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EFFECT OF GAMMA RADIATION ON SURVIVAL AND GROWTH OF SOME GRAM - NEGATIVE BACTERIA ISOLATED FROM ANIMAL FEED STUFF AND FEED ADDITIVES

By

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ABSTRACT

Bacteriological examination for some Gram-negative bacteria (*E.coli, Salmonella* and *Pseudomonas aeruginosa*) from 200 samples of bone meal-protein meal, fish meal and poultry residue meal (50 samples for each) obtained from different sources was done. Results revealed that *E.coli* isolates recovered with an incidence 12% while *Pseudomonas aeruginosa* and *Salmonella* isolates were recovered with an incidence (5% and 3%) respectively. *Salmonella* serovars isolated were *S.*Typhimurium, *S.*Enteritidis and *S.* Salamae in percent 1.5, 1 and 0.5 % respectively. *Pseudomonas aeruginosa* serotypes were (A, D and M) while *E. coli* serotypes were O26, O18, O78 and O157. Bacterial suspension of the isolates were prepared in a concentration of ($3x10^8$ cfu/ml) and exposed directly to Gamma rays emitted from cobalt 60 source at room temperature at a dose rate 3-7 kGy/hr, Complete inhibition of bacterial growth was recorded at dose 4 kGy/hr from exposure to Gamma rays .At the same time, the bacterial suspension of the isolates in a concentration of ($3x10^8$ cfu/ml) inoculated in fish meal samples and exposed directly to Gamma rays emitted from cobalt 60 source at room temperature at a dose rate 3-7 kGy/hr, complete sterility of the feed stuff was recorded at a dose 6 kGy/hr exposure to Gamma rays. In conclusion, Gamma radiation of feed samples contaminated with *E. coli*, *P. aeruginosa* and *Salmonella* serovars revealed complete sterilization and bacteria couldn't be detected by culture resolution.

Key words:

E .coli, *P. aeruginosa* and *Salmonella* serovars**,** Gamma rays (cobalt 60).

INTRODUCTION

Feed stuffs contaminated with Gram negative bacteria are important sources of dissemination of the organism among animals and poultry flocks. Protein in the form of meals is usually the

major source of contamination the rations (**Scallan *et al.,* 2011)**. The use of by- product feed stuff in animal feed has increased in recent years. Enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella enterica* are the most critical food borne pathogenic bacteria and are often found asymptomatically in the gastrointestinal tract of farm animals **(Ferens and Hovde 2011, Scallan *et al.,* 2011)**. When these pathogens are transmitted to a human, they cause severe illness or even death **(Scharff, 2010).** Gamma radiation has a narrow range of length and high energy penetration power resulting from the nuclear disintegration of certain radioactive substances such as the isotopes Cobalt 60 (Co 6o) **(Farrag and Saleh 1996 and Gross, 2007).** Ionizing radiation is defined as radiation that has sufficient energy to remove electrons from atoms and molecules and convert them to electrically charged particles called ions **(Abo-State *et al.,* 2014).** Ionizing radiation can damage the nucleic acids and kill microbes by direct and indirect hits. Direct hit occurs when ionizing radiation directly disrupt nucleic acids, especially DNA. Gamma radiation induced three types of damage in DNA, single strand breaks, double strand breaks and nucleotide damage which include base damage and damage in the sugar moiety. Some bacteria can resist to ionizing radiation and others are sensitive depending on D_{10} value. D_{10} value is defined as the radiation dose required reducing the population by 10 fold or required to kill 90% of total viable number **(Billi *et al*., 2000 and Niemira *et al*., 2006).** Ionizing radiation is able to inactive food borne pathogens on a variety of food products and can serve as a final critical control point to ensure the microbiological safety to food **(Kundu *et al.,* 2014).** Therefore, the aim of the present study was to recover and identify some Gram negative bacteria (*E.coli, Salmonella* and *Pseudomonas aeruginosa*) from feed stuffs and study the effect of Gamma-radiation (Cobalt 60) on these isolated bacteria. Different concentrations of Gamma radiation 3-7 kGy/hr were used for complete inhibition of bacterial growth and complete sterility of feed stuff.

MATERIAL AND METHODS

Sampling:

A total of 200 samples of feed stuffs (50 of each), bone meal, protein meal, fish meal, poultry residue meal was collected from different areas of Egypt.

Isolation and Identification of some Gram negative bacteria were performed according to **ISO 6579-2002, Koneman *et al.,* (1997) and Quinn *et al.,* (2002).**

Procedures for isolation of *Salmonella* isolates:

Day. (1): Non selective pre-enrichment: 25gm of feed stuffs were added to 225ml buffer peptone water to be incubated at 37°C overnight (16-20 hours).

Day. (2): Selective enrichment:

(I) One ml of the pre-enrichment was transferred to 10 ml tetrathionate broth (Müller-Kaufmann) tubes were incubated 37°C±0.5°C 24hs.

(II) Also 0.1ml of the pre-enrichment broth was transferred to 10ml Rappaport Vassiliadis soy peptone (RVS) broth - then incubated at 41.5°C±0.5°C (18.24 hours).

Day. (3): Spread on selective agar plates:

A loop full from the inoculated and incubated tetrathionate broth (I) and RVS broth (II) were spread onto XLD (xylose – lysine Deoxycholate) and onto BGA (Brilliant Green) agar plates and were incubated at 37°C overnight (18-24hours).

Day. (4): A typical *Salmonella* colony on XLD has as lightly transparent zone of reddish colour and a black centre; a pink-red zone may be seen in the media surrounding the colonies. Typical *Salmonella* colonies on a BGA plate cause the colour of the medium to be red/pink. The colonies are grey - red dish pink and slightly convex.

Day. (5): *Salmonella* suspected colonies were picked up and inoculated onto nutrient agar plate's media for biochemical confirmation of *Salmonella* and serotyping.

Day. (5-7): Biochemical confirmation and serotyping of *Salmonella*.

Suspected colonies were confirmed using TSI agar-urea agar- lysine decarboxylation medium - isolates giving typical *Salmonella* spp. reaction and isolates which are suggestive but not typical *Salmonella* spp. should be confirmed by commercially available API 20E or API 10S. The biochemically identified isolates as *Salmonella* were subjected to serological identification according to **Kauffman White Scheme (Kauffman, 1973)** using O and H antisera. **(Minor and Popoff, 2000).**

Isolation and Identification of *P. aeruginosa* (I.C.M.S.F. 1978).

25 grams of each sample were mixed with 225ml louffered peptone water (pH 7.2) and incubated at 37°C for 24 hours then streaked onto Pseudomonas agar base with 0.1% cetramide and inoculated at 37°C for 24 to 48 hours. Pure culture was from colonies suspected to be *P. aeruginosa* and was later identified biochemically according to**)** and

through using API-20E (Biomerieux). *P. aeruginosa* isolates were serogrouped according to **Homma, (1982)** using antisera from Denka Seiken Co. Ltd, Lokyo, Japan.

Isolation and identification of *E. coli* using solid selective media such as MacConkey agar, Brilliant agar and XLD agar (Xylose-Lysine-Deoxycholate) agar.

Serological identification of *E.coli* were serotyped by using the *E. coli* immune O. sera (8 polyvalent sera vials and 43 monovalent antisera vials that were obtained from DENK SEIKEN CO. LTD. TOKYO. Japan. The procedure outlined of *E. coli* serotyping was carried out according to **Edward and Ewing (1972).**

Preparation of Bacterial suspension:

The well grown separated colony of each isolated pathogenic bacteria was picked up and inoculated into nutrient broth medium and incubated at 37°C for 24 hours. The well grow cultures were centrifuged at 8000 rpm for 10 min. The pellet of each isolated strain was washed by sterile saline (0.8% Nacl) twice and resuspended in sterile saline to from homogenous bacterial suspension (≈ $3.0x10^8$ cfu/ml) using turbidity tube (McFarland tube, no.1). The procedure was carried out according to **Martin *et al.,* (1981).** Homogenous bacterial suspension of the pathogenic bacteria (*Salmonella*, *P. aeruginosa* and *E. coli*) $3.0x10^8$ cfu/ml were inoculated into fish meal samples (25 gm each).

Irradiation process on bacterial suspension and contaminated bacterial samples:

Several trials were carried out to detect the optimum dose of Gamma radiation required for complete sterilization of the isolated microorganisms. The bacterial suspension of the isolated microorganisms and inoculated feed stuff with microorganisms were exposed to Gamma rays emitted from cobalt 60 source at room temperature at a dose rate of 3-7 KGy/hr.the irradiation process was carried out using the facilities of the National Centre for Radiation Research and Technology. Egypt. After treatment with Gamma radiation at different doses 3-7 KGy/hr. One loopful from each was cultivated for resolution on nutrient agar to determine the optional sterilization dose **(Diaa-Aldeen and Farag 1996).**

RESULTS

Results revealed that *E. coli* isolates recovered with an incidence 12% while *Pseudomonas aeruginosa* and *Salmonella* isolates were recovered with an incidence (5% and 3%) respectively.

Table (1): Incidence of some Gram negative bacteria recovered from 200 samples

Type of samples	No.	*Salmonella* isolates	*%	*Pseudomonas aeruginosa*	*%	*E. coli*	*%	Total	*%
-bone meal	50	2	4	2	4	8	16	12	24
-Protein meal	50	1	2	2	4	6	12	9	18
-Fish meal	50	1	2	5	10	4	8	10	20
-Poultry residue meal	50	2	4	1	2	6	12	9	18
Total	200	6	3**	10	5**	24	12**	40	20**

***% was calculated according 50 samples.**

****% was calculated according to total No. 200 samples.**

***Salmonella* serovars isolated were *S.* Typhimurium, *S.* Enteritidis and *S.* Salamae in percent (1.5, 1 and 0.5 %) respectively. *Pseudomonas aeruginosa* serotypes were (A, D and M) while *E. coli* serotypes were O26, O18, O78 and O157.**

Table (2): *Salmonella* serovars isolated from different feed stuffs

Salmonella serovars	bone meal *(50)	Protein meal *(50)	Fish meal *(50)	Poultry residue meal *(50)	Total	**%
S. Typhimurium	2	-	-	1	3	1.5
S. Enteritidis	-	1	-	1	2	1
S. Salamae	-	-	1	-	1	0.5
**Total	2	1	1	2	6	3

Table (3): Serotyping of *Pseudomonas aeruginosa* strains isolated from different origins

P. aeruginosa serogroups	bone meal *(50)	Protein meal *(50)	Fish meal *(50)	Poultry residue meal *(50)	Total	**%
P. aeruginosa serogroups A	1	1	2	-	4	2
P. aeruginosa serogroups D	-	1	1	-	2	1
P. aeruginosa serogroups M	1	-	2	1	4	2
**Total	2	2	5	1	10	5

Table (4): Serotyping of *E. coli* strains isolated from different origins

E. coli	bone meal *(50)	Protein meal *(50)	Fish meal *(50)	Poultry residue meal *(50)	Total	**%
O26	2	2	-	-	4	2
O18	2	1	1	2	6	3
O78	2	1	1	4	8	4
O157	2	2	2	-	6	3
**Total	8	6	4	6	24	12

*** No. of samples**
**** Total no. of samples**

Table (5): Effect of Gamma rays emitted from cobalt 60 source at a dose rate 3-7 KGy/hr in comparison on 6 of both bacterial suspension and inoculated fish meal with Gram negative bacteria.

Isolated microorganisms	Bacterial suspension						Inoculated fish meal					
	Doses of ray by KGy/h						Doses of ray by KGy/h					
	3	3.5	4	5	6	7	3	3.5	4	5	6	7
***Salmonella* serovars**												
***S.* Typhimurium**	+	+	-	-	-	-	+	+	+	-	-	-
S. Enteritidis	+	+	-	-	-	-	+	+	+	-	-	-
***S.* Salamae**	+	+	-	-	-	-	+	+	+	-	-	-
***Pseudomonas* serovars**												
***P. aeruginosa* A**	+	+	-	-	-	-	+	+	+	+	-	-
***P. aeruginosa* D**	+	-	-	-	-	-	+	+	+	+	-	-
***P. aeruginosa* M**	+	-	-	-	-	-	+	+	+	+	-	-
E. coli												
O26	+	+	-	-	-	-	+	+	+	+	-	-
O18	-	-	-	-	-	-	-	-	-	-	-	-
O78	-	-	-	-	-	-	-	-	-	-	-	-
O157	-	-	-	-	-	-	-	-	-	-	-	-

+: means growth of bacteria after radiation on selective media.
-: means No. growth of bacteria after radiation on selective media.

DISCUSSION

Gram-negative bacteria are spread worldwide, in virtually all environments that support life. The Gram negative bacteria include the model organism, *Escherichia coli* as well as several bacteria in animal and human diseases such as *Salmonella, Pseudomonas aeruginosa.* Gram-negative bacteria cause infections including pneumonia blood strain infections, wound or surgical site

infections and meningitis in health care settings. Gram-negative bacteria are resistant to multiple drugs and are increasingly resistant to most available antibiotics **(Yoshito *et al.*, 2006)**. Animal feed ingredients of animal origin may be contaminated with Gram-negative bacteria and variable differences results regarding the estimated bacteria prevalence was reported by different studies assessing contamination of feed ingredients originating from animals have always been considered major source of *Salmonella* in feeds and several studies have provided varied data on the prevalence on *Salmonella* in these feed ingredients **(FSA 2008c).** The results outlined as in (Table 1) revealed that the prevalence of *Salmonella* in (200 samples) of bone weal, protein meal, fish meal and poultry residue meal was 3% which was lower than **Li *et al.,* (2012)** results who tested animal feed samples for presence of *Salmonella.* Those were positive for *Salmonella* underwent serotyping. 12.5% of the samples, while **Papadopoulou *et al.,* (2009)** recoded that *Salmonella* isolation from feed stuff were 1.1%. National surveillance programs in Europe recorded that level of *Salmonella* contamination between these years in the EU (1.9% to 2.9%) **(EFSA 2010)**. As shown in (Table 2) *Salmonella* serovars isolated from different feed stuffs were *S.*Typhimurium*, S.* Enteritidis and *S.* Salamae*. S.* Typhimurium in an incidence of 1.5%, 1% and 0.5% respectively. A wide variety of *Samonella* serovars have been identified in feeding stuffs. Surveillance performed by European Member states shows that occurrence of *S.* Typhimurium and *S.* Enteritidis in feeding stuffs was low and mainly other *Salmonella* serovars were detected. *S.* Enteritidis accounted for 6.2% and *S.* Typhimurium accounted for 4.1% of the *Salmonella* isolated from compound feed for poultry in the EU. A Dutch study identified 28 different serovars in poultry feed samples. No *Salmonella.* Enteritidis was found. Other studies did isolate *S.* Enteritidis and *S.* Typhimurium from feeding stuffs and 20 strains of *S.* Enteritidis were recovered from Japanese commercial layer feeds. These points were mentioned before by **Tine *et al.,* (2012)** that *S.*Montevideo was the most commonly isolated serovar recovered from meat meal in a Canadian study assessing *Salmonella* prevalence in feed mills. Also Contamination of feed with *Salmonella* can originate from different sources and a variety of routes. Various feeding stuffs, production facilities and systems, and conservation conditions may be used, offering different opportunities for contamination of feed with zoonotic pathogens. Main sources of contamination are fertilizers on the pasture/fields,

ingredients,Co-products, duts, wild animals e.g (birds, rodents) and contaminated equipment. **(Tine *et al.,* 2012).** According to (Table 1) *Pseudomona aeruginosa* was detected in 5% of the samples. The results agreed with **El-Gohary *et al.,* (2012)** who studied the incidence of *P. aeruginosa* in different feed stuffs samples. Out of 642 samples, 33 were positive for *P. aeruginosa* with an incidence of 5.1%. The isolated strains belonged to (8) serogroups (A-B - D- F - H - K- L and M). The present results were in accordance with **Ibrahim, (2002)** who found that the percent of *P. aeruginosa* in feed stuffs was 5% and disagree with **Alexander Boyer *et al.,* (*2011*)** who isolated *P.aeruginosa* from environmental samples with an incidence (31%). **El-Gohary, (2004)** recorded that,the most prevalent serogroups were H and B from water and ration. According to (Table 4) our study revealed that *E. coli* isolation rate was in 12% of different samples. Serotyping of isolated *E. coli* revealed that, the most dominant serotypes were (O78, O18, O26 and O157) **Davis *et al.,* (2003)** reported that feed was a vehicle for transmission of *Salmonells enterica* in cattle and several lives of evidence suggest that feed can be a vehicle for transmitting *Escherichia coli* O157:H7 as well. They compared isolates from feed samples to bovine fecal isolates from the same farm using pulsed field gel electrophoresis (PFGE). 0.2% of component feed samples and 0.4% of feed mill samples were positive for *E. coli* O157:H7 while 0.8% of component feed samples and none of feed mill samples were positive for *Salmonella*. PFGE profiles from *E. coli* O157:H7 isolated from a component feed samples closely resembled that from a fecal isolate collected later from the same farm. There were indistinguishable PFGE profiles from component feed *Salmonella* Typhimurium isolates and fecal isolates from the same farm. According to (Table 5) irradiation was more effective in case of bacterial suspension as all bacteria was killed at 4.0 KGy at the same time, irradiation treatment in contaminated samples was less effective as all bacteria killed at 6 KGy, that may attributed to the protective effect of the feed stuff nutrition as mentioned before with **Trampuz *et al.,* (2006)**.Some reduction of the nutritional values of the feed must be expected from irradiation, thus irradiation dose at 25KGy can destroy amino acids and lower dose (10KGy) can destroy thiamine and riboflavin and requires additional supplementation. Irradiation changes the structure of the bacterial DNA as discussed by **Maciorowski *et al.,* (2004).** Also said that a synergistic combination of heat, irradiation and chemical treatment was suggested to reduce the microflora on animal feed and maximum of 20 KGy would be sufficient for irradiation of pelleted feed.

While **Diaa-Aldeen and Farag (1996)** used Gamma radiation with doses 15-50KGy/h on animal fish meal and recorded that there is no change in the nutritive value of the fish meals with high doses of Gamma radiation (50KGy/h). On the other hand **Abostate *et al.*, (2014)** determined the close response curve of Gram-negative bacteria as *E. coli, P. aeruginosa* and *Salmonella* spp. which have been isolated from water against increasing doses of Gama radiation; 4KGy reduced the viability of *P. aerugenosa* and *E. coli* completely. However, 3.0KGy reduced the viable count of *Salmonella* spp., 10.0KGy reduced the viable count of all Gram negative bacteria and water become safe.

CONCLUSION

From results obtained in this study, feed has been reported as a vehicle for transmission of some Gram-negative bacteria which effect on poultry industry. The main sources of contamination feed stuffs are fertilizer on the pasture/fields ingredients, co. products, dust, wild animals. Preservation and storage of the feed stuffs is very important and must be controlled. Periodical examination of the stored feed stuffs must be done. So, Radiation at suitable dose (4-6KGy/h) may be recommended as it is easy and inexpensive. Also, synergistic combination of heat irradiation and chemical treatment was suggested to reduce the microflora on animal pelleted feed.

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