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Bio-activities of Tetracycline and Algae Food Supplement Algo-Bio® on *Escherichia coli* Antimicrobial Resistance Isolated from Piglet's Intestinal Flora

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AD and NKG designed the study. Author ANTK performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ANTK, FKN, EK and IKK managed the analyses of the study. Authors BMO and BT managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed at evaluating the effect of Algo-Bio® use on *Escherichia coli* resistance strains isolated from piglets intestinal flora.
Study Design: Bacteriological study.
Place and Duration of Study: Laboratory of the National Reference Center for antibiotics at Institute Pasteur Côte d'Ivoire, between March 2018 and June 2018.

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Methodology: A breeding of three batches of two piglets was carried out, then treatments with tetracycline and Algo-Bio® were administered to them. Enterobacteria was isolated on Mac Conkey medium added up with tetracycline and resistance rates were determined. *Escherichia coli* resistant strains have been identified and antibiotic susceptibility test was performed using disk diffusion method on Müller-Hinton agar.
Results: Enterobacteria resistance rates increased ranging from 18.4% (D0) to 81.5% (D4) to tetracycline-treated piglets and respectively from 25.7% (D0) to 29% (D4) and from 22.3% (D0) to 24.5% (D4), in control piglets and those treated with Algo-Bio®. Antibiotic susceptibility test of *Escherichia coli* strains isolated from piglets treated with tetracycline showed high resistance to ceftazidime (83.3%), amoxicillin (76.9%) and tetracycline (92.3%) with 39.4% strains producing ESBL, 23.7% producing PHL and 5.2% of producing CHP on D4. *Escherichia coli* strains isolated from control piglets and Algo-Bio® treated piglets revealed a decrease of ESBL respectively from 17.6% (D0) to 13.7% (D4) and from 12.5% (D0) to 6.4% (D4).
Conclusion: The study showed that the use of Algo-Bio® does not induce an evolution of antimicrobial resistance in Microbiota strains and consequently this dietary supplement can be used as a good alternative to antibiotics.

Keywords: Antibioresistance; Algo-Bio®; piglets; Escherichia coli; tetracycline; phenotype.

1. INTRODUCTION

Antibiotics are chemicals originally produced by microorganisms. They are used in human and veterinary medicine where various treatment modalities may be encountered, depending on infectious stages of animals [1,2]. There are preventive treatments, metaphylactic and curative. Preventive treatment is part of risk practices in breeding that should be abandoned, given an unfavorable benefit-risk ratio. This zootechnical use has been banned in European Union since 2006 [3]. Indeed, antibiotics are used in intensive breeding as additives to improve animal's growth and performance, creating environments in which selection pressure will favor survival of resistant bacteria in microbiota, and spreading the most commonly used antibiotic resistance genes. Animal's digestive system is a real bioreactor in which multiple gene exchanges can occur, sometimes leading emergence to new multi-resistant pathogens [4,5]. Resistant bacteria of animal origin (pathogenic or not) can be directly transmitted to humans by food, leading food borne infection or resistance genes propagation to commensal and infectious bacteria of human origin in non-pathogenic bacteria case [6]. Considering actual and potential consequences of antibiotics in veterinary medicine for public health, new alternatives must be found in order to limit antibiotics use in breeding. Algae based dietary supplements use could be a promising natural alternative in livestock farming. Algae are marine plants distributed through the world [7,8]. They are used in various fields of activities such as food, cosmetics, textiles and are an important source of polysaccharides [9,10]. Their antibiotic and anti-inflammatory properties give them an appreciated value in medicine. In addition, algae stimulate animal's immune system and became an important source of natural bioactive compounds [11,12,13]. The objective of this work was to determine the effect of Algo-Bio®, an algae-based food supplement use on enterobacteriaceae resistance in piglets' intestinal microflora and on *Escherichia coli* sensitivity to antibiotics.

2. MATERIALS AND METHODS

2.1 Piglets Treatment

A breeding operation of three batches of two recently weaned piglets of mixed race who had never received antibiotic treatment before this study was carried out on a farm in the locality of Adiopodoumé in Côte d'Ivoire. The piglets were weighed to determine their live weight for the administration of treatments per day.

Piglets in batch 1 were used as controls while those in batches 2 and 3 were administered respectively with tetracycline in drinking water at a dose of 1 g in 5 l of water and with an algae-based food supplement (Algo-Bio®) in drinking water at a dose of 1 ml per litre of water per 10 kg by weight.

2.2 Sampling

Fresh faeces from all piglets batches were collected every morning for 5 days: one day before the start of the treatments and four days of treatment corresponding to days D0, D1, D2,

D3, D4, in well labelled sterile stools pots. These stools were kept cold in a cooler containing for transport to laboratory. Isolation, identification and antibiotic susceptibility testing were carried out at the laboratory of National Antibiotic Reference Centre of Institut Pasteur on Adiopodoumé site (Côte d'Ivoire).

2.3 Isolation and Identification of Resistant Enterobacteria

5 g of faeces of each piglets were homogenized in 45 mL of peptone water, and 1/10 serial dilutions were made from stock solution. Then, 100 µL of these dilutions were seeded on Mac conkey gel medium. For each sample, two Petri dishes were seeded on Mac Conkey (MC) medium previously immersed with 9 µg/ml of tetracycline (MC+TET) according to CASFM standard. In addition, enterobacteria that proliferated on that Mac Conkey medium were qualified as resistant. Bacteria on different media MC, MC+TET have allowed to calculate the percentage of resistant enterobacteria according to the formula below:

$$R\,(\%) = \frac{n_{MC+TET}}{n_{MC}} X100$$

R (%): percentage of resistant enterobacteria

n_{MC+TET}: Number of colonies counted on Mac Conkey + Tetracycline medium

n_{MC} : Number of colonies counted on Mac Conkey medium

For each sample, suspect colonies of resistant Escherichia coli from Mac Conkey media with tetracycline added (small pink to red-pink colonies with or without halo) were collected and streaked on a Rapid *E. coli* 2 (REC2) agar. The Petri dishes were incubated at 37°C for 24 hours and then the characteristic colonies of *Escherichia coli*, from violet to pink, were selected for confirmation with tests to identify morphological and biochemical characteristics.

2.4 Antibiotic Susceptibility Testing of Escherichia Coli Strains from Piglets

Antibiotic susceptibility tests were performed on *Escherichia coli* strains isolated according to the type of treatment (control, tetracycline treated piglets and Algo-Bio® treated piglets), using Müller-Hinton agar diffusion method according to the recommendations of the Antibiotic Committee of French Society of Microbiology (CA-SFM veterinary, 2017). Then, the diameters of the inhibition zones around the antibiotic discs were read using a caliper. These values have been interpreted as sensitive (S), intermediate (I) and resistant (R), in comparison with the critical concentration tables for the interpretation of inhibition zone diameters for Enterobacteriaceae. Seventeen (17) antibiotic disks were used for this study: amoxicillin (20 µg); amoxicillin/clavulanic acid (10-20 µg); piperacillin (30 µg); ticarcillin (75 µg); cefepime (30 µg); cefotaxime (5 µg); ceftazidime (10 µg); cefoxitin (30 µg); imipenem (10 µg); aztreonam (30 µg); amikacin (30 µg); gentamicin (10 µg); netilmicin (10 µg); tetracycline (30 µg); chloramphenicol (30 µg); fosfomycin (20 µg); trimethoprim-sulfamethoxazole (1.25-23.75 µg). The extended spectrum beta-lactamase production was confirmed by a standard double-disk synergy test illustrating by a specific image of synergy between antibiotic disks.

2.5 Statistical Analysis

Statistical analysis of data was carried out using XLSTAT software 2017 version. A variance analysis was performed and the significance of the differences between averages from resistances was determined with an error risk of $\alpha < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Enterobacteria Resistance Rates to Tetracycline in Control and Treated Piglets

Tetracycline resistance rates of enterobacteria in control, tetracycline treated piglets and Algo-Bio® treated piglets are shown in Fig. 1. Resistance rates increased rapidly and significantly in tetracycline treated piglets, ranging from 18.4% to 52.1%, 75.2%, 91.7% and 81.5%, respectively, on days D0, D1, D2, D3 and D4. However, resistance rates in control piglets and those treated with Algo-Bio® increased slowly ranging from 22.3% the day before treatment to 24.5% at the end of treatment in piglets treated with Algo -Bio® and from 25.7% to 29% in control piglets. A significant variation of resistance levels in tetracycline treated piglets during days of treatment was observed.

The evolution of enterobacteria during oral treatment with tetracycline showed an increase in resistance rates. Indeed enterobacteria resistance increases ranging from 18.4% on day

before treatment (D0) to 81.5% on the last day of treatment (D4). This is because the use of antibiotics in livestock farming is a factor in the emergence of enterobacteria resistance. These results are similar to those of Sanders and al. [14] and Bibbal [15]. According to these authors, numerous studies have shown that any antibiotic treatment creates a selection pressure favorable to the development of resistant bacteria. This selection pressure is mainly exerted on the bacteria present in the digestive tract, which are very numerous in the terminal part of the intestine. The acquisition of resistance by a bacterium is done either by mutation of the bacterial genome or by acquisition of resistance genes from already resistant strains according to Salyers and al. [16], Muylaert and Mainil [17] and Fabio and al. [18]. The resistant bacteria created by this selection pressure are likely to transmit their resistance genes to other bacteria in the microbiota, leading to an increase in resistance rates observed during treatment. In addition, these resistance genes could be disseminated into the environment and contaminate both the piglet's immediate environment and humans. It should be noted that enterobacteria resistance rates in control piglets increases little ranging from 25.7% to 29% on days D0 and D4 respectively. The slow evolution of these resistance rates is due to the fact that these piglets have not been exposed to antibiotics. Otherwise, Higher is the level of antibiotic use, greater is the proportion of bacteria resistant in piglet's faecal flora according to Sunde and al. [19]. In addition, resistance rates of isolated enterobacteria in piglets treated with the Algo-Bio® feed supplement are close to those observed in control piglets. Indeed, these two batches of piglets presented the best results in terms of resistance during this study. Algo-Bio® is essentially composed of marine algae rich in sulphated polysaccharides with antibacterial and immunomodulatory properties [20,21,22], would exert a bactericidal action on some resistant bacteria. In addition, this food supplement would stimulate immune defenses in piglets. Similar results have been reported in the work done by Chbani and al. [23] and Berri and al. [24]. According to these authors, green algae extract would have capacity to stimulate in vitro cytokines expression involved in lymphocytes activation, recruitment and migration as well as dendritic cells to modulate immune response. In addition, the stimulation of host immune response mediators could be evaluated by these authors using an in vitro system of porcine intestinal epithelial cells. Similar results were observed by Kouadio and al., [25], on slow evolution of resistance rates in untreated piglets and in those treated with Algo-Bio®.

3.2 Antibiotic Susceptibility Test

Fig. 2 and Fig. 3 display the antibiotics susceptibility test results of *Escherichia coli* strains isolated in control, tetracycline treated piglets and Algo-Bio® treated piglets. This results show that no resistance strain has been observed for imipenem in the different piglets batches. *Escherichia coli* strains isolated from

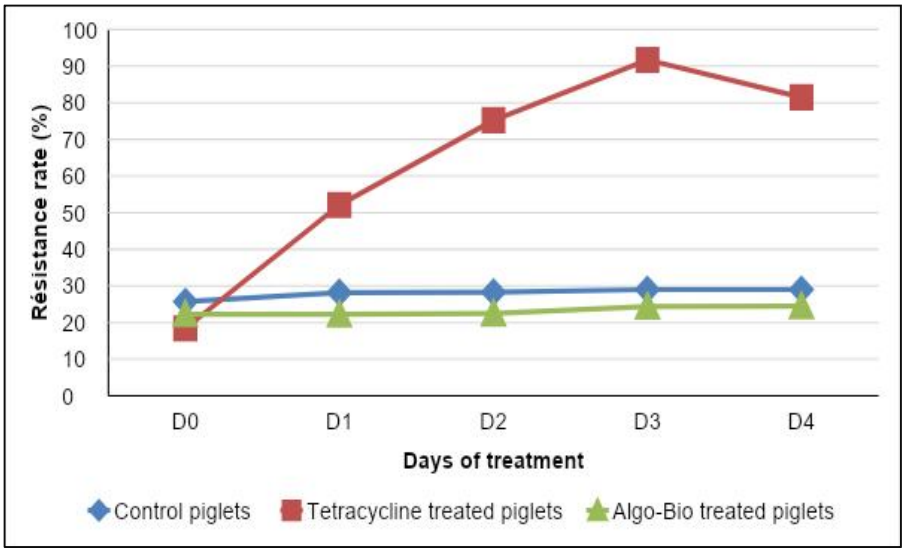

Fig. 1. Tetracycline bacteria resistance rates in piglets

tetracycline treated piglets showed high levels of resistance to beta-lactam antibiotics with 83.3% for ceftazidime, 76.9% for amoxicillin and 61.5% for amoxicillin / clavulanic acid, compared to the other 2 batches in which the strains are relatively sensitive. Among other antibiotics, *Escherichia coli* strains showed high resistance for tetracycline (92.3%) and fosfomycin (66.7%) with tetracycline treated piglets. The best activities were observed in control piglets and those treated with Algo-Bio®.

Antibiotic resistance rates of *Escherichia coli* strains isolated from the 3 piglet batches, namely untreated piglets, tetracycline treated piglets and Algo-Bio® treated piglets, indicate that no resistant strains have been detected for imipenem. In control piglets and those treated with Algo-Bio®, the antibiotic susceptibility test revealed, in addition to the sensitivity of *Escherichia coli* to imipenem, a sensitivity to amikacin and netilmicin with low chloramphenicol resistance.It should be noticed that isolated strains from control piglets and Algo-Bio® treated piglets showed less resistance compared to strains isolated from piglets treated with tetracycline. In addition, the antibiotics used showed the best activity on strains isolated from piglets treated with Algo-Bio®. This would be explained on the one hand by the fact that piglets treated with Algo-Bio® were not exposed to antibiotics in this study and therefore had fewer resistant strains and on the other hand, by the fact that the algae-based feed supplement would have an activity on bacterial resistance. These results are similar to those of Berri and al. [24]. According to these authors, the use of algae in animal nutrition is a promising alternative to overcome antibiotic resistance. Piglets treated with tetracycline showed more resistance to antibiotics tested with high resistance rates to tetracycline (92.3%), ceftazidime (83.3%) and amoxicillin (76.9%). Indeed, the prevalence of antibiotic resistance in Escherichia coli strains is increased by tetracycline treatments in these piglets. Bacterial resistance to tetracycline has co-selected resistance to other classes of antibiotics. These results are similar to those of Courvalin [26] and those of Giguère and al. [27]. According to these authors, a bacterial strain may involve the same plasmid in genes that are resistant to different families of antibiotics. The use of an antibiotic co-selects resistance to other antibiotic families, gradually enriching the bacterial population with multi-resistant strains. In addition, whatever antibiotic is used, it contributes overall to the selection of resistant strains [14,28].

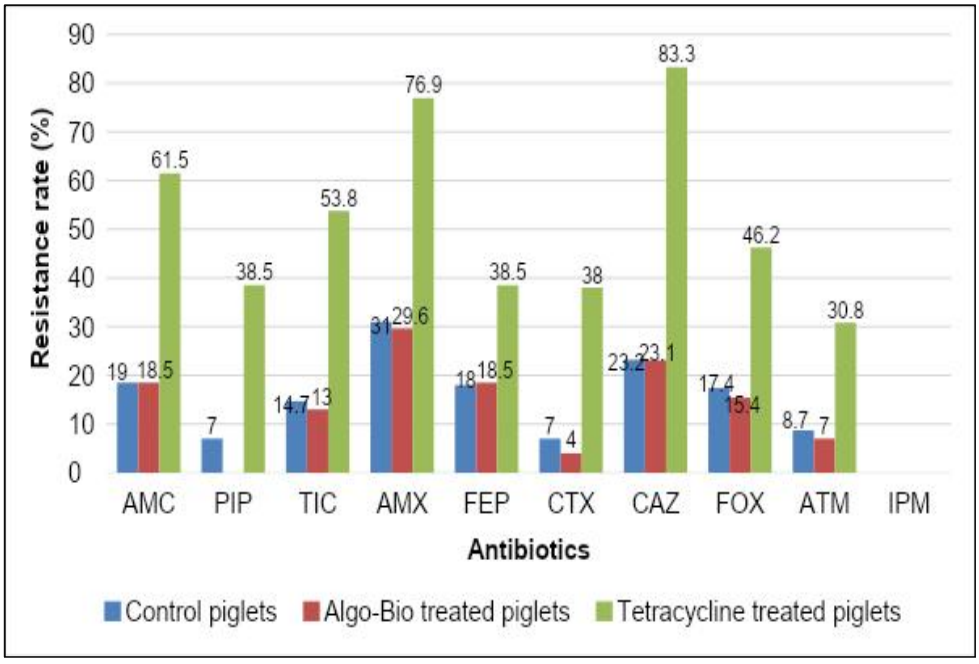

Fig. 2. Antibiotics susceptibility test of *Escherichia coli* strains to betalactam

Amoxicillin / clavulanic acid (AMC); Piperacillin (PIP); Amoxicillin (AMX) Cefepime (FEP); Cefotaxime (CTX); Ceftazidime (CAZ); Cefoxitin (FOX); Imipenem (IPM); Aztreonam (ATM)

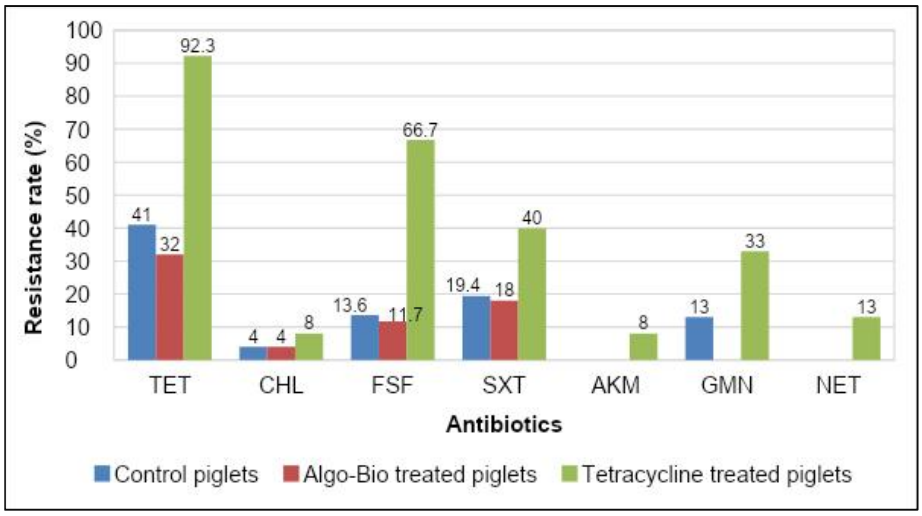

Fig. 3. Antibiotics susceptibility test of *Escherichia coli* strains to other antibiotics
Tetracycline (TET); Chloramphenicol (CHL); Fosfomycin (FSF); Trimethoprim-sulfamethoxazole (SXT); Amikacin (AKM); Gentamicin (GMN); Netilmicin (NET)

3.3 Antibiotic Resistance Phenotypes of *Escherichia coli* Strains Isolated in Piglets

The different phenotypes rates observed at the end of treatment are shown in Table I below. Generally, higher proportions of ESBL, PHL and CHP are observed in piglets treated with tetracycline at the end of treatment compared to those observed in control and Algo-bio® treated piglets. ESBL rates decreased ranging from 17.6% (D0) to 13.7% (D4) in control piglets and from 12.5% (D0) to 6.4% (D4) in Algo-bio® treated piglets. Higher ESBL, PHL and CHP levels are observed in piglets treated with tetracycline at D4 compared to other batches of piglets. *Escherichia coli* strains producing extended-spectrum beta-lactamase (ESBL), penicillinase low-level (PLL), penicillinase high-level (PHL) were observed in the different piglet batches. However, ESBL and PHL levels were increased by the use of tetracycline compared to control piglets and those treated with Algo-Bio® in which there was a decrease in ESBL on day D4. In addition, only piglets treated with tetracycline showed cephalosporinases hyper-production (CHP) at the end of treatment. This could be explained by the phenomenon of co-resistance in bacteria exposed to antibiotics. ESBL-producing bacteria constitute a major concern in hospitals due to their epidemic spread and multi-resistance to antibiotics. Indeed, ESBL are found in a large proportion of gram-negative bacilli, but enterobacteria represent the most incriminated germs according to Gniadkowski [29] and sader et al. [30]. The presence of *Escherichia coli* producing ESBL in weaned piglets who have never received antibiotic treatment before the study could be explained by contamination of piglets by ESBL-producing bacteria under their mother. Indeed, the selection pressure induced by high consumption of broad-spectrum antibiotics is a risk factor for the emergence of multi-resistant bacteria that produce ESBL [31], which confer resistance to penicillin and cephalosporin. The decrease in ESBL bacteria prevalence in piglets treated with Algo-Bio® is explained by the bactericidal action of green algae contained in the feed supplement [24].

Table 1. Proportion of *Escherichia coli* resistance phenotypes in piglets

	Control		Algo-Bio®		Tetracycline	
Phenotypes	**D0**	**D4**	**D0**	**D4**	**D0**	**D4**
ESBL	17,6	13,7	12,5	6,4	13,3	39 ,4
PLL	79,4	82,3	84,3	90,3	83,3	36,8
PHL	2,9	3,9	3,1	3,2	3,3	23,7
CHP	0	0	0	0	0	5,2

ESBL: Extended-Spectrum Beta-Lactamase; PLL: Penicillinase Low Level; PHL: Penicillinase High Level; CHP: Cephalosporinase Hyper-Production

4. CONCLUSION

The algae-based dietary supplement has an impact on increasing antibiotic resistance of enterobacteria isolated from piglet stools. While resistant enterobacteria isolated from tetracycline-treated piglets increase rapidly during tetracycline treatment, those from control and Algo-Bio® treated piglets did not increase significantly. In addition, *Escherichia coli* isolated from these piglets showed high sensitivity to antibiotics tested during the antibiotic susceptibility test and a decrease in ESBL levels compared to piglets exposed to tetracycline. Better control of antibiotic consumption by substituting them with the Algo-Bio® feed supplement in pig farming could be a factor that could help to better control the risks of antibiotic resistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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