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Research Article

Isolation, physiological and biochemical identification of keratinolytic Actinomyces spp. strains capable of inhibiting some pathogenic bacteria encountered in livestock feed

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Abstract

The poultry industry produces an average of 9 million tonnes of keratinous waste per year. This waste is rich in essential amino acids and is an important ingredient in the manufacture of more balanced animal feeds. However, access to keratin amino acids that are difficult to hydrolyse by conventional proteases such as papain, pepsin and trypsin means that we have to use physico-chemical methods that consume a lot of energy and, what's more, destroy these essential amino acids. Furthermore, livestock feed contains pathogenic micro-organisms whose inhibition by the abusive use of synthetic antibiotics is leading to the emergence of antibiotic resistance. The aim of this study was to isolate keratinolytic *Actinomyces* sp. capable of inhibiting the growth of pathogenic bacteria commonly found in animal feed. Soil samples from keratin waste dumps were used as a matrix for the isolation of Actinomyces sp. These were then inoculated onto solid and liquid media enriched with keratin in order to screen and compare their keratinolytic activity. Finally, the antagonism of the best keratolytic isolates to *Escherichia coli*, *Salmonella enteritidis* and *Staphylococcus aureus* was assessed. The study shows that 19 of the 22 actinobacteria isolated produced keratinase hydrolysis diameters ranging from 9.5±3.5mm to 15.5±0.7mm. Seven isolates were able to inhibit the growth of the three pathogenic strains and isolate KER-2 was the most active isolate with inhibition diameters ranging from 15.8±4.6mm to 22.5±0.7mm. This work shows the potential of soil-isolated bacteria to convert bird feather waste into a potential pathogen-free animal feed.

1. Introduction

The poultry industry has expanded rapidly worldwide from 1961, where it was estimated at 9 million tonnes, to 2020 (estimated at 133 million tonnes). Although it falls below 100 tonnes after the COVID-19 crisis, it is clearly on the rise (102 million tonnes) and could reach 143.3 million tonnes in 2030 [1]. Cameroon, with 84 thousand tonnes in 2019, is not to be outdone. One of the main types of waste from this sector of the food industry is feathers, the global production of which has been estimated at 9 million tonnes/year [2].

Poultry feathers contain keratin, a proteinaceous substance that contains a wide variety of amino acids, dominated by essential amino acids such as cysteine, lysine, proline and serine, making poultry feathers an important ingredient in the manufacture of more

balanced animal feeds [3-5]. The amino acids in keratin are linked together by hydrogen and ionic bonds, hydrophobic interactions and disulphide bridges, making it difficult to be hydrolyse by conventional proteases such as papain, pepsin and trypsin [6]. The aforementioned point is the outcome by the use of physical and chemical methods which unfortunately are energy-intensive and destroy some of the essential amino acids needed to enrich animal feed [7].

Furthermore, livestock feed often contains pathogenic micro-organisms such as *Salmonella enteritidis* and *Escherichia coli*, which can cause neonatal diarrhea in cattle calves, piglets and foals. In addition to these diarrheas, other diseases such as mastitis are caused by *Staphylococcus aureus* and *Escherichia coli* [8]. To combat these diseases, livestock farmers add antibiotics to their feed. These antibiotics not only inhibit pathogens and prevent disease, but also promote livestock growth. According to the WHO, 80% of antibiotics used in human medicine are consumed in the animal sector. This overuse of synthetic antibiotics, which are also highly toxic, is leading to the emergence of antibiotic resistance, which has led to a major concentration of efforts in the search for new natural bioactive molecules, the most widely used of which are isolated from microorganisms, including actinobacteria [9]. Actinobacteria are known to produce a wide range of bioactive compounds [10], hydrolysing bird feathers and inhibiting the growth of several pathogenic bacteria found in animal feed. With the aim of using feather waste in animal feed as a source of protein and essential amino acids, while eliminating the pathogens present in this feed, the objective of this work was to isolate, identify biochemically and physiologically keratinolytic Actinobacteria from poultry dumps capable of inhibiting the growth of several pathogenic bacteria.

2. Materials and methods

2.1 Isolation of Actinomycetes

Actinomycetes were isolated using the method described by [11], with slight modifications. To this end, 100 g of a mixture of soil samples taken from different dumps consisting of poultry feathers, horns and ox hooves from the city of Yaoundé-Cameroon (latitude 3°50'29.04'' North and longitude 11°29'31.56'' East) were collected and forwarded to the laboratory of Microbiology (Department of Microbiology, Faculty of Science, University of Yaoundé I), for isolation. Once at the laboratory, the samples were first mixed with $CaCO_3$ in the proportions 1:10 (m/m) and then dried in a dark place for two days until a water content of less than 15% was obtained. Once dried, the mixture was ground using a mortar and then sieved through a sieve with a mesh size of 0.1µm in diameter. One gram of the sieved sample was mixed with 9 ml of sterile physiological water (0.9% NaCl) to form the stock solution. A series of decimal dilutions ranging from 10^{-1} to 10^{-4} was then made from the stock solution. 100µl of each dilution was inoculated onto the surface of Kuster-starch agar (potato starch 5g; casein 0.3g, KNO_3 2g, $K2HPO_4$ 2g; NaCl 2g; trace elements 1ml; agar 15g; Vf 1000ml) previously poured onto Petri dishes. The plates were incubated at room temperature (28 ± 2°C) for 7 days.

The various isolates belonging to Actinomyces spp. recognizable by their macroscopic (embedded in the agar, pigmentation, having a felting form), microscopic (filamentous, Gram+) and biochemical (catalase +) characteristics were purified after three to four replicates in Kuster-starch agar. The spores of the pure isolates obtained were stored at 4°C after harvesting in a solution consisting of 0.01% (v/v) tween-80 according to the method proposed by [12].

2.2 Screening of keratinolytic isolates and evaluation of keratinolytic activity

The screening of keratinolytic isolates was carried out according to the method described by [13] with slight modification. Briefly, four agar cylinders containing isolates previously preserved and then revived by culture on Kuster-starch medium at room temperature for 7 days, were added to 30 ml of a sterile mineral medium (Casein 0.3g, KNO_3 2g, $K2HPO_4$ 2g; NaCl 2g; oligo-elements 1ml; Agar 15g; Vf 1000ml) containing two poultry feathers 3cm long and 0.3g mass each. The resulting mixture was shaken at 110 rpm for 7 days using a Biobase China Sk-3432 electric shaker. The keratinolytic activity of the different isolates capable of degrading poultry feathers was assessed by depositing Kuster-keratin agar in the centre (Keratin 20g; casein 0.3g, KNO_3 2g, $K2HPO_4$ 2g; NaCl 2g; oligo-element 1ml Agar 15g; Vf

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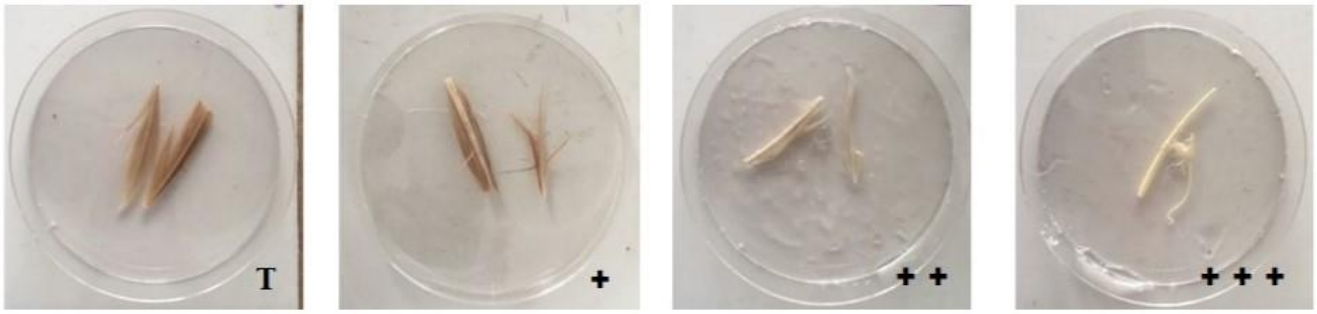

Figure 1. Feather degradation levels after 7 days of culture: T= control (no degradation); (+) = primary; (++) = secondary; (+++) = tertiary.

1000ml), one cylinder of agar from a 7-day culture of each isolate. The plates were then incubated at 4°C for 18 hours, then at 28 ± 2°C for three days, at the end of which time a clear hydrolysis halo appeared around the agar disc, the diameter of which was proportional to the enzymatic activity of the isolate, thus enabling the isolates to be compared according to their keratinolytic activity.

2.3. Antimicrobial activity of keratinolytic actinomycete isolates

The agar cylinder method of [14] was used to evaluate the antibacterial activity of keratinolytic actinomycete isolates against reference strains of *Escherichia coli* ATCC 25922; *Salmonella enteritidis* 155A and *Staphylococcus aureus* SR 231 contained in the strain bank of the Microbiology laboratory of the University of Yaoundé 1. Here, 100µl of a microbial suspension of 6 Log cells/ml of each reference strain was spread on the surface of Mueller Hinton agar by swabbing, then 6mm diameter agar cylinders of 7-day-old cultures of actinomycetes were deposited on the surface of the agars inoculated with each reference strain, at a rate of 4 cylinders per Petri dish. The plates were then incubated at 4°C for 18 hours and then at 37°C for 48 hours, after which time clear halos of inhibition appeared around the agar discs. By measuring the size of the diameters, it was possible to compare the antimicrobial activity of the keratinolytic actinomycetes isolates.

2.4. Physiological and biochemical characterisation keratinolytic actinomycetes isolates

The physiological characterisation of keratinolytic actinomycetes isolates capable of inhibiting bacterial growth was carried out by assessing salt tolerance in nutrient broths with different salt concentrations. Briefly, 100µl of microbial suspension at 6 Log spores/ml was inoculated into broths with different NaCl concentrations: 0, 2.5, 5, 7.5 and 10% (m/v). Once the nutrient media with different NaCl concentrations had been seeded, NaCl tolerance was demonstrated by the observation of a cloudiness in the tube after 48 hours of culture at 28 ± 2°C. Biochemical characterisation was carried out by evaluating the growth of isolates in the presence of different carbon sources (ONPG, urea, gelatine, glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose) from the API 20 gallery.

2.5. Statistical analysis

All the tests were carried out in three repetitions per treatment. The data from these experiments were analysed using XLSTAT version 2020, where they were subjected to analysis of variance (ANOVA). Duncan's post-hoc test was used to compare the means with each other at the 5% threshold, in order to detect the smallest significant differences between the means and classify them into homogeneous groups.

3. Results

3.1 Isolates of keratinolytic actinomycetes

A total of 22 actinomycete isolates were isolated from the soil samples a. Only 19 of the 22 actinobacteria isolated showed keratinolytic activity on keratin-enriched agar media with hydrolysis diameters ranging from 9.5±3.5mm to 15.5 ± 0.7mm (Table 1). As keratinolytic activity varies from one isolate to another, three (03) categories of actinomycete isolates were identified, depending on the level of degradation of the poultry feather after 7 days, as shown in Fig. 1. The primary level of degradation, characterised by detachment of the spine beard,

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Table 1. Keratinolytic activity of Actinomyces spp. isolates.

Isolates	Diameters of hydrolysis	Level of degradation
KER-1	9.5 ± 3.5^{e}	+++
KER-E	14.5 ± 3.5^{abc}	
KER-4	14.5 ± 2.1^{abc}	
KER-T2	14.0 ± 0.0^{abcd}	++
KER-26	15.0 ± 1.4^{ab}	
KER-15	15.5 ± 0.7^{a}	
KER-T3	12.0 ± 2.8^{abcde}	
KER-16	15.5 ± 0.7^{a}	+
KER-A	14.0 ± 1.4^{abcd}	
KER-13	13.0 ± 1.4^{abcde}	
KER-T1	$14.0 \pm 1.^{4abcd}$	
KER-2	15.0 ± 1.4^{ab}	
KER-14	13.0 ± 1.4^{abcde}	
KER-3	10.0 ± 1.4^{de}	
KER-J	12.5 ± 0.7^{abcde}	
KER-19	11.0 ± 0.0^{bcde}	
KER-i	10.5 ± 2.1^{cde}	
KER-18	13.5 ± 2.1^{abcde}	

Data are presented Means ± Standard deviation. Values with different superscript letters in the same column are significantly different at $p < 0.05$. Poultry feather degradation levels: (+) = primary; (++) = secondary; (+++) = tertiary.

involved 12 isolates, including KER-16, KER-A, KER-13, KER-T1, KER-2, KER-14, KER-3, KER-J, KER-19, KER-i, KER-18 and KER-24. The secondary level of degradation was characterised by detachment and partial hydrolysis of the beard and includes 4 isolates: KER-T2, KER-26, KER-15 and KER-T3. The tertiary level of degradation involves 3 isolates: KER-1, KER-E and KER-4, which were capable of detaching and completely hydrolysing the beard from the feather spine.

3.2. Antimicrobial activity of keratinolytic actinomycetes isolates

Out of the 19 isolates having keratinolytic activity, seven isolates were selected on the basis of their high keratinolytic activity and macroscopic difference in order to assess their ability to inhibit the growth of pathogenic bacteria. The results of this inhibitory activity are recorded in Table 2 and some illustrated in Fig. 2. It can be highlighted from Table 2 that 4 isolates (KER-2. KER-T2. KER-I and KER-4) of the 7 actinobacteria isolates tested inhibited all three pathogenic strains. The inhibition diameters ranged from 15.8±4.6mm to 22.5±0.7mm and KER-2 was the isolate that inhibited most of the pathogen growth. Also, *E. coli* appears to be the most inhibited bacterium followed by *S. aureus*, while *S. enteritidis* was the least inhibited.

3.3 Isolate characteristics

The primary identification of the phenotypic characteristics of the seven Actinomycetes isolates used in the antagonism test against the reference strains gave the results shown in Table 3. Three of the 7 isolates produced pigments of different colours in the culture medium. Concerning salt tolerance which is a tool for classifying actinobacteria, the table shows that isolate KER-i exhibits intolerance to NaCl above 5% and can therefore be classified as a species with low salt tolerance. The 6 other isolates (KER-2. KER-T2. KER 4. KER-15. KER-26 and KER-1) showed salt tolerance above 7% with a maximum of 5%; these isolates can be classified in the high salt tolerance group.

4. Discussion

The accumulation of keratin-rich poultry feather waste is among the major led of environmental pollution problems. However, after hydrolysis this waste can be reused in animal feed. Furthermore, the use of antibiotics in animal feed to prevent disease and improve productivity in modern livestock farming is a common practice. Nonetheless, the inappropriate use of antibiotics in livestock farming may be at the root of the emergence of antibiotic resistance [9]. One of the less energy-intensive solutions to the problems of environmental pollution and antibiotic resistance is the use of microorganisms capable of both degrading keratin and producing new antibiotic molecules. Soil bacteria belonging to the Streptomyces genus remain a source of a wide variety of bioactive molecules. This study showed that only 14% of the 22 isolates of Actinomycetes were incapable of degrading poultry feathers indicating that these isolates do not possess keratinolytic activity. According to [4], keratin is a substrate that is recalcitrant to degradation due to the orientation and interactions inherent in its structure and is therefore not hydrolysable by all actinomycete genera. In contrast, the 19 other isolates showed different levels of keratin hydrolysis. This difference in activity between actinomycete isolates can be explained by the quantity and type of keratinase produced in the

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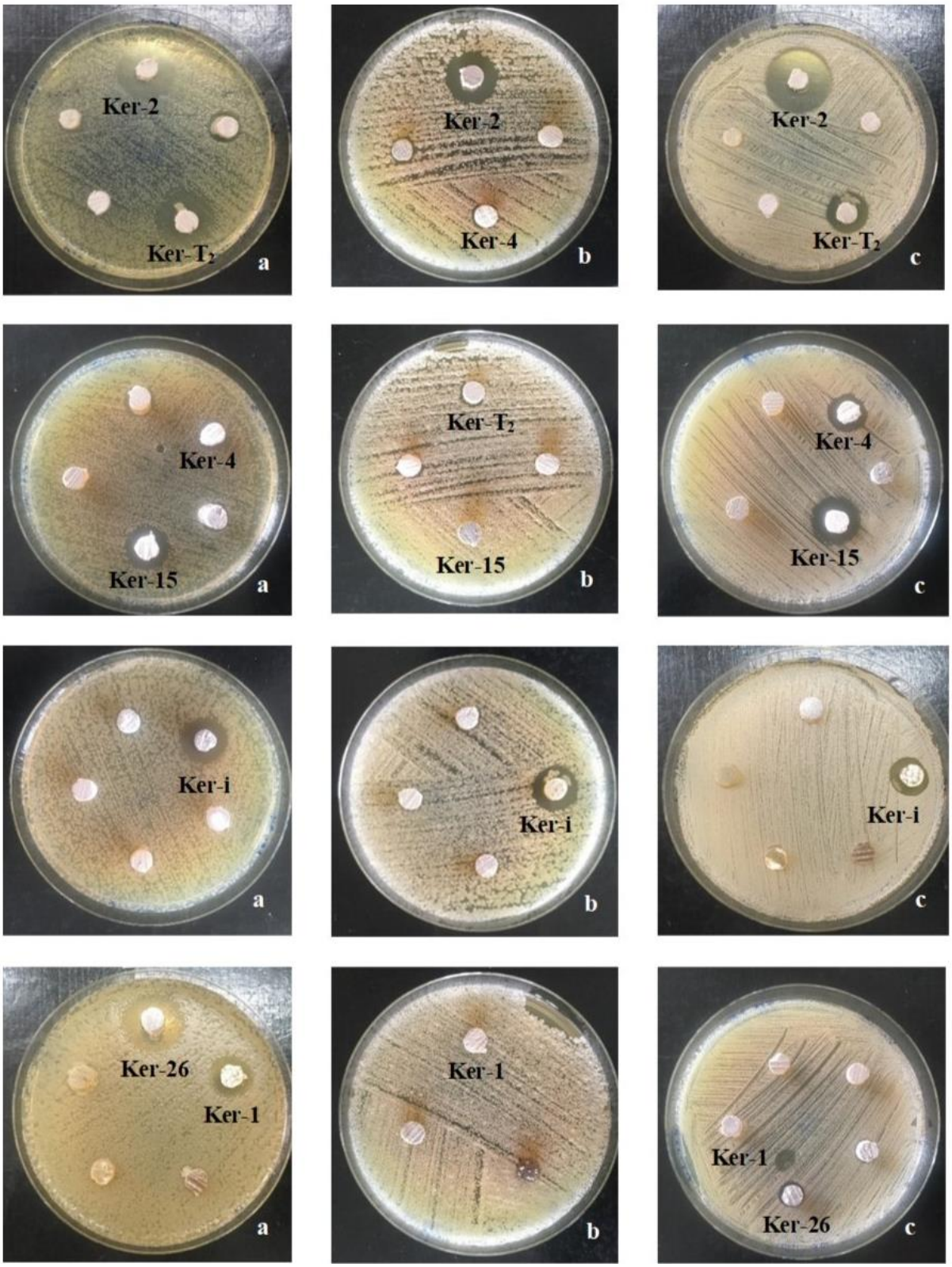

Figure 2. Antagonism example of three isolates Actinomyces spp against (a) = *Escherichia coli*; (b) = *Salmonella enteritidis* and (c) =*Staphylococcus aureus.*

medium by these isolates. According to [15] keratin is hydrolysed by enzymes called keratinases which depending on the type of keratin are of two types: α-keratinase for keratins with high rigidity and β-keratinase for less rigid keratins. Isolates of actinomycetes that were able to detach the beard and completely hydrolyse the spine (tertiary degradation) would therefore be capable of producing both types

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Table 2. Inhibition zone diameter of Actinomyces spp. isolates against pathogenic strains.

Isolates	Diameters of inhibition (mm)		
	E. coli ATCC 25922	*S. enteritidis* 155A	*S. aureus* SR 231
KER-2	22.5 ± 0.7^{a}	15.8 ± 4.6^{a}	20.0 ± 7.1^{a}
KER-T_2	21.5 ± 0.7^{a}	14.0 ± 2.8^{ab}	16.5 ± 2.1^{ab}
KER-i	12.8 ± 3.2^{bc}	11.3 ± 2.5^{bc}	12.0 ± 2.8^{b}
KER-4	11.3 ± 2.5^{c}	8.3 ± 1.1^{c}	13.0 ± 2.8^{b}
KER-15	14.3 ± 3.9^{bc}	0.0 ± 0.0^{d}	13.0 ± 2.8^{b}
KER-26	14.3 ± 3.9^{bc}	0.0 ± 0.0^{d}	7.0 ± 1.4^{c}
KER-1	11.3 ± 2.5^{c}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}

Data are presented Means ± Standard deviation. Values with different superscript letters in the same column are significantly different at $p < 0.05$.

Table 3. Phenotypic and biochemical characteristics of Actinomyces spp. isolates.

Characteristics		Actinomyces isolates						
		KER-2	KER-T_2	KER-i	KER-4	KER-15	KER-26	KER-1
Microscopic and macroscopic characteristics	Aerial mycelium	Gray	Gray	Gray	Orange	Red	White	Brown
	Basal mycelium	Beige	Brown	White	Orange	Red	Gray	Brown
	Pigmentation	-	Beige	-	Yellow	Red	-	-
	Grouping	Filament	Filament	Filament	Filament	Filament	Filament	Filament
	Mobility	Immobile	Immobile	Immobile	Immobile	Immobile	Immobile	Immobile
Tolerance to Na Cl	0%	+	+	+	+	+	+	+
	2.5%	+	+	+	+	+	+	+
	5%	+	+	+	+	+	+	+
	7.5%	+	+	-	+	+	+	+
	10%	+	+	-	+	+	+	+
Biochemical tests	ONPG	+	+	-	+	+	+	-
	Urea	+	+	+	+	+	+	+
	Gelatine	+	+	+	+	+	+	+
	Glucose	-	-	+	+	-	-	-
	Mannitol	-	-	-	-	-	-	-
	Inositol	-	+	-	-	-	-	-
	Sorbitol	-	-	-	+	-	-	-
	Rhamnose	-	-	-	+	-	-	+
	Saccharose	-	-	-	+	-	-	-
	Melibiose	-	-	-	+	+	-	-
	Amygdaline	-	-	-	+	+	+	-
	Arabinose	+	-	-	+	+	+	-

+ : growth presence ; - : growth absent.

of keratinases. The genus Streptomyces and more specifically Actinomyces are generally known to be important sources of antibiotic production against several multi-resistant bacteria [16,17]. In order to evaluate the ability of isolates of keratinolytic actinobacteria to inhibit the growth of several pathogens present in animal feed, it was demonstrated in this study that Actinomyces isolates were more active on S. aureus (Gram +) than on S. enteritidis (Gram -). This result is in line with the work of [18]. This can be explained by the absence of the external lipopolysaccharide membrane cell wall in Gram-positive bacteria. However, the results obtained for E. coli are contrary to the hypotheses previously put forward concerning the high sensitivity of Gram+ bacteria compared to Gram-

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bacteria and to those of [19] and can be explained by the specificity of the bioactive molecules produced by the isolates against *E. coli*.

5. Conclusions

This study revealed that 19 isolates were keratinolytic *Actinomycetes* spp. strains with seven (KER-T2, KER-26, KER-15 and KER-T3, KER-1, KER-E and KER-4) having a high keratinolytic activity. Also, KER-2. KER-T2. KER-I and KER-4 isolates inhibited all the three pathogenic strains (*S. aureus*, *S. enteritidis* and *E. coli*) usually encountered in animal feed with isolate KER-2 having the highest diameter of inhibition of the pathogen growth. Based on the result obtained, isolate KER-2 could be used to fight against pathogenic strains in livestock feed. Thus, the use of these isolates would help contribute to antibiotic resistance encountered in animals.

Authors' contributions

Conducted the experiments and edited the manuscript, G.J.; Conception, data analysis and edited the manuscript, N.Z.N.; Data analysis and edited the manuscript, B.B.D.O.; Translated and edited the manuscript, N.B.; Designed the work and edited the manuscript, J.J.E.N.

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Availability of data and materials

All data will be made available on request according to the journal policy

Conflicts of interest

The authors declare no conflict of interest

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