureD*. It is resistant to colistin, with a colistin MIC of 16 mg/L, and to sulfamethoxazole, trimethoprim, tetracycline, ampicillin, gentamicin, chloramphenicol, and rifampicin. In addition to the *mcr-1* gene, and mutations in the PhoP and PhoQ genes of unknown impact on colistin resistance, we could detect the following resistance genes: *sul1*, *sul2*, *sul3*, *dfrA1*, *tetA*, *cat*, *cmlA1*, *bla*_{TEM-1a}, *aac(3)-IIa*, *aac(3)-IVa*, *aadA1*, *aadA3*, *aph(3')-Ia*, *aph(4)-Ia*, *strA*, and *strB*.

The inoculum was prepared by culture in Mueller Hinton (MH) broth containing colistin (2 mg/L) for 18 h at 37°C, under gentle agitation. The bacterial pellet obtained after centrifugation (5,180 g, 5 min) was re-suspended in peptone buffered solution in order to obtain a titer of approximately 10⁸ colony-forming units (CFU)/mL. The titer of the suspension was checked by plating serial dilutions on MH media.

2.2. Preparation and characterization of colistin-loaded on alginate nanoparticles

The alginate nanoparticles (Alg NPs) were obtained using a top-down process with the BALL MILL RETSCH (PM100S10, Eragny, France). To this end, 2 g of sodium alginate powder (Sigma Aldrich, St Louis, MA USA) and 112 g of zirconium oxide beads were placed in a grinding pot of 1.050 kg, for 10 h with a stirring at 440 rpm, and a pause every 5 min, followed by a reverse rotation every 10 min. The Alg NPs were collected and dispersed in Milli-Q water (500 µg/mL) under ultrasonication (BRANSON 2800, frequency: 40 KHz) for 1 h at 25°C.

Then, a solution containing colistin (60 µg) was adjusted at pH 6.5 with 1 M sodium hydroxide prior to addition of 500 µg/mL of Alg NPs. The solution was introduced in an ultrasound bath

(FS100B, Decon Laboratories Ltd, Hove, UK) for 45 min at 50/60 Hz. The temperature of the bath was maintained below 30°C, in order to limit the aggregation of Alg NPs.

The amount of colistin adsorbed on the surface of the Alg NPs was determined by HPLC. To this end, a solution of colistin loaded onto Alg NPs was previously dialyzed on an 8 kDa membrane in order to remove non-associated colistin with the nanoparticles. The sample was filtered through a 0.45 µm regenerated cellulose membrane before loading the column (injected volume 40 µL). Equipment used during this experiment was the Shimadzu LC2010-HT (Shimadzu, Tokyo, Japan) with a column C4 of 5 µm QS Uptisphere® 300 Å, 250 × 4.6 mm (Interchim, Montluçon, France). The eluent composition was a mixture of two solvents A and B (A: 0.1% trifluoroacetic acid in deionized water, and for B: 0.1% trifluoroacetic acid in acetonitrile) with a flow rate of 1 mL/min. The column was heated to 40°C, and the elution of the compounds was carried out by a linear gradient of 0 to 80% during 30 min. Samples detection was performed at 215 and 254 nm. The concentration of colistin was determined using quantification of obtained peaks area comparatively to samples of colistin with known concentrations analyzed under the same conditions. The amount of colistin loaded on Alg NPs was calculated as the difference between the initial amount of colistin used for the formulation and the amount of colistin remaining in the supernatant. The percent of loading efficiency was calculated by the following formula:

$$\% \text{ Entrapment efficiency} = \frac{\text{Total amount of added colistin} - \text{unbound colistin}}{\text{Total amount of colistin}} \times 100$$

Particles size and zeta potential were determined by dynamic light scattering (DLS) measurements using a Malvern Zeta sizer Nano ZS instrument (Malvern Instruments Inc., Worcestershire, UK), equipped with a 4 mV He-Ne laser emitting at 633 nm. The capillary cell

was pre-rinsed with ethanol and purified water prior to loading samples. Samples were dissolved in Milli-Q water at a concentration of 500 µg/mL.

FTIR analysis was performed on a Thermo Fisher Scientific, Nicolet 380 (Waltham, MA, USA).

The colistin, Alg NPs and colistin/Alg NPs were analyzed, separately, in a pellet form, which is made by mixing 1 mg of the sample with 175 mg of potassium bromide (KBr). After that, the mixture was grounded properly to ensure uniform distribution in the KBr base. Finally, the grounded mixture was pressed in a hydraulic press by applying a pressure of ~7 to 9 tons.

2.3. Animal experimental design

The experiment was performed in accordance with French animal welfare regulations and the protocol was approved by the Ethics Committee authorization (APAFiS no. 2019010215289917). The experiment was conducted in the ANSES Ploufragan animal facilities (ANSES registration No. C-22-754-1). Strict biosecurity measures were implemented to avoid contamination of the pigs, including the use of an air filtration system and airlocks for each unit, unit-specific clothes, and compulsory showering after visiting the pigs. The trial was conducted with 34 specific-pathogen-free (SPF) Large White piglets, males and females, selected after genotyping the SPF available piglets for the polymorphism linked to the intestinal receptors of *E. coli* F4 (Le Devendec et al., 2018). Then, at six weeks of age, the piglets were randomized, on sex and weight, into four experimental groups, the F4-susceptible piglets being in priority placed into the inoculated groups (Table 1). The experimental groups were as follows: NINT (non-infected, non-treated group), INT (infected, non-treated group), ICC (infected, commercial colistin group), and ICNP (infected, colistin/Alg NPs). The animals did not receive any antibiotic treatment prior to the trial and were given the same non-supplemented feed. Each group was housed in a separate room, containing two pens of four or five animals.

On the morning of Day 0, the ten NINT piglets were orally inoculated with 10 mL of sterile broth. On the same day, the 24 piglets of the infected groups were orally inoculated with 10 mL

of the inoculum. For all treated groups, treatment began on the evening of Day 0 and ended on the morning of Day 5. Pigs from the ICC group received colistin (Colivet solution, 2 000 000 IU/mL, CEVA, Libourne, France) by oral gavage at the recommended dosage (50 000 IU/kg of body weight twice a day). Pigs from groups ICNP were also treated twice a day by oral gavage (40,415 IU/kg per day, 50 mL per pig each time).

The weight of each animal was recorded once a week. During the week, daily clinical examinations consisted of looking for general clinical signs and taking rectal temperatures. Individual fecal samples were collected from pigs on Days -3, 1, 2, 3, 4, 5, 7, 10, 14 and 17. The pigs were sacrificed on Day 21 (groups ICC and ICNP), Day 22 (two pigs from group INT), Day 23 (group NINT) or Day 24 (six pigs from group INT). The sacrificed animals were autopsied and samples of the jejunum, ileum, cecum, colon, and rectum were collected. The samples collected post-mortem (PM) contained both fecal material and scraped mucosa.

2.4. Analysis of fecal and PM samples

For culture analysis, all the samples were diluted 1/10 in peptone buffered solution containing 20% glycerol. The 1/10 diluted aliquots were stored at -70°C. After thawing, decimal dilutions were prepared. One hundred microliters of decimal dilutions was inoculated using EasySpiral® Dilute (Interscience, Saint-Nom-la-Bretèche, France) onto MacConkey media (MC) to determine the titers of the total *E. coli* population and onto MC supplemented with rifampicin (250 mg/L)(MCRif) to assess the titers of the inoculated strain. The plates were incubated overnight at 37°C. Moreover, for samples negative on MCRif, one mL of the 1/10 thawed dilutions was enriched overnight in 9 mL of colistin-supplemented Brain Heart Infusion broth (colistin 2 mg/L) before plating 100 µL on MCRif plates. The limit of detection was 10 CFU/g. Whenever possible, one colony obtained on MCRif per sample was stored. PCR was used to confirm that it belonged to the *E. coli* species (Bej et al., 1991), to detect the presence of the *mcr-1* gene (Liu et al., 2016), to check that a positive result was obtained with a F4 antiserum

(Laboce, Ploufragan, France). For isolates that did not exhibit the expected results (presence of *mcr-I*, and positive F4 agglutination test), we determined the phylogenetic group (Clermont et al., 2013), and studied ERIC-PCR profiles (Rivera et al., 1995). The susceptibility of some of these isolates was compared to the susceptibility of the inoculated strain by determining the MIC of colistin using a broth microdilution method on EUVSEC plates (Sensititre, ThermoFisher Scientific, Dardilly, France).

In addition, fecal samples collected on Days 4, 10 and 17 were inoculated, after thawing, onto the Chromagar Orientation™ agar medium (i2a, Montpellier, France) supplemented with colistin (8 mg/L) and vancomycin (8 mg/L) (CAO-CV) (Mourand et al., 2018). When *E. coli* colonies developed, two colonies per sample were stored and further analyzed. Their identity and the presence of the *mcr-I* gene were checked by PCR, their resistance to rifampicin was tested by culture on rifampicin-supplemented MH media (250 mg/L) and they were tested by agglutination with the F4 antiserum.

2.5. Statistical analysis

The Kruskal-Wallis non-parametric test was used to compare weight gains or titers. The distributions of pigs or isolates were analyzed using the Chi2-test or the Fisher exact test. Analyses of variance were performed to evaluate the impact of the treatments on the bacterial titers in fecal and PM samples.

3. Results and Discussion

3.1. SEM and Dynamic Light Scattering of alginate nanoparticles and colistin-loaded on alginate nanoparticles (colistin/Alg NPs)

Scanning electron microscopy (SEM) imaging revealed nanostructures of different shapes with an average size of 87 nm (Figure S1). Dynamic light scattering (DLS) permitted to determine the hydrodynamic size of the Alg NPs before and after colistin loading acquired at pH 6.5. The

prepared Alg NPs (500 $\mu\text{g/mL}$) displayed a hydrodynamic size of 111 nm . The difference observed in size of Alg NPs using the two techniques was ascribed to the presence a hydration shell in the liquid phase (DLS), as compared to SEM recorded in a dry state. After loading of colistin (60 $\mu\text{g/mL}$) on Alg NPs (500 $\mu\text{g/mL}$), the hydrodynamic size remained almost unchanged (112 nm) while the zeta potential value changed from negative (-32.0 mV) for the naked nanoparticles to slightly positive (+0.7 mV). With a pKa of ~ 10 , colistin behaves as a polycation under our experimental conditions. Therefore, loading of colistin on Alg NPs neutralizes the negative charge and turns the surface charge to slightly positive. The result clearly indicates the successful adsorption of colistin on the Alg NPs surface through electrostatic interactions. However, other types of interactions such as hydrogen bonding and dipole interactions, owing to the presence of many polar groups in colistin and Alg NPs cannot be discarded.

The amount of colistin loaded on Alg NPs was about 60 μg , as determined using HPLC analyses. Finally, the successful loading of colistin on the surface of Alg NPs was confirmed by Fourier transform infrared (FTIR) spectroscopy (Figure S2). The FTIR spectrum of Alg NPs comprises a broad band at around 3413 cm^{-1} ascribed to surface stretching vibrations of -OH and adsorbed water along with bands at 2921 cm^{-1} (CH_2 stretching), 1617 cm^{-1} (asymmetric stretching vibration of COO groups), 1414 cm^{-1} (symmetric stretching vibration of COO groups), and 1032 cm^{-1} (elongation of C-O groups). The FTIR spectrum of pure colistin is displayed in Figure S2 and consists of a broad stretching vibration band at $\sim 3387\text{ cm}^{-1}$ assigned to -OH and -NH stretching vibrations, and the characteristic amide I and II bands at respectively 1655 and 1533 cm^{-1} . The band at $\sim 1114\text{ cm}^{-1}$ is attributed to antisymmetric stretching of the sulphate counter ion. The results are in full agreement with the literature data (Freudenthal et al., 2016). After colistin loading on Alg NPs, the overlap between the stretching vibrations of both components makes it difficult to discriminate the peaks due to colistin (Figure S2).

However, the shift of the main peak of Alg NPs from 1617 to 1612 cm^{-1} suggests strong interactions between colistin and the nanoparticles' surface.

3.2. Inoculum titer, pathology and bodyweight gains

The aim of this experimental study was to evaluate the impact of colistin, either its classical formulation (free form) or loaded on Alg NPs, on the *E. coli* fecal population, and the efficacy on PWD. The experimental model was based on the results previously obtained with the 12-269M strain (Le Devendec et al., 2018). The inoculum titer was 1.68×10^8 CFU/mL, thus 1.68×10^9 CFU per pig instead of 7.68×10^7 CFU, in an attempt to obtain a more severe model. No clinical signs were observed in the NINT group. Diarrhea and signs of discomfort were detected in the other groups a few hours after inoculation, and were present on four out of eight different pigs in the INT group ($p=0.02$). The distributions of affected/non affected pigs in the two other groups (3/8 affected pigs in the ICC group and 0/8 affected pigs in the ICNP group) were not significantly different between them and in comparison with the NINT and INT groups ($p>0.05$). The numbers of individual diarrheic samples collected during the twelve days following inoculation varied from 0 out of 120 in the NINT, and 0 out of 96 in the ICNP group, to 3 out of 96 in the ICC group and 9 out of 96 in the INT group. Thus the numbers of diarrheic samples were significantly higher in the INT group compared to the NINT ($p=0.0005$), and ICNP groups ($p=0.003$).

The mean bodyweight gains for the different periods (Day -3 to Day 4, Day 11 or Day 18) are shown in Table S1. The mean body weight gains of the treated groups were lower, but there was no significant difference between groups.

Thus, as previously reported, severe diarrhea was observed during the week after inoculation. Similarly, Rhouma et al. (Rhouma et al., 2016) inoculated four-week-old piglets, previously selected on the presence of the F4 receptor gene, with a colistin-susceptible ETEC:F4 strain, and observed severe diarrhea scores, hypothermia and occasional deaths. The apparent higher

severity of their model may be linked to the lower age of the animals at inoculation, or to other experimental conditions. Concerning the clinical efficacy of the treatments, according to our results, pigs of the ICNP group had a lower number of diarrheic samples, the other criteria (number of affected pigs and weight gains) being not significantly different. In the experiment described by Rhouma et al. (Rhouma et al., 2016) on ETEC:F4 challenged piglets, administration of colistin, at the doses of 100 000 or 50 000 IU/kg, resulted in lower diarrhea scores in challenged treated pigs compared to challenged untreated ones, but only during the treatment period, and colistin administration did not affect the decreased body growth rate, resulting from the ETEC:F4 strain inoculation

3.3. Evolution of the titers of the dominant *E. coli* population and of the inoculated strain

Mean *E. coli* titers obtained on MC for fecal samples were globally stable, with a mean of 7.0 and ranging from 5.8 to 7.9 (Table 2). In comparison with the INT group, the titer of the ICC group was significantly lower on Days 2, 4 and 5 but higher on Day 10, the titer of the ICNP was significantly lower on Day 2 and higher on Day 17.

The total numbers of samples positive on MCRif without enrichment ranged from 62 out of 72 (INT group) to 66 out of 72 (ICNP group) ($p>0.05$), whereas after enrichment, positive samples were observed in 67 out of 72 (INT group) to 69 out of 72 (ICC group) ($p>0.05$) (Table 3). No statistically significant difference between the fecal sample titers of the groups on each sampling day was observed.

The colistin minimum inhibitory concentration (MIC) of 186 isolates obtained on MC and MCRif media from thawed fecal samples collected just at the end of the treatment period (Day 5) and at the end of the trial (Day 17), from INT, ICC and ICNP pigs were determined (Table 4). All isolates positive for the *mcr-I* gene had a MIC of 8 or 16 mg/L, as the inoculated strain, whereas those negative for the *mcr-I* gene were susceptible, with a MIC of 0.5-2 mg/L. Just at the end of the treatment, many isolates obtained on non-supplemented media from colistin-

treated pigs, either ICC or ICNP, were resistant to colistin, contrary to isolates from the INT group ($p < 0.001$ for each comparison). On the final sampling, all isolates obtained on non-supplemented media, thus belonging to the dominant *E. coli* population were *mcr*-negative. Most isolates obtained on MCRif contained the *mcr-I* gene and were resistant to colistin, except for the ICNP group on Day 17, but only five isolates were available.

The mean titers of PM samples obtained on MC are shown in Table S2. The lowest titers were observed for the jejunum. No significant difference between groups was observed between the titers of the different organs.

The titers obtained on MCRif and the numbers of positive PM samples are summarized in Table 5. Concerning jejunum samples, only two jejunum samples, one from the ICC and one from the ICNP groups were found positive on MCRif. The numbers of positive pigs did not differ significantly. There was no significant differences between the mean titers of the different organs of the groups.

These different results concerning the impact of the colistin treatments on the *E. coli* population can be compared to those of previous trials of our group or others. We previously studied the impact of a colistin treatment on the fecal *E. coli* population of non-inoculated pigs (Fleury et al., 2016). We noticed a significant but transitory decrease in the total *E. coli* population during treatment, but all the *E. coli* isolates remained susceptible to colistin. In the study of Viel et al. (Viel et al., 2018), the pigs were orally inoculated with a non-pathogenic *mcr-I*-positive *E. coli*, with a MIC of 8 mg/L. The colistin treatment given at the recommended dose (100 000 IU/kg/day for five days) led to a non-significant decrease of the *E. coli* population, which returned to its initial level within a few days after the last colistin administration. The *mcr-I*-positive *E. coli* population remained relatively constant, at a low level during and after the treatment, and no selection of this resistant population was observed. According to Rhouma et al. (Rhouma et al., 2016) on ETEC:F4 challenged piglets, administered or not colistin at the

doses of 100 000 or 50 000 IU/kg, both doses significantly reduced the fecal *E. coli* total population and also, contrary to our results, the excretion of the inoculated ETEC:F4 during the treatment period. In our conditions, using a limited number of animals placed in strict biosecurity conditions, and with individual administration of the medications, we observed a non-significant impact of colistin at the 100,000 IU /kg on the excretion of the 12-269M strain. This result is probably in relation with its colistin resistance (MIC of 16 mg/L), whereas the strain used by Rhouma et al. had a colistin MIC of <0.06 mg/L. We could also observe that the dominant *E. coli* population on Day 5 had a colistin wild-type phenotype in INT pigs, whereas a non-wild-type phenotype, with *mcr-1* positive results, was obtained for the dominant *E. coli* population excreted by ICC pigs, and to a lower extent by ICNP ones. Nevertheless, on Day 17, the dominant *E. coli* population was of the wild-type phenotype, and *mcr-1* negative in all groups, suggesting once again that the modifications of the *E. coli* populations caused by colistin are transitory.

To overcome colistin resistance, many authors have studied *in vitro* synergistic effect of combination of colistin with other antibiotics (MacNair et al., 2018; Yahav et al., 2012) or antimicrobials (Cannatelli et al., 2018; Naghmouchi et al., 2013, Madi-Moussa et al. 2022), but *in vivo* studies are still rare. In Europe, the marketing authorizations for veterinary oral products containing colistin in combination with other antimicrobial substances was withdrawn (EMA/CVMP/CHMP, 2016). Thus, other strategies to enhance the antimicrobial activity of colistin or other compounds are needed. An option is to optimize the delivery of the drug and increase its *in vivo* half-life by using nanoparticles. Thus, colistin-loaded lipid nanoparticles were found to be much more efficient for eradication of *Pseudomonas aeruginosa* biofilms compared to free colistin, although they had the same activity against planktonic bacteria (Sans-Serramitjana et al., 2016). Similarly, colistin nanoparticles prepared via coacervate complexation had equal or better *in vitro* antimicrobial activity compared to free colistin and could be

used in animal models of bacteremia or pneumoniae: the results revealed that they had lower liver toxicity and their antimicrobial efficacy was comparable to free colistin (Liu et al., 2018). Our results showed that the use of colistin/Alg NPs yielded globally similar clinical and biological results as the commercial colistin formulation. The two detected differences were a significantly lower number of diarrheic samples compared to INT pigs, which was not observed in the ICC group, and the reduction of the total *E. coli* population was observed only on Day 2, whereas it was observed on three sampling times in the ICC group. The efficacy of colistin/Alg NPs should now be studied in various experimental and field conditions, including infections caused by *E. coli* strains with a large range of antimicrobial susceptibility, as it could decrease the total amount of colistin that is used, in line with the national (EcoAntibio plans) and international recommendations (EMA/CVMP/CHMP, 2016). The decrease of the use of colistin in animals would lead to a decrease of the amount of colistin spread *via* manures in the environment, as colistin is not or only poorly absorbed in the intestinal tract (Rhouma et al., 2016).

3.4. Loss and transfer of colistin resistance

Our experiment was also an opportunity to evaluate the loss of colistin resistance between *E. coli* strains. Analysis of 196 fecal isolates obtained on MCRif without enrichment showed that 190 (97%) shared the characteristics of the inoculated strain 12-269M (presence of the *mcr-I* gene and positive reaction with the F4 antiserum). Four isolates, one from the INT group, one from the ICC group and two from the ICNP group) were F4-positive and *mcr-I*-negative. They belonged to the phylogenetic group A like the inoculated strain, and shared the same ERIC-PCR profile, one band excepted; the colistin MICs of these isolates were 0.25 or 0.5 mg/L. Two isolates were F4-negative, and *mcr-I*-negative; one of these shared the phylogenetic group and the ERIC-PCR profile of the inoculated strain, one band excepted, but the other one had another ERIC-PCR profile.

Among the 75 studied PM isolates obtained on MCRif without enrichment, 56 were *mcr-I*-positive and F4-positive, 17 were *mcr-I*-negative, and F4-positive and 2 were *mcr-I*-negative and F4-negative. Thus the ratio of *mcr*-negative isolates was higher for PM samples (19 out of 75) compared to fecal ones (4 out of 196, $p < 0.001$). These 19 *mcr-I*-negative isolates were obtained from pigs from the three inoculated groups; they had a colistin MIC of 0.25 or 0.5 mg/L and shared an ERIC profile similar, one band excepted, to the one of the inoculated strain. The detection of isolates rifampicin-resistant, *mcr-I* negative, F4-positive, with an ERIC-PCR profile similar to *E. coli* 12-269M, and with a colistin MIC of 0.25-0.5 mg/L suggested that the loss of the *mcr-I* gene from the strain 12-269M occurred on several occasions, as it was observed for isolates from the three inoculated groups. In comparison with the MIC determined for the 12-269M strain, the *mcr-I*-negative isolates had decreased MICs, which are comparable to those of wild-type *E. coli* strains (<https://mic.eucast.org/search>), suggesting that the colistin resistance in *E. coli* 12-269M was associated only with the *mcr-I* gene and not with the mutations in the PhoP and PhoQ genes. This resistance gene loss had already been observed during our previous experiments (Le Devendec et al., 2018), and we did not further analyse the *mcr-I*-negative isolates. The ratio of isolates missing the *mcr-I* gene was higher for PM samples compared to fecal samples; this difference may be due to a later sampling time for PM samples, or to the differences between fecal and PM samples which were collected at different levels of the digestive tract, and contained both digesta and scrapped mucosa. Further trials with sacrifice of animals at different times and separate analysis of feces, digesta and mucosa could help us to understand this phenomenon. As up to a quarter of PM 12-269M isolates had lost the *mcr-I* gene after three weeks, it would be interesting to evaluate whether this ratio would further increase with an extended observation period. This would indicate that, *in vivo*, for this studied strain, the *mcr-I* resistance, or the plasmid bearing this gene, is associated with a substantial biological cost (Choi et al., 2020).

All fecal samples collected on Day 4, except one sample from the ICC group, gave *E. coli* colonies on Chromagar Orientation™ agar medium; supplemented with colistin and vancomycin (CAO-CV), presenting the characteristics of the inoculated strain (presence of *mcr-I*, F4 agglutination and resistance to rifampicin). This result confirms our previous *in vitro* and *in vivo* observations of the non-transferability of the *mcr-I* gene of *E. coli* 12-269M (Le Devendec et al., 2018). None of the samples collected on Days 10 and 17 enabled the detection of the inoculated strain, nor of *mcr-I*-positive *E. coli*.

4. Conclusion

In conclusion, our study revealed that, under our experimental conditions, colistin/Alg NPs had a slightly better therapeutic effect than free colistin, on the diarrhea induced by the colistin-resistant ETEC 12-269M. Both treatments led to a transitory decrease of the total *E. coli* fecal population, with a majority of resistant *E. coli* isolates during treatment, but the dominant *E. coli* population was found susceptible at the end of the trial. The loss of the *mcr-I* gene of the inoculated strain was detected more often in PM samples, and may indicate an *in vivo* biological cost of this resistance for this strain. Further studies are needed to evaluate, in experimental or field conditions, the therapeutic efficacy of colistin/Alg NPs against infections caused by various *E. coli* strains, as such an approach could lead to a decrease of colistin use.

Supporting information

Figure S1: Characterization of alginate nanoparticles by scanning electron microscopy (SEM)

Figure S2: FTIR spectra of (a) alginate nanoparticles, (b) colistin, (c) alginate nanoparticles loaded with colistin (colistin/Alg NPs).

Table S1: Growth: mean bodyweight gains (kg) in the different groups

Table S2. Titers obtained on MacConkey media from the post-mortem samples

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Experimental design

Group (n)	Room	<i>E. coli</i>	12-	Treatment	F4 status
		269M		Day 0-Day 4	
		inoculation on			
		Day 0			
NINT (10*)	C2	No		No	7 resistant, 3 susceptible up to Day 14, then 5 resistant and 3 susceptible
INT (8)	C3	Yes		No	8 susceptible
ICC (8)	B1	Yes		Colistin (Colivet 100 000 IU/ kg)	8 susceptible
ICNP (8)	B2	Yes		Colistin-loaded Alg NP (40,415 IU/kg)	8 susceptible

*The NINT group contained ten piglets up to Day 14, then two piglets were removed from this group, to be included into another assay. NINT: non-infected, non-treated group; INT: infected, non-treated group; ICC: infected, treated with commercial colistin; ICNP: infected, treated with Colistin-loaded Alg NP

Table 2. Mean *E. coli* titers obtained on MacConkey media

Days										
Group (n)	D-3	D1	D2	D3	D4	D5	D7	D10	D14	D17
NINT (10)*	6.43±0.61 ^a	6.77±0.77 ^b	6.79±0.76 ^{ab}	6.79±0.88 ^a	6.66±0.66 ^{ab}	6.83±0.46 ^a	6.38±0.42 ^{bc}	6.37±0.59 ^b	6.45±0.33 ^b	6.48±0.38 ^b
INT (8)	7.25±0.66 ^a	7.94±1.06 ^a	7.67±0.90 ^a	7.66±1.13 ^a	7.75±1.24 ^a	7.06±0.82 ^a	6.71±0.52 ^{abc}	6.90±0.39 ^b	6.94±0.57 ^{ab}	6.57±0.28 ^b
ICC (8)	6.99±0.73 ^a	7.41±0.45 ^{ab}	6.06±1.09 ^b	6.56±0.89 ^a	6.11±1.40 ^b	5.77±1.07 ^b	6.20±0.58 ^c	7.73±0.55 ^a	7.03±0.51 ^{ab}	6.99±0.41 ^{ab}
ICNP (8)	6.44±0.35 ^a	7.36±0.49 ^{ab}	6.45±0.85 ^b	7.20±0.75 ^a	7.40±0.66 ^{ab}	6.43±0.62 ^{ab}	6.63±0.51 ^{abc}	6.66±0.26 ^b	7.60±0.91 ^a	7.40.37± ^a

*The NINT group contained ten piglets up to Day 14, and then two piglets were removed from this group, to be included into another assay For each day, titers sharing a common superscript are not significantly different. NINT: non-infected, non-treated group; INT: infected, non-treated group; ICC: infected, treated with commercial colistin; ICNP: infected, treated with colistin-loaded alginate nanoparticles

Table 3. Culture on MCRif: numbers of positive samples and mean *E. coli* titers

		Days									
Group		D1	D2	D3	D4	D5	D7	D10	D14	D17	Total
INT	PS-WO-E ^a	7	8	8	8	8	8	5	5	5	62 ^d
	PS-W-E ^b	7	8	8	8	8	8	7	6	7	67 ^e
	Mean ^c	4.74±2.51	5.56±1.87	6.62±2.00	6.90±1.84	5.40±1.67	3.04±0.84	1.99±1.25	1.68±1.19	1.91±1.12	
ICC	PS-WO-E	6	8	8	8	8	8	7	6	6	65
	PS-W-E	6	8	8	8	8	8	8	8	7	69
	Mean	3.89±2.51	4.86±1.32	5.76±1.38	6.00±1.42	5.56±1.33	3.62±0.93	3.21±1.17	2.30±0.94	1.85±0.90	
ICNP	PS-WO-E	8	8	8	8	8	8	6	7	5	66
	PS-W-E	8	8	8	8	8	8	7	8	5	68
	Mean	5.94±1.25	5.52±1.35	6.61±1.57	7.18±1.02	5.88±0.98	4.02±0.63	2.48±1.36	2.90±1.17	1.74±1.50	

^aNumber of samples positive without enrichment out of eight analyzed samples; ^bNumber of samples positive with or without enrichment out of eight analyzed samples; ^cMean titer on MCRif; ^dTotal number of positive samples without enrichment out of 72 tested samples; ^eTotal number of positive samples with or without enrichment out of 72 tested samples; NINT: non-infected, non-treated group; INT: infected, non-treated group; ICC: infected, treated with commercial colistin; ICNP: infected, treated with colistin-loaded alginate nanoparticles; MCRif: MacConkey media

supplemented with rifampicin. All fecal samples from the NINT group were negative on MCRif, as well as those obtained on Day -3 from the other groups. For each day, mean titers are not significantly different.

Table 4. Colistin susceptibility of *E. coli* isolates obtained on MC and MCRif media

Day	Group	Isolates obtained on MC		Isolates obtained on MCRif	
		<i>mcr-I</i> -negative and MIC of 0.5- 2 mg/L	<i>mcr-I</i> -positive and MIC of 8- 16 mg/L	<i>mcr-I</i> -negative and MIC of 0.5- 2 mg/L	<i>mcr-I</i> -positive and MIC of 8- 16 mg/L
5	INT	21	3	1	7
	ICC	2	22	0	8
	ICNP	8	16	0	8
17	INT	24	0	0	7
	ICC	24	0	0	6
	ICNP	24	0	3	2

NINT: non-infected, non-treated group; INT: infected, non-treated group; ICC: infected, treated with commercial colistin; ICNP: infected, treated with colistin-loaded alginate nanoparticles; MC: MacConkey media; MCRif: MC supplemented with rifampicin.

Table 5. Titers and numbers of positive results obtained on MCRif media from post-mortem samples

Groups	Jejunum	Ileon	Caecum	Colon	Rectum	Total of positive organs ^c	Total of positive pigs ^d
INT	0 ± 0.00^a	0.75 ± 0.71	2.32 ± 0.67	2.58 ± 0.47	2.36 ± 0.72		
	0 ^b	5	8	8	8	29	8
ICC	0.25 ± 0.71	1.76 ± 1.00	2.13 ± 1.11	1.96 ± 1.31	1.45 ± 1.01		
	1	7	7	6	7	28	8
ICNP	0.125 ± 0.35	1.69 ± 1.42	1.81 ± 1.31	1.34 ± 1.37	1.24 ± 1.38		
	1	6	6	5	4	22	6

^aMean of eight samples for each group and each organ; ^bNumber of positive samples out of eight analyzed samples; ^cTotal of positive samples out of 40 analyzed samples for each group; ^dTotal of pigs positive for at least one organ, out of eight analyzed pigs. NINT: non-infected, non-treated group; INT: infected, non-treated group; ICC: infected, treated with commercial colistin; ICNP: infected, treated with colistin-loaded alginate nanoparticles; MCRif: MacConkey media supplemented with rifampicin; all the samples from the NINT group were negative on MCRif