

African Journal of Biotechnology

Volume 17 Number 2, 12 January, 2018

ISSN 1684-5315



*Academic
Journals*

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ARTICLES

- Influence of spacing and application of biofertilizer on growth and yield of okra (*Abelmoschus esculentus* (L.) Moench)** 17
Juliete Araújo da Silva Nunes, Járisson Cavalcante Nunes, Jandiê Araújo da Silva, Ademar Pereira de Oliveira, Lourival Ferreira Cavalcante, Denizard Oresca and Ovídio Paulo Rodrigues da Silva
- Semen quality characteristics and testosterone levels of rabbit bucks fed *Costus afer* leaf** 24
P. K. Ajuogu, U. Herbert, P. C. Ibeh, M. B. Nodu, U. H. Ukpabio, C. G. Onyegbule and A. O. Akintola
- Isolation, characterization and antibiotics susceptibility of β -glucuronidase producing *Escherichia coli* and other enteric bacteria from ground beef** 29
Abdel-Hamied M. Rasmey, Salha G. Desoki and Mohamed Deabes

Full Length Research Paper

Influence of spacing and application of biofertilizer on growth and yield of okra (*Abelmoschus esculentus* (L.) Moench)

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Received 13 October, 2017; Accepted 10 January, 2018

The adequate spacing in okra (*Abelmoschus esculentus*) influences its growth and fruit production. The objective of this study was to evaluate the growth, the leaf composition and okra production in different spacing with and without biofertilizer. This research was carried out from October, 2012 to March, 2013, at the Federal University of Paraíba, Areia county, Paraíba State, Brazil. The experimental design was a randomized complete block design with three replications, using the 3 × 4 × 2 factorial scheme, referring to three spacing between rows (0.80, 1.00 and 1.20 m), four spacing between plants (0.30, 0.40, 0.50 and 0.60 m), with and without bovine biofertilizer. The leaf area, mean fruit mass, number of plant⁻¹ fruits, plant⁻¹ fruit yield and N, P and K contents were significantly influenced by the spacing × biofertilizer interaction. The commercial productivity of the okra was altered only by the spacing. The application of biofertilizer at spacing of 1.00 × 0.50 m and 1.20 × 0.50 m increased the leaf area of okra. The smaller spacing, associated with the presence of biofertilizer, increased the productive characteristics of okra.

Key words: Organic input, population density, yield.

INTRODUCTION

Okra (*Abelmoschus esculentus*) is an annual vegetable, of easy cultivation; it has erect, upright and semi-woody stem, being able to reach up to 3 m in height. The main

producing regions of Brazil are the Northeast and Southeast (Oliveira et al., 2013). The crop is very suitable for family agriculture practiced in Brazil, because it has

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Table 1. Soil chemical characteristics in the 0-20 cm depth, biofertilizer and the of cattle of manure used in the experiment.

Soil characteristic	Value	Bovine biofertilizer	Value	Bovine manure	Value
OM (g kg ⁻¹)	17.4	N (g kg ⁻¹)	1.04	N (g kg ⁻¹)	10.96
pH em água (1:2.5)	5.8	P (g kg ⁻¹)	0.33	P (g kg ⁻¹)	3.44
P (mg dm ⁻³)	87.6	K (g kg ⁻¹)	0.54	K (g kg ⁻¹)	11.33
K ⁺ (mg dm ⁻³)	109.5	Ca ⁺² (mmol L ⁻¹)	0.29	OM (g kg ⁻¹)	72.14
Na ⁺ (cmol _c dm ⁻³)	0.18	Mg ⁺² (mmol L ⁻¹)	0.49		
Ca ⁺² (cmol _c dm ⁻³)	3.5	Na ⁺ (mmol L ⁻¹)	38.00		
Mg ⁺² (cmol _c dm ⁻³)	1.30	OM (g kg ⁻¹)	21.04		
Al ⁺³ (cmol _c dm ⁻³)	0,00				

OM = organic matter.

good adaptability to the tropical climate, resistance to drought and provide employment in the harvest, classification and packing operations, besides the precocity in the production and relatively long period of harvest, being a good alternative of income for the small scale farmer (Oliveira et al., 2007; Kumar et al., 2015).

The ideal plant density to be used in a given crop is that sufficient to achieve the optimum leaf area index to intercept the maximum solar radiation useful for photosynthesis (Oliveira et al., 2010; Paranhos et al., 2016). In this sense, the plant population acts on the penetration of solar radiation and on the balance between growth of the vegetative parts and the fruits. Thus, changes in the plant population or increased availability of solar radiation indirectly affect the distribution of dry matter between plant organs (Kunz et al., 2007).

The spacing used for the cultivation of okra varies with the producer's system of conduction and interest, with recommendations of high and low population densities, such as: 90 × 30, 100 × 50 and 150 × 50 cm, the last one with two plants by pit (Sedyama et al., 2009). The use of adequate spacing is very important because it exerts influence on flowering, number of productive stems, yield per plant and crop productivity, which may exceed 15,000 kg ha⁻¹ (Silva et al., 2007; Gaion et al., 2013). In the literature it is possible to find scientific information of works evaluating the effect of spacing on the okra culture in Nigeria (Makinde, 2014) and India (Brar and Singh, 2016). However, the scientific information for Brazil is still incipient. Philip et al. (2010) by evaluating the effect of NPK spacing and fertilization on okra in Nigeria, verified that planting density affects crop production components, and concluded that the spacing of 0.9 m × 0.3 m provides the higher yield for the crop.

The crop usually requires high doses of organic fertilization, which is of fundamental importance for adequate plant nutrition, fruit quality and productivity improvement with less or no use of nitrogen fertilizers (Sedyama et al., 2009). In view of these aspects, it is necessary to use organic fertilizers, solid or liquid, in adequate quantities, since the okra has a good vegetative and productive development in the organic

system of production, reaching quality adequate to market demands (Cardoso and Berni, 2012).

Among the liquid organic fertilizers, the bovine biofertilizer stands out because it is an organic source of low cost, mainly due to the increasing demand for new production technologies that present cost reduction. These facts have stimulated researchers and farmers to experiment biofertilizer prepared from the aerobic or anaerobic digestion of organic materials as fertilizer to replace mineral fertilizers (Silva et al., 2012). In vegetables, the biofertilizer can be used by spraying weekly on soil or leaf, diluted in water in proportions ranging from 10 to 30% to allow a perfect development of the plants, since it has a short vegetative and reproductive cycle, requiring a faster and more efficient complementation (Oliveira et al., 2013). In this sense, this work aimed to evaluate the growth, the leaf composition and okra production in different spacing with and without biofertilizer.

MATERIALS AND METHODS

The work was carried out from October, 2012 to March, 2013, at the Federal University of Paraiba (UFPB), Areia, Paraiba States, Brazil. According to Köppen's classification, the climate of the research area is type As' (Alvares et al., 2014), which is characterized as hot and humid, with autumn-winter rains, and average annual temperature ranging between 23 and 24°C. Altitude of the research site is 574.5 m. The soil of the experimental area, according to the criteria of the Brazilian System of Soil Classification-SiBCS (Embrapa, 2013), is classified as a RegoliticNeosol. The chemical analyzes of the soil in the 0 to 20 cm layer, bovine biofertilizer and bovine manure (Table 1) were performed according to Embrapa (2011), by the Laboratory of Chemistry and Soil Fertility of the UFPB.

The experimental design was a randomized complete block design with three replications, in a 3 × 4 × 2 factorial scheme, referring to three spacing between rows (0.80, 1.00 and 1.20 m), four spacing between plants (0.30, 0.40, 0.50 and 0.60 m) and presence and absence of bovine biofertilizer. The number of plants of the experimental plot varied according to the spacing, and the two central rows were collected for evaluation purposes. The soil was prepared by means of plowing and harrowing, in order to provide favorable conditions for planting and development of the

Table 2. Summary of analysis of variance for leaf area (LA), average fruit mass (MMF), number of fruits per plant (NFP), nitrogen (N), phosphorus (P) and potassium (K) contents, and yield per plant (PP) and commercial yield (PC) of okra cultivated at different spacing with and without bovine biofertilizer.

FV	GL	LA	MMF	NFP	N	P	K	PP	PC
Block	2	147193.93*	1.73ns	26.33ns	22.74ns	1.81ns	29.68ns	1.28ns	52.58**
Spacing (S)	11	1074142.49**	2.47ns	315.18**	25.32**	3.66**	57.61**	0.28**	194.20**
Biofertilizer (B)	1	284457.84**	0.57ns	280.64**	26.69ns	11.42**	59.67**	0.34**	9.25ns
S × B	11	231307.25**	3.93**	57.12*	21.93**	2.01*	27.26*	0.10**	10.04ns
Residue	46	35735.51	1.25	27.02	8.14	1.02	9.51	0.03	9.31
Total	71								
CV (%)		16.00	5.77	17.17	7.75	19.98	12.81	23.84	15.82

FV, Source of variation; GL, Freedom degree; **significant at $P < 0.01$ probability error; *significant at $P < 0.05$ probability error; ns, non-significant; CV, coefficient of variation.

plants, and then opening of pits. The planting of the crop was by means of direct sowing by placing five seeds per pit of the Xingó hybrid, with thinning at 15 days leaving only one plant per pit. The fertilization consisted of 100 kg ha⁻¹ of P₂O₅ (single superphosphate), 70 kg ha⁻¹ of K₂O (potassium chloride) and 15 t ha⁻¹ of bovine manure in planting fertilization. The coverage was supplied 100 kg ha⁻¹ de N (ammonium sulfate) in equal parts at 30 and 60 days after sowing (DAS). The biofertilizer was prepared according to Diniz et al. (2013), obtained by fermentation for 30 days in plastic recipient in the absence of air and mixture containing fresh bovine manure and water in the proportion of 50% (volume/volume). To obtain the anaerobic system, the mixture was placed in a 200 L plastic bottle, leaving a sealed space of 15 to 20 cm in its interior, hermetically sealed, and fitting a hose to the lid, plunging the other end into a recipient with water of 20 cm height, for the exit of gases. In the treatments that received biofertilizer (in the soil), six applications were performed at a concentration of 20% (1 L of biofertilizer and 4 L of water) every 15 days until 90 DAS, in the amount of 0.5 L plant⁻¹.

Manual weeding was performed with the help of hoes, aiming to keep the weed free area. In the periods of absence of rainfall, additional irrigations were carried out by the conventional sprinkler system, with an attempt to maintain the crop with sufficient moisture availability for its normal growth. There was no attack of pests and diseases that could cause economic damage to the crop. Flowering started 52 days after sowing with management of the plots as per the recommendation, leaf area of five plants in each treatment and repetition was determined using the LAI-model 2200 equipment. Twenty fresh leaves were collected on the same day from the middle part of the plants each treatment, as suggested by Malavolta et al. (1997), which were conditioned in Kraft paper bags to oven drying with forced air circulation at 65°C until it reached constant weight. The leaves were ground in a Willey mill and transferred to the Laboratory of Chemistry and Soil Fertility of the Federal University of Paraíba for the determination of the N, P and K contents, according to Embrapa (2011) methodology. Harvesting of the fruits, every two days, started at 52 days after sowing and lasted until 113 DAS, at which time the experiment was closed. In all harvests, the fruits were harvested before their full maturation. The average commercial fruit mass was recorded by weighing all the commercial fruits, divided by the number of fruits harvested. Fruits with length between 10 and 15 cm, straight, without deformations and with intense green coloration were considered commercial fruit (Filgueira, 2008). The number and production of commercial fruits plant⁻¹ corresponded to the number and production of commercial fruits divided by the number of plants harvested in each plot and repetition. Commercial fruit yield was determined by weighing all commercial fruits in each treatment, and

the results were estimated for t ha⁻¹. The data were submitted for analysis of variance by F test, and then the means comparisons by Scott-Knott test, at 5% probability, using the statistical program SISVAR (Ferreira, 2011).

RESULTS AND DISCUSSION

The leaf area, average fruit kneading, the number of fruit per plant (fruit plant⁻¹), fruit production per plant (kg plant⁻¹), and the contents of N, P and K were significantly influenced by the interaction effect of spacing × bovine biofertilizer. The commercial productivity of okra only responded to the isolated effect of spacing (Table 2). The largest leaf areas in the treatments with bovine biofertilizer were obtained at spacing of 1.00 × 0.50 m and 1.20 × 0.50 m, with an area of 2246 and 1978.7 cm², respectively. In the treatments without the organic input, the largest leaf area was observed in the spacing 1.20 × 0.50 m, with a value of 1968.61 cm². When the leaf area values were compared in the treatments with and without biofertilizer in the spacing of 1.00 × 0.50 m, it was observed that the area in the treatments with biofertilizer was superior (103%) (Table 3). When considering the leaf area alone, it was observed that the plants in this spacing presented a larger area to intercept the incident solar radiation. According to Silva et al. (2010), the reduction of plant density increased the leaf area, however, it reflects in lower production per plant (Ramos et al., 2009), as observed in Table 5. In this sense, the plant population acts on the penetration of solar radiation and on the balance between vegetative growths (Kunz et al., 2007; Taiz and Zeiger, 2013).

At the spacing of 1.00 × 0.60 m, without biofertilizer, the maximum average mass of 20.3 g was obtained (Table 3). The average fruit mass is located within the range for commercial fruits in okra, defined by Filgueira (2008), between 20 and 25 g, which may indicate that it is not necessary for high population of plants in the okra to raise the average mass of fruits. It was expected that in this spacing the effect of the biofertilizer on the average

Table 3. Leaf area, average mass of commercial fruits and number of fruits plant⁻¹ in okra cultivated in different spacing with and without bovine biofertilizer.

Spacing (m)	Leaf area (cm ²)		Average mass of fruit (g)		N° of fruit planta ⁻¹	
	Biofertilizer		Biofertilizer		Biofertilizer	
	With	Without	With	Without	With	Without
0.80 × 0.30	702.80 ^{dA}	741.33 ^{dA}	19.5 ^{aA}	19.6 ^{cA}	22.7 ^{bA}	23.8 ^{cA}
0.80 × 0.40	830.53 ^{dA}	785.30 ^{dA}	20.3 ^{aA}	19.9 ^{bA}	38.8 ^{aA}	33.9 ^{bA}
0.80 × 0.50	1083.65 ^{cA}	781.63 ^{dA}	21.0 ^{aA}	19.2 ^{cA}	39.8 ^{aA}	42.5 ^{aA}
0.80 × 0.60	726.09 ^{dA}	682.53 ^{dA}	19.3 ^{aA}	18.6 ^{cA}	42.6 ^{aA}	42.4 ^{aA}
1.00 × 0.30	805.30 ^{dA}	781.06 ^{dA}	20.0 ^{aA}	18.2 ^{cA}	31.9 ^{aA}	23.7 ^{cB}
1.00 × 0.40	764.13 ^{dB}	1224.20 ^{cA}	22.2 ^{aA}	18.7 ^{cB}	26.7 ^{bA}	22.3 ^{cA}
1.00 × 0.50	2246.00 ^{aA}	1104.76 ^{cB}	19.4 ^{aA}	19.4 ^{cA}	28.5 ^{bA}	24.0 ^{cA}
1.00 × 0.60	1232.40 ^{cA}	1021.35 ^{cA}	18.3 ^{aB}	20.3 ^{aA}	35.0 ^{aA}	17.5 ^{cB}
1.20 × 0.30	1722.20 ^{bA}	1278.36 ^{cB}	19.4 ^{aA}	19.2 ^{cA}	19.0 ^{bA}	22.7 ^{cA}
1.20 × 0.40	1072.53 ^{cA}	1241.95 ^{cA}	19.1 ^{aA}	17.9 ^{cA}	29.3 ^{bA}	25.2 ^{cA}
1.20 × 0.50	1978.70 ^{aA}	1968.61 ^{aA}	20.2 ^{aA}	18.2 ^{cB}	37.0 ^{aA}	27.3 ^{cB}
1.20 × 0.60	1570.05 ^{bA}	1615.70 ^{bA}	18.0 ^{aA}	18.5 ^{cA}	35.4 ^{aA}	30.3 ^{cA}
Mean	1272.63	1102.23	19.47	19.29	32.26	28.31

Means followed by the same lowercase letter in the columns and upper case in the lines, do not differ by the Scott-Knott test, 5% probability.

fruit mass would be higher, because the okra responds positively to its use (Oliveira et al., 2013), due to its easy assimilation, providing an increase in infiltration velocity of water, due to the organic input contributing to improvement of soil conditions, especially the physical properties of the soil (Silva et al., 2012), taking into account the nutritional requirements of the crop, due to the balanced supply of macro and micronutrients, which allowed development of their genetic potential and results in higher yields. On the other hand, the highest average mass of fruits in the treatments without biofertilizer can be attributed to the initial soil characterization (Table 1), associated to organomineral fertilization with 100 kg ha⁻¹ of N, 100 kg ha⁻¹ of P₂O₅, 70 kg ha⁻¹ of K₂O and 15 t ha⁻¹ of bovine manure. In addition, although the fruits were harvested every two days, and fruits with a length between 12 and 18 cm (Filgueira, 2008) were adopted as the criterion for harvesting, possibly the fruits collected in the treatments without the biofertilizer presented a longer length, which may have contributed to increase the average mass of the fruits in the treatments without the organic input.

The highest numbers of okra plant⁻¹ (42.6, 39.8, 38.8, 37.0, 35.4, 35.0, 31.9) fruits with biofertilizer were verified at the spacing 0.80 × 0.60 m, 0.80 × 0.50 m, 0.80 × 0.40 m, 1.20 × 0.50 m, 1.20 × 0.60 m, 1.00 × 0.60 m and 1.00 × 0.30 m, respectively. Without biofertilizer, the spacing 0.80 × 0.50 m and 0.80 × 0.60 m produced 42.5 fruits plant⁻¹ (Table 3). The results obtained for fruit numbers are similar to those obtained by Sedyama et al. (2009) and Wu et al. (2003) evaluating the effect of spacing in the okra, where they found a number of fruits plant⁻¹ of

45.09 and 42.76, respectively. Oliveira et al. (2013) working with the same crop registered a number of lower fruits plant⁻¹, with a value of 33 fruits plant⁻¹.

The maximum levels of leaf N in okra were 42.9, 42.6 and 40.2 g kg⁻¹ obtained at the spacing 0.80 × 0.30 m (41666 plants ha⁻¹), 0.80 × 0.40 m (31250 plants ha⁻¹) and 1.00 × 0.60 m (16666 plants ha⁻¹) in the treatments without biofertilizer (Table 4). According to Malavolta (1987), these contents were within the species-specific range (35 to 50 g kg⁻¹) and were similar to the levels of 35.8 and 32.6 g kg⁻¹ obtained by Sedyama et al. (2009) in the populations of 23809 and 35714 plants ha⁻¹. The highest leaf content of N in the 0.80 × 0.30 m and 0.80 × 0.40 m spacing in the treatments without biofertilizer is the response of chemical fertilization with 100 kg ha⁻¹ of N and organic fertilization with bovine manure (10, 96 g kg⁻¹), associated to lower fruit yield per plant (Table 5), which may have contributed to increased leaf N content of okra plants even without the application of bovine biofertilizer.

The spacing 1.20 × 0.40 m, 1.20 × 0.50 m and 1.20 × 0.60 m, with biofertilizer, were responsible for the highest levels of P in okra at 6.69 g kg⁻¹, 6.32 g kg⁻¹ and 6.39 g kg⁻¹. However, these levels were above the appropriate range (3.0 to 5.0 g kg⁻¹) for the species (Malavolta, 1987; Trani and Raji, 1996). The maximum K contents with biofertilizer use were verified at spacing 0.80 × 0.50 m, 29.4 g kg⁻¹, 0.80 × 0.60 m, 26.5 g kg⁻¹, 1.00 × 0.30 m, 27.3 g kg⁻¹, 1.20 × 0.50 m, 27.0 g kg⁻¹ and 1.20 × 0.60 m 27.6 g kg⁻¹ (Table 4). Without the addition of the organic input, the highest K contents in the leaves were verified at spacing 0.80 × 0.60 m, 29.7 g kg⁻¹, 1.00 × 0.30 m, 27.6,

Table 4. Nitrogen, phosphorus and potassium contents in okra leaves in okra grown at different spacing with and without bovine biofertilizer.

Spacing (m)	Nitrogen (g kg ⁻¹)		Phosphorus (g kg ⁻¹)		Potassium (g kg ⁻¹)	
	Biofertilizer		Biofertilizer		Biofertilizer	
	With	Without	With	Without	With	Without
0.80 × 0.30	36.2 ^{aB}	42.9 ^{aA}	5.99 ^{aA}	5.38 ^{aA}	22.6 ^{bA}	25.3 ^{bA}
0.80 × 0.40	34.4 ^{aB}	40.2 ^{aA}	4.08 ^{bA}	4.07 ^{bA}	23.8 ^{bA}	21.5 ^{cA}
0.80 × 0.50	40.0 ^{aA}	35.9 ^{bA}	5.98 ^{aA}	4.00 ^{bB}	29.4 ^{aA}	24.1 ^{bB}
0.80 × 0.60	33.6 ^{aA}	37.6 ^{bA}	5.41 ^{aA}	5.54 ^{aA}	26.5 ^{aA}	29.7 ^{aA}
1.00 × 0.30	34.3 ^{aA}	38.5 ^{bA}	4.81 ^{bA}	5.39 ^{aA}	27.3 ^{aA}	27.6 ^{aA}
1.00 × 0.40	35.0 ^{aA}	32.1 ^{bA}	6.47 ^{aA}	4.95 ^{aA}	22.6 ^{bA}	21.1 ^{cA}
1.00 × 0.50	33.2 ^{aA}	34.6 ^{bA}	4.63 ^{bA}	4.73 ^{aA}	23.8 ^{bA}	23.5 ^{bA}
1.00 × 0.60	38.6 ^{aA}	42.6 ^{aA}	3.60 ^{bA}	3.98 ^{bA}	24.1 ^{bA}	14.0 ^{dB}
1.20 × 0.30	38.9 ^{aA}	35.4 ^{bA}	5.26 ^{aA}	2.85 ^{bB}	23.8 ^{bA}	17.0 ^{dB}
1.20 × 0.40	37.0 ^{aA}	34.0 ^{bA}	6.69 ^{aA}	4.23 ^{bB}	20.6 ^{bA}	22.0 ^{cA}
1.20 × 0.50	36.7 ^{aA}	37.1 ^{bA}	6.32 ^{aA}	6.41 ^{aA}	27.0 ^{aA}	29.4 ^{aA}
1.20 × 0.60	36.2 ^{aA}	37.8 ^{bA}	6.39 ^{aA}	4.49 ^{bB}	27.6 ^{aA}	22.0 ^{cB}
Mean	36.21	37.43	5.46	4.67	24.98	23.16

Means followed by the same lowercase letter in the columns and upper case in the lines do not differ by the Scott-Knott test, 5% probability.

Table 5. Production and commercial productivity of okra cultivated at different spacing with and without bovine biofertilizer.

Spacing (m)	Production of fruit plant ⁻¹ (kg plant ⁻¹)		Comercial productivity (t ha ⁻¹)
	Biofertilizer		
	With	Without	
0.80 × 0.30	0.44 ^{CA}	0.47 ^{bA}	20.48 ^c
0.80 × 0.40	1.35 ^{aA}	0.80 ^{bB}	29.31 ^a
0.80 × 0.50	1.03 ^{bA}	1.00 ^{aA}	28.88 ^a
0.80 × 0.60	0.99 ^{bA}	1.14 ^{aA}	25.37 ^b
1.00 × 0.30	0.74 ^{CA}	0.52 ^{bA}	19.97 ^c
1.00 × 0.40	0.54 ^{CA}	0.56 ^{bA}	16.75 ^d
1.00 × 0.50	0.67 ^{CA}	0.47 ^{bA}	15.76 ^d
1.00 × 0.60	1.01 ^{bA}	0.43 ^{bB}	17.33 ^d
1.20 × 0.30	0.42 ^{cB}	0.68 ^{bA}	14.73 ^d
1.20 × 0.40	0.65 ^{CA}	0.52 ^{bA}	13.56 ^d
1.20 × 0.50	0.93 ^{bA}	0.75 ^{bA}	16.25 ^d
1.20 × 0.60	0.76 ^{CA}	0.66 ^{bA}	13.03 ^d
Mean	0.80	0.66	19.28

Means followed by the same lowercase letter in the columns and upper case in the lines, do not differ by the Scott-Knott test, 5% probability.

g kg⁻¹ and 1.20 × 0.50 m, 29.4 g kg⁻¹ (Table 4). These contents were within the range suitable for okra (20.5 to 30 g kg⁻¹) according to Malavolta (1987). Values similar to the maximum K content obtained in this study were verified by Sedyama et al. (2009), which obtained K leaf content in okra of 25.14 kg⁻¹, in the population of 23,809 plants ha⁻¹.

In general, the presence of the biofertilizer increased P and K contents, possibly by improving soil properties (Mellek et al., 2010), providing better conditions for assimilation by the plants, together with the contents of these nutrients in their initial composition, of 0.33 and 0.54 g kg⁻¹ respectively. The highest production of plant⁻¹ fruit was achieved in the 0.80 × 0.40 m spacing in the

presence of the biofertilizer and in its absence, the 0.80 × 0.50 m and 0.80 × 0.60 m spacing produced the highest production of plant⁻¹ fruits, respectively, of 1.00 and 1.14 kg (Table 5). These results demonstrate that, regardless of the use of the biofertilizer to increase fruit production in okra, a population of over 31000 ha⁻¹ plants is required. The efficiency of okra plant in harnessing higher soil volume and, consequently, higher amount of water and nutrients occurs in smaller spacing, consequently larger plant populations. Sediyaama et al. (2009) and Ijoyah et al. (2010) reported that in this vegetable the highest yields of plant⁻¹ fruits are obtained when it is cultivated with smaller spacing.

The maximum commercial fruit yields of 29.31 and 28.88 t ha⁻¹ were obtained at the smallest spacing of 0.80 × 0.40 m and 0.80 × 0.50 m (Table 5). These productivities are considered good for the conditions of the present study, because according to Sediyaama et al. (2009), it is within the range for maximum commercial productivity obtained with plant population of 23,809 and 35,714 plants ha⁻¹, which is 21.9 and 31.3 t ha⁻¹, respectively. The commercial fruit yield was reduced with the increase of the spacing, possibly due to the pressures exerted by the population of plants that markedly affected its development. According to Gebologlu and Sagllam (2002), when the density of plants per unit area increases, it is likely to reach a point at which plants compete for essential growth factors such as nutrients, light and water, reducing their productive capacity. Some authors have observed a reduction in fruit productivity with spacing elevation in some vegetables, such as tomato (Wamser et al., 2009), maxixe (Oliveira et al., 2010) and okra (Sediyaama et al., 2009).

The increase in commercial fruit yield obtained in smaller spacing may be due to the greater interception of photosynthetically active light and photosynthesis in the canopy, which stimulates the growth of the crop and increases the total assimilates available for the fruits. Ibeawuchi et al. (2005) argue that one of the major aspects of ecology and management that often limits agricultural production is improper spacing in the field. Changes in the plant population or increased availability of solar radiation indirectly affect the distribution of dry mass between plant organs (Kunz et al., 2007).

Conclusion

The largest leaf areas of okra were obtained at spacing of 1.00 × 0.50 m and 1.20 × 0.50 m in the presence of biofertilizer. The plants were adequately supplied in nitrogen and phosphorus. The smaller spacing, associated to the presence of biofertilizer, increase the production characteristics of okra. To increase the productivity of commercial fruits in okra, management of the crop is more effective than the use of organic fertilizer.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Semen quality characteristics and testosterone levels of rabbit bucks fed *Costus afer* leaf

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Received 14 December, 2016; Accepted 25 October, 2017

Previous studies have shown *Costus afer* to possess ethno-medical and ethno-veterinary properties, however, the link between *C. afer* and reproductive activities are not clear. The aim of this research was to assess the impact of *C. afer* leaf on semen quality characteristics and testosterone levels of adult buck rabbits. Eighteen adult crossbred New Zealand and Chinchilla breeds were divided into three treatments (A, B and C) (n=6) in a completely randomized design (CRD) for 12 weeks. The results showed significant ($P<0.05$) decrease in sperm motility, sperm count, and normal morphology due to treatment effect. Sperm abnormalities, dead cells, sluggishly motile, and pus cells were significantly ($P<0.05$) increased in treated group. The treatment had no significant impact ($P>0.05$) on parameters like appearance (normal), semen volume, and viscosity between the groups. Serum testosterone increased significantly ($P < 0.05$) in treated groups (C: 21.0 ng/mL; B: 11.6 ng/mL) than control (A: 11.0 ng/mL), respectively. These results indicate that *C. afer* has a negative fertility impact on buck rabbits.

Key words: *Costus afer*, semen, testosterone, rabbit, buck.

INTRODUCTION

In recent times, the use of herbal medicines has gradually acquired a more vital therapeutic role to replace the synthetic once for animals and humans due to

increased incidence of drug resistance (Olowosulu and Ibrahim, 2006). In Africa particularly in Nigeria, several plants have been identified to have medicinal and

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nutritional importance (Egba et al., 2014). The diverse African herbal plants afford the trado-medical practitioner best opportunities in the selection of herbs for various human and animal diseases (John, 2004). In folk and traditional medicine practices, several herbal plants and/or their extracts have been used to enhance fertility in male and female animals (Vasudeva and Sharma, 2007; Singh and Singh, 2009) in their unaltered form.

Some of the herbs and/or its extracts have been reported to improve libido, sexual behaviour, mating performance and spermatogenesis (Tomova et al., 1981; Chauhan et al., 2007), while others balance the levels of hormone such as testosterone, luteinizing hormone, and follicle stimulating hormones (Koumanov et al., 1982) in hypothalamic-pituitary gonadal axis (reproductive axis) of male and female animals (Gamache and Acworth, 1998; Asuquo et al., 2013).

Costus afer (Costaceae) is an important indigenous West Africa herbal plants with unique medicinal properties commonly used throughout its area of distribution. It is tropical perennial herbaceous rhizomatous plants found in the forest belt of Africa (Burkili, 1985; Edeoga and Okoli, 2000). Experimental evidences have shown that *C. afer* possess important bioactive and medicinal potentials (Etukudo, 2003; Akpan et al., 2012; Ukpabi et al., 2012). Several studies have revealed its ethno-veterinary and ethno-biotic importance such as antimicrobial properties (Akpan et al., 2012; Ijioma et al., 2014), hypoglycemic effect (Momoh et al., 2011), treatment of stomach aches and rheumatic pains (Etukudo, 2003), treatment of hepatic oxidative stress and toxicity (Ukpabi et al., 2012), anti-diabetic (Soladoye and Oyesika, 2008), treatment of eye inflammation, headache, oedema, and fever (Omokhua, 2011). This is due to its numerous phytochemical properties such as steroids, flavonoids, alkaloids phynols, saponins, and phenols (Oliver-Bever, 1986; Tchamgoue et al., 2015; Ukpabi et al., 2012). Previously, tropical ethno-medical and ethno veterinary plants and other products with propensity to limit and/or enhance reproductive function in animals have been reported (Yahaya and Ajuogu, 2014; Yahaya et al., 2015). Therefore, this study was designed to assess the impact of *C. afer* on the fertility status (semen quality characteristics and testosterone levels) in buck rabbits.

MATERIALS AND METHODS

All experimental procedures involving animals and the ethical issues of University of Port Harcourt were strictly followed. The experiment was done at the Research and Demonstration Farm of Faculty of Agriculture, University of Port Harcourt, Rivers State. Eighteen adult cross breed of New Zealand and Chinchilla bucks with an average weight of 1.5 to 2.0 kg were assigned randomly to three groups (A, B, and C) (n=6) and were further subdivided into three replicates (n=2) per group in a completely randomized design (CRD). The study lasted for 12 weeks.

The *C. afer* leaves were harvested fresh and fed along with

growers mash *ad libitum* to the experimental animals (buck rabbits) as follows: Treatment group A (control group), concentrate feed only; Group B, concentrate feed with *C. afer*; treatment group C, fresh *C. afer* only. All the groups were fed on *ad libitum* bases.

Prior to the treatment exposure, blood samples were collected from each animal through the ear vein in all the treatment groups using sterile syringes and hypodermic needles and decanted into well labelled sterile sample bottles for preliminary analysis of serum testosterone levels. Similarly, at the termination of the study, blood samples were also collected from all the bucks in all the treatments groups using the sample procedure mentioned earlier and were used to assess serum testosterone levels using commercial test kits BioCheck, Inc, 323 Vintage Park Dr Foster City CA 94404. The protocol was as described by the manufacturer.

Semen samples were collected from the bucks in all the groups with the aid of artificial vagina (AV) using the method described by Herbert and Adejumo (1995) and modified method of Ajuogu and Ajayi (2010).

The samples were collected twice at the beginning of the study and at the expiration of the study. Group C could not mount due to exhortation; as a result, no semen was collected. The semen quality characteristics were assessed by microscopic examination.

Data obtained from testosterone and semen samples were analysed using the analysis of variance (ANOVA). According to Steel and Torrie (1980), means with significant differences were separated using Duncan's Multiple Range Test (DMRT, 1955).

RESULTS

Preliminary semen analysis of buck rabbit before exposure to *C. afer*

The preliminary semen analysis result of rabbit buck exposed to *C. afer* is presented in Table 1. The results revealed no significant changes ($P>0.05$) on the semen quality parameters measured. The semen volumes are 1 ml each in treatments A and C, while in treatment B, it was 0.5 ml. The pH was 8.0 in treatment (A, B and C), respectively. The viscosity and progression of the semen in all the treatments (A, B and C) were both good and normal.

Preliminary semen analysis of rabbit buck

The statistical analysis of semen quality assessment parameters revealed no significant difference between the treatment groups. Sperm motility was 80, 70, and 60% for treatments C, B and A. Sperm count was 8×10^6 , 7×10^6 and 6×10^6 ml^{-1} for treatments A, B and C, respectively (Table 1). The pH, viscosity and progression were 8.0, normal, and good in all the treatments. The semen appearance was milky in all the groups. For normal morphology, pus cells, was not significantly different between control and treatment groups.

Also, Table 2 shows the preliminary semen analysis of rabbit buck before being exposed to *C. afer*. From the results, serum testosterone of the bucks in all the groups showed no significant difference ($P>0.05$) between the treatment groups.

Table 1. Preliminary semen analysis of rabbit buck before exposure to *Costus afer*.

Parameter	A	B	C
Semen appearance	Milky	Milky	Milky
Semen volume	1 ml	0.5 ml	1 ml
Semen pH	8.0	8.0	8.0
Viscosity	Normal	Normal	Normal
Progression	Good	Good	Good
Motility (%)	6±0.05	7±0.08	8.0±0.03
Sperm count (ml)	8×10 ⁶	7×10 ⁶	6×10 ⁶
Normal morphology (%)	20±0.05	20±0.01	20±0.05
Abnormalities (%)	80±0.08	80±0.05	90±0.03
Pus cells	3-4±0.02	4-5±0.03	3-4±0.04
Dead cells (%)	20±0.03	10±0.02	10±0.01
Sluggish motile (%)	20±0.05	20±0.04	10±0.02

A: Treatment group A (control group) concentrated feed only; B: Treatment group B, concentrated feed with *C. afer*; C: Treatment group C, fresh *Costus afer* only.

Table 2. Preliminary testosterone analysis of rabbit bucks before exposed to *Costus afer*.

Parameter	A	B	C
Testosterone nmol/L	6.80±0.03	7.6±0.05	6.9±0.03

A: Treatment group A (control group), concentrated feed only; B: treatment group B, concentrated feed with *Costus afer*; C: treatment group C, fresh *C. afer* only.

Table 3. Influence of *C. afer* on semen quality characteristics and serum testosterone levels of buck rabbit.

Parameter	A	B	C
Semen appearance	Milky	Milky	-
Semen volume	1 ml	1 ml	-
Semen pH	8.0	8.0	-
Viscosity	Normal	Normal	-
Progression	Good	Poor	-
Motility (%)	80±0.05 ^a	10±0.02 ^b	-
Sperm count	9×10 ^{6a}	2×10 ^{5b}	-
Normal morphology (%)	90±0.05 ^a	20±0.02 ^b	-
Abnormalities (%)	10±0.03 ^b	80±0.01 ^a	-
Pus cells (hpt)	2-3±0.01 ^b	4-5±0.03 ^a	-
Dead cells (%)	10±0.02 ^b	70±0.05 ^a	-
Sluggishly motile (%)	10±0.04 ^b	20±0.01 ^a	-
Testosterone ng/mL	11.0±0.02 ^b	11.6±0.01 ^b	21.0±0.04 ^a
Mortality	0	0	0

Mean ± SD in the same row with different superscripts differs significantly (P<0.05). A: Treatment group A (control group), concentrated feed only; B: treatment group B, concentrated feed with *Costus afer*; C: treatment group C, fresh *C. afer* only.

The results of the effect of *C. afer* on semen characteristics of buck rabbit are shown in Table 3. There was no record of the semen characteristics of buck

in treatment C because the bucks were so weak that they could not mount. The statistical analysis of the motility and sperm count was significantly different (P<0.05)

between the treatment group. Treatment group A (80%, 9×10^6) was significantly better ($P < 0.05$) in semen motility and sperm count than group B 10% (2×10^5). Other semen parameters: semen appearance (normal), semen volume (1 ml) and viscosity (normal) were the same for both groups. Progression was good in treatment group A and poor in group B.

Normal morphology was significantly ($P < 0.05$) better in treatment group A (90%) than group B (20%). Effect on semen abnormalities was significantly ($P < 0.05$) higher in group B (80%) than in group A (20%). Pus cells were more in group B (4 to 5 hpt) than group A (2 to 3). Dead cells were significantly ($P < 0.05$) more in group B (70%) than group A (10%). Percentage of sluggish motile sperm cells was significantly higher in group B (20%) than group A (10%). Serum testosterone was significantly ($P < 0.05$) higher in treatment group C (21.0 Mg/mol) than in groups B (11.6 Mg/mol) and A (11.0 Mg/mol), respectively. No mortality was recorded in all the groups.

DISCUSSION

The semen status of rabbits is a mixture of spermatozoa suspended in a liquid medium secreted at different locations by the epididymis and at various glands. Its analysis or evaluations provide index for prediction of fertility status in animals (Quintero-Moreno et al., 2007; Ajayi et al., 2011). *C. afer* is one of the several tropical medicinal plants used against various ailments. The effect of the treatments on semen pH, appearance and volume is within the normal range according to International Rabbit Reproduction Group (2005). The treatment dependent depressed influence ($P > 0.05$) of *C. afer* on sperm motility, sperm count and morphology between the treatment groups although within normal levels of fertilizable semen status of rabbit bucks (International Rabbits Reproduction Group 2005), may indicate that *C. afer* have a negative influence on sperm fertility index (motility, sperm count, and morphology). The reduction of sperm count, mal-formation of spermatozoa, and insufficient motility have been reported as the leading cause of fertility failure or infertility in male (Chauhan and Dixit, 2008).

This result is similar to the findings of Rao and Alice (2001) who reported the antifertility effect of *Phyllanthus amarus* at dosage level of 100 mg/kg body weight of male and female mice which was attributed to changes in hormonal profile that regulates reproductive function in animals. Similarly, Adedapo et al. (2003) reported different degrees of testicular degeneration and reduction in diameter of seminiferous tubular of rats treated with extracts of *P. amarus*.

On the contrary, Arhoghro et al. (2014) reported that *C. afer* leaf extract can be applied for the enhancement of immune system and treatment of serum cholesterol concentration which is a vital precursor in steroidal

hormonal secretion. This was evidenced on the significant impact it had on testosterone concentration recorded in treatment group C (21.0) and this may be suggestive of the fact that its reproductive enhancement activity could be on tissues such as testes and prostate as well as promoting secondary sexual characteristics as it is major role of testosterone in determining fertility status in animals.

However, *C. afer* treatment in this study was observed to increase serum testosterone levels (C: 21.0 nmol/L). This is in line with previous studies that reported increased testosterone profile in animals treated with herbal plants (Chen et al., 1993). Testosterone is a steroid hormone secreted from Leydig cells of the testes in males and it play critical role in the reproductive function and promotion of secondary sex characteristics such as muscle growth and strength, bone mass, and growth of body hairs (Zouboulis and Degitz, 2004). Chen et al. (1993) observed that Chinese yam increases testosterone levels in animals. Yahaya and Ajuogu (2014) exposed adult rabbits to *Laguclaria racemosa* leaves (white mangrove) and revealed significant increase in testosterone production. It is therefore suggested that graded levels of the test plant be investigated to determine the levels at which it affects other areas of male reproduction such as sexual behavior, reproductive hormonal profile, etc.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation, characterization and antibiotics susceptibility of β -glucuronidase producing *Escherichia coli* and other enteric bacteria from ground beef

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Received 30 July, 2017; Accepted 24 October, 2017

The current work aimed to determine the prevalence of *Escherichia coli* in fresh ground beef purchased from butchers' shops in Suez Governorate, Egypt, and the antibiotics susceptibility pattern of the isolated bacteria. *E. coli* was isolated and detected on tryptone bile glucuronide agar (TBGA) plates as chromogenic selective medium for this species. The sensitivity and resistance of the isolated bacteria to antibiotics were performed according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS). A total of 299 bacterial isolates were recovered from 130 ground beef and *E. coli* had the highest frequency of occurrence (81.5%). The isolated enteric bacteria were identified phenotypically and genotypically as *Serratia marcescens*, *E. coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae* and deposited in the GenBank nucleotide sequence database under accession numbers KU237235, KU237236, KU237237 and KU237238, respectively. Antibiotic susceptibility test showed that the four isolated species were susceptible to norfloxacin, pefloxacin, kanamycin and ceftriaxone, and resistant to clindamycin and the other tested antibiotics showed different susceptibility pattern with each tested species. Precautions and strict hygienic measures should be taken during the processing stages of ground beef in order to avoid contamination by enteric bacteria.

Key words: Contamination, Enterobacteriaceae, genotype, resistance, susceptibility, meat.

INTRODUCTION

Over the past decade, increase of bacterial contamination in meat intended for human consumption in many countries is one of different factors responsible for human illness. The trend of some researchers

nowadays is to establish a structured model for improving the food control systems for producing safer food by reducing the number of food-borne pathogens. Ground beef is a processed meat product that might be widely

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exposed and handled during the manufacture process and the probability of contamination of the processed meat is high. The different causes of ground beef contamination may be the use of raw meat, different processing steps during production and the large surface area due to reduction in particle size (Kang et al., 2001; Gundogan and Avci, 2013). The contamination of meat surface by members of the family Enterobacteriaceae will make the produced meat hazardous to human due to their potentiality in causing food poisoning and also they show the hygienic standard of the slaughterers' shops (Nossair et al., 2014).

Enterobacteriaceae are Gram negative bacteria used as indicator microorganisms and a measure of the hygienic status of food products (Jordan et al., 2007; Gundogan et al., 2011). The members of this family have been known to cause health hazard for human and lead to spoilage and deterioration of food products, particularly meat (Ayhan et al., 2000). Some bacterial species of Enterobacteriaceae including *Escherichia coli*, *Citrobacter* spp., *Enterobacter* spp. and *Klebsiella* spp. may be recognized using standard methods that detect their ability to ferment glucose or lactose quickly (usually within 24 and 48 h) generating acid and gas (Molina et al., 2015). These species are known as coliform bacteria and are often used as faecal indicator organisms by food and water, due to their normal habitat which is the gastrointestinal tract of human and animals. These bacterial species have a high spoilage potentiality for food under the favorable conditions for their growth and multiplication (Ray and Bhunia, 2008).

Enterobacteriaceae are the main cause of severe infections from contaminated food, and many commensal species of this family are widely exposed to an extensive use of the currently used antibiotics, consequently, they have developed resistance (Paterson, 2006). Because of self-medication, the suboptimal quality of bacteria can be transferred to pathogenic species (Doucet et al., 2001; Schoeder et al., 2004). Bacterial species of the genus, *Klebsiella* and *Enterobacter* spp. are among the most common Gram-negative bacteria next to *Escherichia coli* that cause nosocomial infections and often give rise to urinary and respiratory tract infections (Saidi et al., 2014).

The main goal of this study was to investigate the best detection method by using selective differential medium for isolation of *E. coli*, *Klebsiella*, *Enterobacter* and *Serratia* spp. from ground beef and to determine their susceptibility to some common antibiotics.

MATERIALS AND METHODS

Samples collection

A total of 130 fresh ground beef samples were collected from different fresh meat shops located in Suez Governorate, Egypt. All samples were collected aseptically in sterilized plastic bags, transported to the laboratory under chilled conditions and processed immediately for microbiological analysis.

Isolation of bacteria

Enrichment culture

Samples were enriched using tryptone soy broth (TSB) to enhance the appearance and growth of the different species belonging to Enterobacteriaceae. Twenty five grams from each food sample were weighed out and homogenized into 225 ml of sterile buffered peptone water in a stomach bag for 5 min (Hara-Kudo et al., 2008) and incubated at 37°C for 4 h. 1 ml was transferred to TSB broth tubes and incubated at 37°C for 18 h.

Plating on selective media

A loopful inoculum of each sample was streaked in triplicate onto tryptone bile glucuronide agar (TBGA) plates and incubated overnight at 37°C. The different colored colonies were selected on the chromogenic selective agar and the well separated pure colonies were picked up on nutrient agar slants as pure cultures and stored at 4°C.

Phenotypic characterization of the isolated bacteria

Morphologically typical colonies were verified by Gram staining, IMViC tests, fermentation of sugars like glucose and lactose, triple sugar iron agar test, oxidase test, urease test, catalase test, pigment production and motility test. Also, the selected colonies were streaked on nutrient agar (NA), Mac Conkey agar (MAC), xylose lysine deoxycholate agar (XLD agar) and Brilliant Green Agar (BGA) media to differentiate between the selected isolates according to the colonies colour.

Genotypic characterization of the selected isolated bacteria

The isolated strains were characterized genotypically with reference to virulence marker genes employing polymerase chain reaction.

DNA extraction

The isolates were grown in 2 ml of BHI broth overnight at 37°C. Cultures were harvested by centrifugation (8000 g for 10 min) and suspended in 400 µl of TE solution (10 mM Tris HCL; 1 mM EDTA, pH 8.0). Bacteria were lysed by addition of 10 µl (20 mg/ml) proteinase K and 100 µl of 10% SDS followed by incubation at 37°C for 1 h. The preparation of total genomic DNA was conducted according to the method of Abd-Alla et al. (2012).

PCR Amplification

The 16S rRNA encoding gene was amplified by the polymerase chain reaction (PCR) from purified genomic DNA using the bacterial universal PCR primers 16S F: 5'-GAGTTTGATCCTGGCTTAG-3' and 16S R: 5'-GGTTACCTTGTTACGACTT-3'. The reaction was performed in a thermal cycler (Eppendorf, Germany) with a pre-heated lid. The cycling conditions included an initial denaturation at 94°C for 5 min followed by 37 cycles each of 30 s denaturation at 94°C, 30 s annealing at 51°C and 30 s extension at 72°C. It was followed by final extension of 5 min at 72°C. After the reaction, PCR products were kept at -20°C until further analysis by agarose gel electrophoresis.

PFGE agarose gel documentation

PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose TBE-gels (Tris-base Boric EDTA-gel) and the gels were

Table 1. Information of antibiotics discs used in disc diffusion assay

Antibiotic	Abbreviation	Dosage (μg)	Mode of action
Norfloxacin	NOR	10	Blocking DNA replication
Pefloxacin	PEF	5	DNA gyrase inhibitor
Rifampicin	RD	5	RNA synthesis inhibitor
Kanamycin	K	30	30S inhibitor
Streptomycin	S	10	30S inhibitor
Clindamycin	DA	2	50S inhibitor
Ceftriaxone	CRO	30	Cell wall synthesis inhibitor
Cephalothin	KF	30	Cell wall synthesis inhibitor
Cefuroxime sodium	CXM	30	Cell wall synthesis inhibitor
Cephalexin	CL	30	Cell wall synthesis inhibitor

Table 2. Frequency and number of isolates recovered from 130 ground beef samples and their grouping according to the colonies colour on TBGA medium.

Group	Colony colour	Frequency	Percent	Number of isolates
A	Red	7	5.4	14
B	Green	106	81.5	125
C	White	56	43	60
D	Colourless	87	66.9	100

visualized and pictured under UV light. Gels photos were captured using gel documentation system then analyzed by Gel Docu advanced ver.2 software. PCR products of about 1500 bp were purified from gel with the QIA quick PCR purification kit (Qiagen, Hilden, Germany).

DNA sequencing

Purified PCR products were sequenced by cycle sequencing with dideoxy mediated chain-termination (Sanger et al., 1977). DNA sequencing was applied by 3500 Genetic Analyzer, Applied Biosystems (Biotechnology Research Center, Suez Canal University, Ismailia, Egypt). For sequencing of the purified PCR products, the same primers were used. Sequences of the 16S rRNA of isolates were first analyzed using the advanced BLAST search program at the NCBI website: <http://www.ncbi.nlm.nih.gov/BLAST/> in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny was evaluated using CLUSTALW program (<http://clustalw.ddbj.nig.ac.jp/top-eh.html>). The phylogenetic tree was displayed using the TREE VIEW program. Phylogenetic tree derived from 16S rRNA gene sequence was generated in comparison with 16S rRNA gene sequences from different standard bacteria strains obtained from GenBank.

Antibiotic sensitivity testing

Antibiotics sensitivity of the selected bacterial isolates was studied against 10 different antibiotics according to methods of Bauer et al. (1966). Bacterial inocula of the tested bacteria were prepared by growing the bacteria in nutrient broth medium for 18 h at 37°C. One hundred microliter of the bacterial inoculum was seeded individually into sterilized plates and the melted nutrient agar medium was poured and left for solidification. The discs impregnated with

various commercially available antibiotics with known dosage were placed on the surface of the nutrient agar plates, incubated at 37°C for 18 to 24 h and the formed inhibition zones were measured and recorded. Larger zones of inhibition indicate a higher level of antibiotic effectiveness and the tested bacterial strain is designated as sensitive to the antibiotic. The absence of apparent zone of inhibition indicates that the bacterial strain is resistant to this particular antibiotic drug. The specific information of each antibiotic including its abbreviation, name, dosage and inhibition mechanism are listed in Table 1.

RESULTS AND DISCUSSION

A total of 299 bacterial isolates were recovered from 130 ground beef samples on TBGA medium as selective differential medium for isolation of *E. coli* (Table 2). The isolation of *E. coli* from different food products and clinical samples on the chromogenic selective TBGA medium was performed by different researchers (Kodaka et al., 1995; Popovic et al., 2010). Tryptone bile glucuronide agar (TBGA) is a selective differential medium recommended for detection of β -glucuronidase-positive *E. coli* in food products prepared for human consumption. The medium contains bile salts that are selective against Gram-positive bacteria and X-glucuronide (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) as a chromogen for the detection of β -D-glucuronidase enzyme which is present in *E. coli* only of coliforms. *E. coli* absorb the chromogenic substrate, 5-bromo-4-indolyl-D-glucuronide, X-glucuronide. The intracellular



Figure 1. Colonies morphology of the coliform bacteria isolated from ground beef samples on TBGA medium.

enzyme β -D-glucuronidase of *E. coli* is able to break the bond between the substrate and the chromophore which dimerises in the presence of oxygen to form an insoluble coloured compound within the cell. The released chromophore is colored and accumulates inside the cells (Feng and Hartmann, 1982). The accumulation of the coloured chromophore results in blue colonies on the agar. The chromogenic substrate is concentrated within the colony facilitating their enumeration visually among the other organisms on the plate without UV lamp.

In the current study, it was observed that there are three others colonies colour on this medium besides *E. coli* colonies. The recovered bacterial isolates were divided into four different groups: A, B, C and D on the basis of their colonies colour on TBGA plates. The bacterial groups: A, B, C and D were characterized by colony colour as red, green, white and colourless, respectively (Figure 1). The appearance of these different coloured colonies besides the *E. coli* colonies might be due to different intracellular enzymes by these bacteria or different reactions between the components of the medium and intracellular metabolites. Generally, this chromogenic medium may be suitable for better detection of these four groups of isolated bacteria. Isolation and enumeration of *E. coli* is used as reliable indicator of fecal contamination and probability of toxigenic microorganisms' presence in this food. Group B which represents *E. coli* had the highest frequency of occurrence and represented by 81.5% recovered from 106 samples out of 130 samples, while groups A, C and D is represented by 5.4, 43 and 66.9%, respectively (Table 1). The results are almost in line with that of Greeson et al. (2013) who studied the prevalence of Enterobacteriaceae in 36 samples of meat and reported that *E. coli* was the most frequent contaminant and its prevalence was 72.2%. *E. coli* is known as a fecal

contamination indicator in foods due to its presence in the intestinal tract. The gastrointestinal tract and the hands of personnel were recorded as major transferors of *Klebsiella* spp. and *E. coli* (Gundogan and Yakar, 2007). On the other hand, the current study results are in contrast with those of Mohammed et al. (2014) who recorded low frequency (15.89%) of *E. coli* isolated from 384 meat samples. Also, a similar study on isolation of *E. coli* from retail meat was performed by Nossair et al. (2014) who isolated different members of Enterobacteriaceae from 50 samples of retail meat collected from buffaloes and it was found that different species of bacteria were isolated at different rates, where *E. coli* is the highest isolated bacterium (40%) followed by *K. pneumoniae*. The obtained results showed that the examined ground beef of cow origin were highly contaminated with *E. coli* and other enteric bacteria which showed fecal contamination potential for severe hazard (Mohammed et al., 2014).

The isolated bacteria were members of the intestinal flora of human and animals and many of them might lead to food deterioration and toxicities (Gundogan and Yakar, 2007; Haryani et al., 2007). These results emphasized the role played by meat in transmission of *E. coli* that could constitute public hazard and food poisoning outbreaks (Reuben and Gyar, 2015; Kabiru et al., 2015). Contamination of both carcasses and the environment by *E. coli* from the intestinal contents of cattle during slaughter is one of the most significant risk factors in transmission to humans (Koohmaraie et al., 2005; Bosilevac et al., 2009). Moreover, Enterobacteriaceae contaminating ground beef in butchers' shops may originate from human carriers (workers) who handle and prepare the meat during cutting and grinding. Also, infected rodents that may be present in the butchers' shops or slaughterhouse could represent a neglected

Table 3. Phenotypic characterization of the isolated coliform bacteria.

Test	Group A	Group B	Group C	Group D
Gram stain	-ve	-ve	-ve	-ve
Cell shape	Short rods	Short rods	Short rods	Short rods
Oxidase	-	-	-	-
Methyl red	-	+	-	+
VP	+	-	+	-
Citrate test	+	-	+	+
Glucose ferm.	+	+ and gas	+ and gas	+
Lactose ferm.	-	+ and gas	+	+
TSI	+	+ and gas	+ and gas	+
H ₂ S	-	-	-	-
Urease	-	-	-	-
Motility	+	+	-	-
Indole	-	+	-	-
Pigment	Red	Negative	Negative	Negative
Nutrient agar	Red	Colourless	Colourless	Colourless
MAC agar	Red	Pink	Pink	Pink
XLD agar	Red	Pink with yellowish zone	Pink with yellowish zone	Pink with yellowish zone
BG agar	Red	Colourless	Colourless	Colourless

nsource of contamination by *E. coli* and other coliforms bacteria (Okonko et al., 2010).

The four isolated coloured bacterial groups were characterized morphologically, biochemically and genotypically. The morphological and biochemical characterization of the total recovered bacterial isolates belonging to the different four groups: A, B, C and D were performed by conventional methods. All the tested isolates belonging to the four groups were negative for Gram staining and have short rods shape. Also, the four groups were negative for oxidase and positive for glucose fermentation. On the other hand, the four groups differed in their result for lactose fermentation as the test was positive for the three groups: B, C and D, and was negative for group A. The results of the other biochemical tests showed more differences between the four bacterial groups in this investigation. Group A was characterized by red pigment production and was positive for VP, citrate and motility, whereas, was negative for methyl red, H₂S, urease and indole. Group B was positive for methyl red, motility and indole but negative for VP, citrate, H₂S and urease. Group C was positive for VP and citrate but was negative for methyl red, H₂S, urease, motility and indole. The fourth group showed positive result for each methyl red and citrate and gave result for VP, H₂S, urease, motility and indole. The colonies colour differed on different culture media as presented in Table 3. Based on the morphological and biochemical characterizations, the isolated strains belonging to the four groups: A, B, C and D were related to *Serratia marcescens*, *E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, respectively, according to the Bergey's Manual of Determinative Bacteriology (Holt, 1994).

The phenotypic-based identification was confirmed by genotypic identification. One isolate was selected from each of the four groups and their DNA were extracted, and a fragment of about 1500 bp from each one was amplified using the universal primers 16S F and 16S R (Figure 2). Comparison between 16S rRNA gene sequences of the tested isolates Ras1, Ras2, Ras3 and Ras4 belonging to groups A, B, C and D, respectively and 16S rRNA gene sequences on GenBank database as determined using Blast search analysis, were done. Sequencing of 16S rRNA genes of the tested isolates Ras1, Ras2, Ras3 and Ras4 had 16S rRNA gene with 99% nucleotides identity to that of *S. marcescens* NR102509.1, *E. coli* NR 114042.1, *E. cloacae* NR 118011.1 and *K. pneumoniae* NR 112009.1, respectively, available in Genbank database (Figure 3). The phylogenetic tree was drawn from 16S rRNA sequence data by the neighbor-joining method. The tested isolates Ras1, Ras2, Ras3 and Ras4 were identified as *S. marcescens*, *E. coli*, *E. cloacae* and *K. pneumoniae*, respectively. The nucleotide sequences of *S. marcescens* Ras1, *E. coli* Ras2, *E. cloacae* Ras3 and *K. pneumoniae* Ras4 were deposited in the GenBank nucleotide sequence database under accession numbers KU237235, KU237236, KU237237 and KU237238, respectively.

Five isolates of each of *S. marcescens*, *E. coli*, *E. cloacae* and *K. pneumoniae* recovered in this investigation were selected and tested for their susceptibility to ten antibiotics (Figure 4). The analysis of the strain resistance to antimicrobial agents showed that norfloxacin, pefloxacin, kanamycin and ceftriaxone inhibited the growth of all tested isolates belonging to *S.*

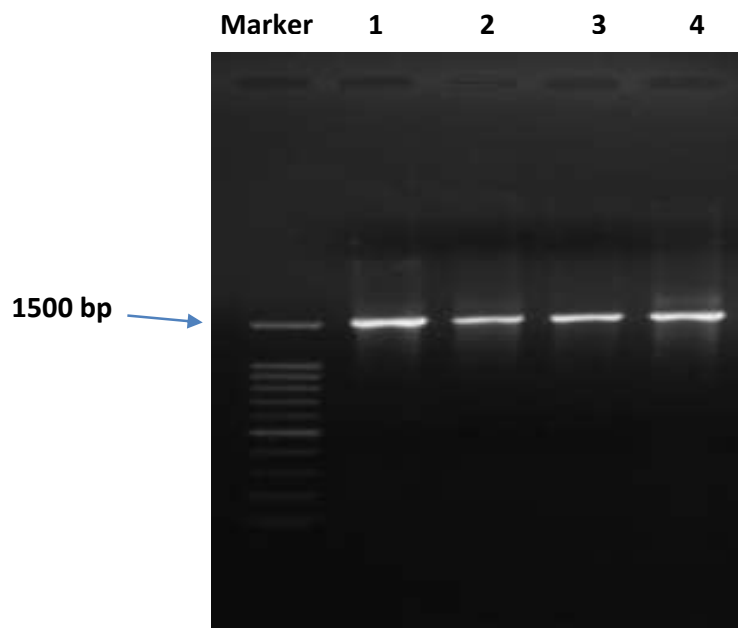


Figure 2. PCR gel electrophoresis of 16S rRNA gene of the four isolated bacterial species showing bands at 1500 bp.

marcescens, *E. coli*, *E. cloacae* and *K. pneumoniae*. Other tested antibiotics showed differential susceptibility. All the tested isolates of the four tested species were resistant to clindamycin. The tested isolates of *S. marcescens* were also resistant to cefuroxime sodium, cephalixin, cephalothin, streptomycin and rifampicin, whereas, were highly sensitive to norfloxacin, pefloxacin, kanamycin and ceftriaxone. The tested isolates of *E. coli* were highly sensitive to norfloxacin, pefloxacin, ceftriaxone and cephalothin but with moderate sensitivity to cefuroxime sodium, cephalixin and streptomycin and had low sensitivity to the others antibiotics. *E. cloacae* isolates were resistant for cephalixin, cephalothin and clindamycin, however, were highly sensitive to norfloxacin, kanamycin and ceftriaxone. On the other hand, *K. pneumoniae* was resistant to cephalothin, rifampicin in addition to clindamycin and was highly sensitive to norfloxacin, pefloxacin and ceftriaxone. Shiga toxin-producing *E. coli* isolated from samples of meat were multi-resistant, exhibiting resistance to ampicillin, ciprofloxacin, tetracycline, sulfamethoxazole-trimethoprim, gentamycin and streptomycin (Li et al., 2011). In a study carried out by Kalmus et al. (2011) to evaluate antibiotic resistance of *E. coli*, ampicillin, streptomycin and tetracycline resistance were observed in 24.3, 15.6 and 13.5%, respectively, among the *E. coli* isolates. While examining the hygienic and sanitary quality of pasteurized cow's milk, *E. coli* was identified in 77.05% of the samples and the highest rates of resistance to antimicrobial agents were obtained for ampicillin (19.2%), cephalothin (18.9%) and tetracycline

(17.1%) (Zanella et al., 2010). *E. coli* and other coliforms recovered from humans and animals had antibiotic resistance and several species were resistant to many antimicrobial agents commonly used in human and veterinary medicine (Greeson et al., 2013). The treatment of cattle with common antibiotics leads to increase in resistance and transfer of these resistant strains to human hosts (Rinsky et al., 2013; Barnett and Linder 2014; Cordoba et al., 2015).

Conclusion

The contamination of food products by bacteria exclusively, Enterobacteriaceae, represents a major problem in food production and leads to food spoilage and human illness. Ground beef processing passes through different steps which may be the route to contamination by *E. coli* and other enteric bacteria. Based on the results obtained in the current investigation, it was observed that ground beef may act as a vector for transmission of *E. coli* and other members of Enterobacteriaceae to man. So, more precautions and strict hygienic measures should be taken to clean and disinfect meat during the processing stages of production in order to avoid contamination by these bacteria. Also, the treatment of cattle with therapeutic antibiotics should be reduced or replaced by natural therapeutic herbs in order to reduce the appearance of resistant enteric bacteria in these animals and the produced food, and reduce the spread of antibiotic resistance among normal

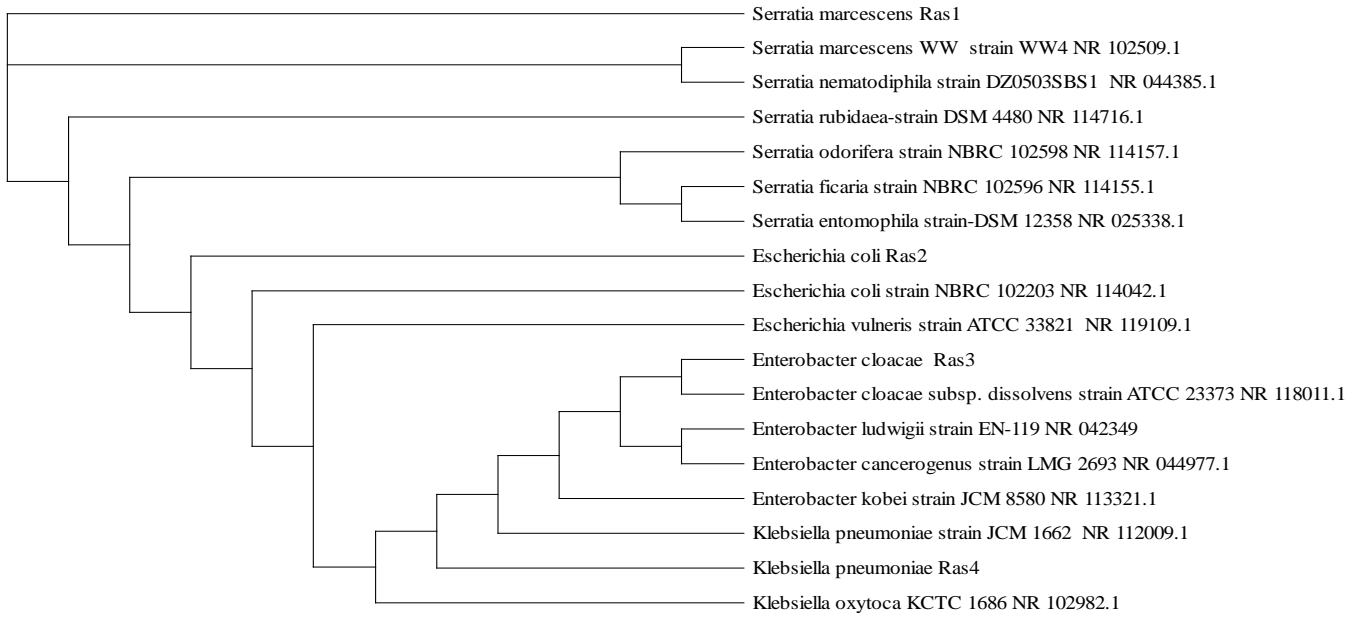


Figure 3. Neighbor-joining tree based on 16S rRNA gene sequences showing positions of the isolated bacteria and related strains.

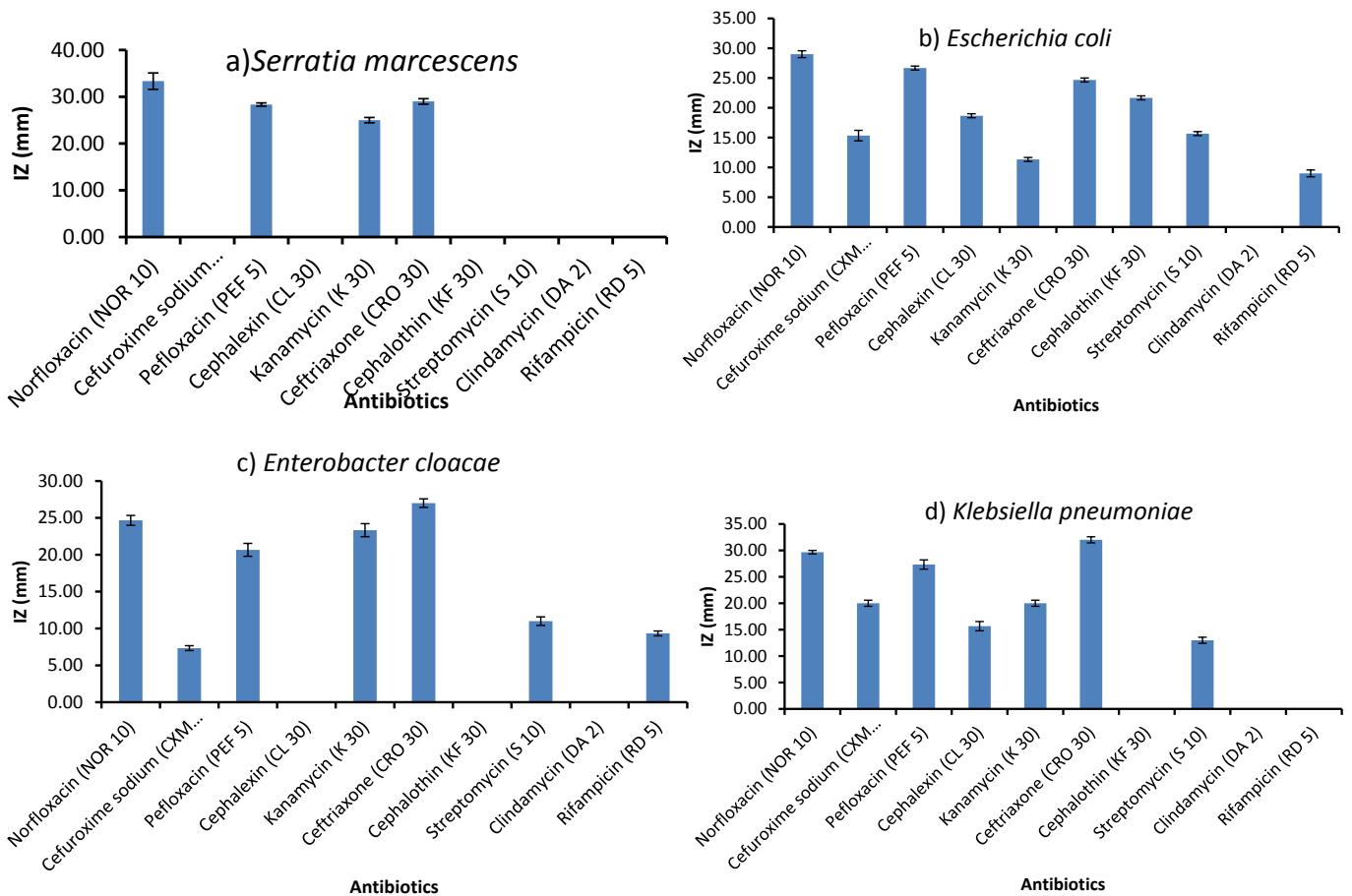


Figure 4. Antibiotic susceptibility indicated by inhibition zones (mm) formed against; a) *S. marcescens*, b) *E. coli*, c) *E. cloacae* and d) *K. pneumoniae*.

flora and pathogenic bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors appreciate the Department of Botany and Microbiology, Faculty of Science, Suez University for providing the research facility.

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