MICROBIOLOGICAL AND RELATED CHANGES DURING FERMENTATION OF POULTRY WASTE

A Thesis Submitted to
The University of Mysore, Mysore
For the award of the degree of
Doctor of Philosophy
in
Food Science

By

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Department of Meat, Fish and Poultry Technology
Central Food Technological Research Institute
Mysore - 570 013, India
December 1996

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Certificate

I *hereby certify that the Thesis entitled* ***`Microbiological and Related changes during fermentation of poultry waste'*** *submitted by Mrs. Doris M. Shaw for the degree of* ***Doctor of Philosophy*** *in Food Science in the University of Mysore, Mysore is the result of the research work carried out by her in the Department of Meat, Fish and Poultry Technology, Central Food Technological Research Institute, Mysore, Under my guidance during 1992 - 1996.*

D. Narasimha Rao

23.12.96

(D. Narashima Rao)
Guide

Declaration

The work incorporated in this thesis entitled ***`Microbiological and Related changes during fermentation of,poultry waste'*** *for the degree of* ***Doctor of Philosophy*** *in* ***Food Science*** *in the University of Mysore, Mysore* ***was*** *carried out by me at the department of the Department of Meat, Fish and Poultry Technology, Central Food Technological Research Institute, Mysore, under the guidance of DR. D. Narashima Rao, during the period 1992 -1996.*

I further declare that the results of the work presented in this thesis have not been submitted previously for the award of any degree, diploma or any other similiar title.

Doris M. Shaw 23.12.96

Doris m. Shaw

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Glossary

BOD	Biological Oxygen Demand
BSM	Back Slop Material
C	Centrigrade
Cal	Calories
cfu	Colony forming unit
cm	centrimeter
df	Degree of Freedom
FCR	Feed Conversion Ration
g	Gram(s)
h	Hour(s)
IBD	Infectious Bursal Disease
kg	Kilogram(s)
l	Litres
LAB	Lactic acid bacteria
LDPE	Low density polyethylene
Log	Logairthum
ME	Metabolizable energy
mg	Milligram(s)
min	Minute(s)
ml	Millilitre
mm	Millimeter
MPN	Most probable number
MRS	de Man, Rogosa and Sharpe
N	Normal
nm	Nanometer
µ	Neu
ppm	Parts per million
rpm	revolutions per minute
SE	Standard Error
SEm	Standard Error Mean
TPC	Total Plate count
TTC	Trichloro acetic acid
v/w	Volume / Weight
wt	Weight
w/w	Weight / Weight
wk	Week

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SYNOPSIS

Fermentation has centuries of` history of achievement without theoretical foundation. Fermentation preserved food through the accumulation of lactic acid in the preparation of cheese, soured milk, sauerkraut and also ensilage. It was Pasteur's demonstration on the role of microbes, that led to his definition of fermentation as "life without air". As Pasteur correctly recognized that in fermentation, the organisms derive metabolic energy by its property of performing its respiratory function, somehow or other, with the oxygen existing combined in sugar.

Superficially it might seem that microorganisms are generally harmful but this is far from the truth. Their tremendous benefits offered to man greatly outweigh their harmful effects. Not only are microbial activities essential in recycling nutrients and energy, but also microbial techniques are applied commercially to provide foods, beverages, pharmaceutical products, chemicals and even feed. Throughout history man has utilized microorganisms. But only during the last century he began to learn about the organisms involved. Continued research and increased understanding of microbial activities undoubtedly has led to various benefits. It is very important that one controls the undesirable microorganisms so that the desirable useful bacteria (which are most wanted) will be provided the best of favorable conditions and nutrition. The desirable microorganisms dominate, so that a pure, uncontaminated

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product can be obtained. Likewise in fermentation the selection of specific microorganism is necessary to derive a particular product. This necessitates understanding and applying the principles of microbial growth and physiology.

The term fermentation is correctly employed to describe the breakdown of carbohydrates and other materials under anaerobic conditions where primary interest is in describing the end rather than the mechanisms of biochemical reactions.

Silage is a process in material that undergoes fermentation where in bacteria produce lactic acid, acetic acid and butyric acid from sugar present in the raw material. The net result is reduction in pH which prevents the growth of spoilage microorganisms.

The efficiency of silage is judged according to the relative proportions of the acids produced during fermentation. The greater the proportion of lactic acid to butyric acid the higher is the efficiency. A good quality silage is one which is favoured by the lactics-producing lactic acid, while one with butyric acid is referred to as silage of poor quality.

Many reports have appeared on the understanding of silage processes as affected by environmental factors. The effects of these factors on microbial succession are yet to be understood during fermentation. The ensilage technique has also been applied for the preservation of fish and fish wastes (Woolford, 1984). It appears that fermentation process was mainly due to homofermentative bacteria in fish and fish waste silage. It is also observed that there is much variation in the type of fermentation from one silage fermentation to another (Woolford. 1984). The significant bacteria in silage fermentation are: Lactic acid bacteria, Endospore

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forming bacteria. Coliform bacteria and the fungi. These groups could be further classified as spoilage, pathogenic and acid producers. The pathogenic microorganisms are the Pseudomonas and fungi. The acid producer mainly is lactic acid bacteria. Yeasts may also contribute to the production of acids.

Fermentation silage has become an important method of preservation of waste materials. Since the poultry industry has enormously expanded in recent years in India, large quantities of waste material such as poultry viscera are generated. These wastes cause environmental pollution and health hazards. This waste material contains large quantities of proteinaceous material. It is therefore necessary to conserve this waste material for useful purposes. Thus an economical and simple process such as fermentation silage process may be a very useful method for preserving poultry intestine, especially in tropical countries like India. There is no available data and information on the type of fermentation (homofermentative and heterofermentative) taking place in the poultry viscera.

Therefore attempts have been made in the present investigation to understand the microorganisms involved in the ensilage of poultry intestine, their interactions with each other, their behaviour towards environmental variables, within the ensiled environment, and their usefulness in preserving the proteinaceous material like poultry intestine.

This thesis comprises of seven chapters covering the microbial aspects of poultry intestine, microbial succession during the fermentation, factors affecting fermentation and development of stable fermented material which can be utilized as a feed ingredient in animal diets.

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The first chapter deals with test material used and its source, different bacterial media used to culture different bacteria and their composition and details about certain fine chemicals, solutions and their composition and bacterial species used in the *in vitro* studies and their sources.

Second chapter highlights the quantitative and qualitative determination of characteristics associated with poultry intestine. The study revealed that poultry intestine harbours pathogenic, spoilage microorganisms and is also rich in lactic acid bacteria. The presence of lactic acid bacteria is advantageous for the preservation of poultry intestine. Pathogenic microorganisms isolated were **Salmonella** and **E.coli.** Species of Salmonella identified where **S.typhimurium, S.gallinarium, S.virchow, S.cerro, S.entritids.** E.coli microorganisms were identified up to serotype level, 38 serotypes were obtained from poultry intestine : **0166, 0144, Rough, 064, 057, 01016, 039, 084, UT, 042, 054, 016, 020, 0116, 0103, 0100, 0101, 0157, 081, 0154, 062, 027, 03, 0146, 0147, 0132, 0159, 0134, 0169, 0116, 09, 0129, 021, 011, 0130, 045, 0109, 08.** The spoilage microorganisms isolated was **Pseudomonas,** and the species identified were Ps.maltophila, Ps.cepacia, **Ps.pseudomalleli, Ps.stutzeri, Ps.diminuta, Ps.mallei.**

The third chapter gives a detailed account of the physiological and biochemical characteristics of lactic acid bacteria. The growth characteristics of **Lactobascillus plantarum** was studied as it was founded to be the main species involved in ensilage in the present studies. Antimicrobiai substances produced by species of **lactobacillus** were tested with pathogenic strains of E.coli and Salmonella isolated from poultry intestine. This gave a clear insight into the interaction of species

among themselves in poultry intestine silage. The antimicrobial substances produced by lactobacilli strains when tested against these pathogenic species revealed the inhibition or growth retardation in the presence of the antimicrobial substances. This showed that the lactic acid bacteria inhibited pathogen present in poultry intestine during ensilage.

Fourth chapter gives an account of optimization of various additives: like molasses, antimycotic agents (Sorbic acid, Benzoic acid and Propionic acid) and sodium chloride. It was evident that 10% molasses(w/w) proved best with propionic acid at 0.5%. Salt addition was not suitable as it results into increased salt level in the feed which would be unpalatable to the poultry.

Temperature, an important factor was also taken into account to develop a rapid technique for ensiling process. Fermentation at ambient temperature did inhibit all spoilage and pathogenic forms in 24 hours but fermentation at 37 °C increased the rapidity of the preocess and within 12 hours, all undesirable organisms were inhibited. Temperature variation also led to develop a faster process for ensiling poultry intestine so as to minimize the loss of nutrients. It was at pH 4.2 that the material attained stability and could be considered safe.

In the fifth chapter succession of microorganisms was studied. It was noticed that aerobic flora consisting of E.coli, Staphylococcus and Enterococci were also present in the early stages of fermentation. But during fermentation, lactic acid bacteria became dominant and has helped in conserving the raw material due to the production of acid. Succession with regard to change in temperature was also studied. At 37 °C, reduction of undesired microorganisms was more than at ambient

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temperature. A study on the survival of **Salmonella** was also conducted. Salmonella inhibition was observed during the fermentation process. This was attributed to the production of lactic acid. Decrease in pH, increase in acid, decrease of undesirable organisms and maintenance of lactic acid bacteria were observed in the microbial succession during fermentation process. A correlation has been drawn between increase in lactic acid bacteria counts and decrease in reducing sugars.

The sixth chapter deals with the effect of addition of backslop material (which is the stabilized fermented material having **a** pH of 4.2) on fermentation process. When the fermented silage has reached a pH of 4.2 at 24 hours, this referred to as *backslop* material. Blackslop material was inoculated into freshly homogenized poultry intestine containing molasses and propionic acid. pH changes and the effect of backslop material on pathogenic bacteria were studied. It was noticed that 5% was sufficient to act as backslop material. At all these three levels of (5%, 25% and 35%) backslop material tested pathogenic and spoilage microorganisms were eliminated in 12 hours. This indicated that addition of backslop material (as culture) has brought rapid inhibition of undesired microorganisms.

In the seventh chapter, the stabilized product was evaluated for its chemical composition and safety. Stabilized product was analysed for the presence of **E.coli, Staphylococcus, Enterobactcria and Salmonella.** Results indicated that fermented product was free from pathogenic microorganisms and was found to be safe microbiologically. Feeding trials have been carried out using poultry intestine silage in broiler chicks feed. Poultry intestine silage was used at 25% and 50% level in the place of fish meal. The chicken fed with poultry intestine silage did not show any

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adverse effects in growth. Histological data of organs from experimental birds showed no abnormalities. Sensory analysis revealed that meat from silage fed birds has been found acceptable. Thus, the studies revealed that poultry intestine silage can be effectively substituted for fish meal up to 50% level.

Signature of the Candidate

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Part - I

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REVIEW OF LITERATURE

Silage

Silage making was traditionally used for preserving wet fodder and storing it in a silo; collectively referred to as silage. Nowadays silage product formation has been extended not only for grass but also for other materials of sufficiently high moisture content (e.g.: forage corn, forage legumes, fish viscera, whole fish and poultry waste and poultry viscera) liable to spoilage by aerobic microorganisms. These materials were stored anaerobically. This process is also referred to as ensilage.

Microaerophilic or anaerobic bacteria naturally present in the raw material utilize the sugars and convert them into acid which lowers the pH sufficiently to prevent spoilage of the product. This process is often called fermentation ensilage. Microorganisms especially lactic acid bacteria play the essential and vital role in fermentation and help preserving the material for useful purposes like feeds. Acids are also used to prevent spoilage of materials. This is called acid ensilage. Both these methods i.e. fermentation ensilage and acid ensilage aim at minimizing nutrient loss and / or increasing the acceptability of the concerned products to livestock.

History of Silage

Silage production is not a recent development. It has its origin in antiquity. Kuchler (1962). Kirstein (1963), and Schukking (1976) stated that

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ensilage was practiced around 3,000 years ago. Excavation and traces obtained from old oil paintings and also paintings in the museum at Naples depict that the ancient Egyptians were familiar with art of ensilage dating back to 1,000 to 1,500 BC. (Kirstein, 1963). Records indicated that silos were uncovered from the carthage ruins, and the findings revealed that fodder was ensiled in 1,200 B.C.

In earlier days, ensilage was mainly used for preservation of grains and fodder when provender was in plenty, for unseen days when pestilence and bad weather led to poor harvest. However, in all probability the stored grain did not undergo fermentation, and therefore not necessarily called silage in the strict sense of the word. But the only apparent similarity between ancient and present day modem silage is the use of silo the vessel or structure constructed to store the raw material.

The first reference to the conservation of green fodder by ensilage was that of Professor John Symonds of the University of Cambridge in 1786, who during his tour of Italy, observed the preservation of green leaves in wooden casts (Yust., 1956). The earliest account of ensilage as it is understood today was given by Grieswald in 1842 (Watson,. 1956). In Germany and Hungary it was Johnston (1843) who described the making of "Sour hay" and drew its similarities to the preparation of saurkraut. It was in Western Europe Goffert a French farmer and writer, who promoted the ensiling process (Yust.,1956). Wrightson (1874) reported silage making in the Austro-Hungarian empire and Jenkins (1884) investigated the practice on behalf of the Royal Agricultural Society of England. Silage making was introduced in the United States by Professor F.H. King in

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1891. It was first introduced in Finland in 1920 by A.I. Virtanen (Raa and Gildberg.. 1982).

The ensilage of animal products was prompted by the success of the AIV principle Virtanen (1933). Thus in Scandinavia, attention was paid towards evaluating the potential of the process for the preservation of fish and fish waste. Slaughter house offal, and other by-products of such establishments has high protein sources of animal feed. Even though the objective of plant and animal product ensiling is the same, the former is viable alternative to hay making a mere means of preserving a primary agricultural product; while the latter is an alternative to the energy dependent drying of some forms of surplus or non marketable sources of protein and means of preservation for other forms which would otherwise be disposed off by burning, dumping off or burying.

Production of Silage

i. Raw Materials

Any material which is unstable in air and has, or is provided with sufficient fermentable carbohydrates can be ensiled. Silage product does hold good not only for ensiling of grass or grain but also animal by-products. Therefore the raw material for ensilage production can be divided into the following categories:

A. Silage from plant materials

Silage is made from grasses, legumes such as alfaalfa or peas, whole crop cereals like corn and sorghum, from potatoes, from wastes and food manufacture such as sugar beet pulp and tops, pea haulms and pods, and fruit residues. A

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number of researchers have worked on the ensiling of grass and fodder crops. They have either worked on the microbiological, chemical or / and bio-chemical aspects of ensiling of the above mentioned raw materials. However, grass and corn are the materials most widely ensiled.

It is well established that the extent of fermentation during ensilage is influenced by the dry matter content of the crop (Watson and Nash., 1960; Brown and Kerr., 1965; Gorden., 1965; Nash., 1959). Thus in unwilted silages there is very little residual soluble carbohydrate. The experiments conducted by Mcdonald *et al,* (1968) was, in the first, wilted Italian ryegrass at two different dry levels (34% and 47%) was ensiled, in the second fresh grass (15.9% dry matter) and similar herbage wilted to 30.3% dry matter were ensiled. It was found that total edible dry matter losses from the wilted grasses were low and ranged from 6.7 to 10.4%. Changes in individual sugars and organic acids were followed. The residual amounts of sugars in the wilted silages were directly related to the degree of wilting. Thus from the knowledge of the sugar lost and the amount of mannitol and ethanol formed it has been possible to examine quantitatively the main chemical changes during the ensilage of the wilted materials. The results confirmed the efficiency with which wilted grass anaerobically conserved.

The advantage of wilting crops, before ensiling them, to a dry matter content of 30% or above have been stressed by many workers (Watson and Nash., 1960; Nash, 1959; Woodward and Shepherd., 1938; Murdoch *et al,* 1955; Brown and Kerr., 1965). Apart from reducing or eliminating effluent losses, the

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reduction in moisture content discouraged clostridial activity and produced a silage which was more acceptable to ruminate animals (Gordon *et al.,* 1965). It has been shown that these 'high dry matter' silages have higher pH values and contain more sugar than silages with unwilted grass have given variable results although most workers agree that, provided the silo is adequately sealed, dry matter losses are low. The major nutrients fermented during ensilage of grass by lactic acid bacteria are glucose and fructose and probably malic and citric acids. The fermentation products resulting from the breakdown of the major hexoses in grass vary depending upon whether the organisms reponsible are of the homolactic or of the heterolactic type. The various pathways have been reviwed in a publication by Whittenbury *et al.,* (1967). It can be seen that in the case of the homolactic microorganisms, lactic acid alone is normally formed, whereas with the heterolactic bacteria, manitol is a major product of fructose fermentation and ethanol of glucose fermentation. In conclusion the ensilage of wilted rye grass under anaerobic conditions results in very little loss of nutrients and high losses occured during ensilage of wilted grass, these losses arise from oxidation and not from fermentation. If any, advantages to be gained in terms of reduced dry matter, loss by wilting crops to dry matter contents of greater than 30 to 34%.

Ensilage on ammonia treated straw with whey has been conducted by the use of lactic acid bacteria which are alkaline adapted. Investigations by Suhaimi *et al.,* (1987) has shown lactic acid bacteria were well equiped to adapt to alkaline media and can counter act clostridal growth effectively during the process of ensilage. The results from the study showed that the association or the individual populations of the alkaline adapted lactic acid bacteria can be used effectively as

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an inoculum for ensilage for mixture of ammonia treated straw and whey. In the silage inoculated with the bacterial association, a pH of 4.0 was reached. At this low pH material having a dry matter content as low as 20% can remain stable quite indefinitely if it is not exposed to the air (Mcdonald, 1981). Such pH reduction was also comparable to lucerne silage inoculated with L.plantarum (Ely *et al,* 1981). Addition of lactic acid bacteria during ensiling of herbages have been conducted by Mcdonald *et al,* (1964).

B. Silage from animal by products

Fish and fish offal probably form the bulk of animal product ensiled. Mussel processing ways have also been used as a fermentation substrate (Murado, Gonzalez and Pastrana., 1994) and shrimp waste too have been ensiled (Tatterson and Windsor., 1974; Ariyani and Buckle.. 1991; Sachindra *et al,* 1994).

Contribution on the various aspects on chemical changes, microbiological changes and bio-chemistry of fish silage have been reported. (Arnesen *et al,* 1981).

The chemical usage in ensiling as reported by Petersen (1951); Hanson *et al* (1951); Freeman *et al* (1956) showed the possibility of preserving surplus fish and fish offal in liquid form for animal feed.

Chemical studies on fish silages where cod viscera were stabilized when preserved with formic acid at pH values below 4.0 (Backhoff., 1976). A decrease of soluble carbohydrates and increase of pH showed the microbial spoilage of the formic acid preserved mixture. The first sign of deterioration of that mixture was

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the appearances of patches of mould growth. An unpleasant odour of amines followed the increase of pH and the mixture gradually attained a dark brown colour.

An identical combination product with a silage preserved with 0.75% (v/w) of propionic acid in addition to 0.75% (v/w) formic acid was quite stable, even though the pH of the moist product was about 4.5 (Gildberg and Raa., 1977). After one year such mixtures had a fresh acidic smell which was better than immediately after preparation. Such a silage also remained sterile but with slight ammonia production due to degradation of 8.0% of protein nitrogen of ensilage after 220 days at 27 °C.

Experiments were also carried out using other acids like phosphoric acid, citric acid, sulphuric acid, hydrochloric acid in order to lower pH drastically (1.0 to 4.3) so as to obtain sterile material (Paul Reece., 1980; Disney *et al, 1978).*

Mineral acid like formic acid was used to prepare fish silage to different carbohydrate sources like ground maize and cassava were used as fillers for the preparation of animal feed. (Disney *et al,* 1978). British patent (1962) described treatment using hydrochloric acid, sulphuric acid, formic acid for production of liquid fishing food.

Ensilage or liquid preservation has been achieved by directly using mineral acids or by producing the required acids indirectly. The ensiled products prepared by fermentation have been considered better, as the rancid odour was not developed (Nilsson *et al,* 1961).

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Jew fish and silver bellies were minced with 10% molasses and pure culture (l8h to 22h old) of L.plantarum NCIB 6165. Thus microorganisms were used in preparation of silage (Arul James., 1966).

Hercules *et al.,* (1985) have reported suitability for fish silage production using different cultures of Lactobacilli (L.plantarum-South Africa, L.plantarum-Spray dried culture, L.alimentarius L123 and L.plantarum L45O, L.plantarum LI533, L.plantarum L1535, L.alimentarius L151). On the basis of this work, lactobacillus would appear to differ in their ability to ferment and stabilize fish silage. Of the eight tested five achieved superior fermentation. These were proprietary sprayed dried cultures and stock cultures L45O and LI553 of L.plantarum also stock cultures L123 and L151 of L.alimentarius. These findings had particular reference in the manufacture of fish silage since the material for fish silage would be either fishery waste and or fish off-cuts and not necessarily absolutely fresh.

Hercules *et al.,* (1985) have also studied for comparative purposes and acid preserved silage by acidifying the fish material with 1.9% sulphuric acid and 0.8% formic acid.

The raw materials for fish silage are of the following categories: pelagic fish, trash fish, viscera by-products and others. In the commercial industrial processing offish for human consumptions, yields are about 50% for direct human consumption. The other 50% consists of by-products from the process, e.g: heads, skin, bones and viscera. In some countries these by-products are converted to fish meal and some into fish silage, some part is still wasted (Arason., 1994).

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Fish meal factories are usually placed at the main fishing ports where it is convenient to use the bulk of waste material for fish meal. Fish silage offers an opportunity to make use of by-catch, viscera and by-products from the fishing vessels and the factory trawlers. By-catch is often thrown overboard of its low price.

Pelagic fish may be caught periodically in quantities exceeding the local fish meal processing or freezing capacities. Preserving and storage as silage is a convenient way of utilizing this resource.

Ensiling of poultry offal has been used for the production of feed from these proteinaceous waste material. (Potter and Fuller., 1967; Bharagava and O. Neil., 1975; Erdos., 1984; Kim and Rhee., 1977; Machin *et al,* 1984; Shrivastava and Singh., 1985; Sahoo and Panda., 1983; EL Boushy *et al,* 1985 (Hazarik and Barukh., 1993). But detailed work on the microbiological aspects of poultry waste during ensiling process are not reported.

Detailed work on microorganisms present in the gut, crop, caeca, excreta, quantitative and qualitative aspects have been described by Jayne-Williams and Fuller., (1971).

Reports were available on the colonization of lactic acid bacteria in the intestinal tract of chicken (Victor *et al.,* 1992), the duodenum, ileum, and the ceacum of young chicks (Salanitro *et al .,* 1978) and crop epithellium (Fuller., 1973).

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Bacterial colonization of intestinal tract of chicks take place soon after hatching when the young animals ingest food. (Ochi *et al,* 1965 and Smith.,1965). During the first two to four days streptococci and enterobacteria colonized the small intestine and ceacum. The work of Ochi *et al,* (1964) also indicated that after the first week the composition of the flora stabilized, in that lactobacilli predominated in the small intestine (with smaller numbers of streptococci andenterobacteria), whereas the ceacum was colonized mainly by anaerobes (bacteroids and bifid bacteria) and fewer numbers of facultative bacteria. Other studies on the development of the intestinal flora in chickens (Barnus *et al,* 1972; Huhtanen and Pensack., 1965; Lev and Briggs., 1956; Timms., 1968), showed qualitatively similar results in that lactobacilli and Streptococci (faecal) could be isolated from duodenum and ileum and Clostridium, bacteroids, anaerobic lactobacilli and E.coli could be recovered from the caecum. Work of Salanitro *et al,* (1974) showed several types of anaerobic and facultatively anaerobic bacteria colonize the small intestine as well as the ceacum.

Salmonella is the commonly found pathogen in the intestinal and ceacal contents (Victor *et al,* (1992).

Ecological studies on the lactobacillus flora associated with the crop epithelium of fowl was conducted by Fuller., (1973), where he confirmed Ochi *et al,* (1964) work of lactobacillus predominating in the small intestine. The lactobacillus flora lining the crop of the chicken became established soon after hatching and adhere to the crop epithelium throughout the life of the bird. It has been suggested that these lactobacilli have formed a symbiotic relationship with

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the chicken intestine and helped to regulate the concentration of its intestinal micro flora.

The anaerobes according to Ochi, Mitsuoak and Siga., (1964) formed a major part of the caecal flora of chickens, occurring at more than 10 / g. With five week old chickens fed on a normal broiler diet, Barnes and Imprey (1970) found that the gram negative non-sporing rods (Bacteriodaceae) formed about 40% of the population and were equaled in numbers by the gram positive non-sporing rods (including bifido-bacteria), while peptostreptococci occurred at about 15%, curved rods (possibly spirilla) were also isolated together with other unidentified anaerobes.

Acid ensilage of poultry offal has been carried out by Mahendrakar *et al.,* (1991). Poultry intestine were seperately homogenised and treated with different combinations of sulphuric, formic and propionic acid. The effectiveness of acid treatement was assesed by pH changes. Autolysis in the acidfied homogenates was followed by periodical changes in the nitrogen fraction, that is water - soluble nitrogen, non protein nitrogen and α-NH_2 nitrogen contents. The studies on the nitrogen fraction indicated that autolysis was completed in 3 to 4 days and that the rate of autolysis was governed by pH of the homogenate and not a type of acid, combination used. Acidification of offals to pH below 3.9 significantly reduced the total microbial count and completely eliminated coliforms.

Natural fermentation and addition of chemicals and cultures in animal by-products

It is a well known fact that all raw materials, contain organisms whether it be plant or animal waste. It is the choice of these organisms that matter, to bring about the success in the fermentation.

Natural fermentation involves the usage of desirable microorganisms native to the raw material to suppress undesired microorganisms to achieve fermentation. Werahadikusumah *et al.,* (1972) have reported on the development of lactic acid bacteria (LAB) during the early stages of fermentation. The most commonly fermented animals were fish and shrimp (Amano., 1962; Tanikawa., 1971). Nilsson and Rydn., (1965) proposed the fermentation offish along with a cereal meal as starch could be preserved by lactic acid bacterial fermentation and stored as silage. (Raa., 1981; Raa and Gildberg., 1982).

Chemicals like propionic acid, formic acid, sulphuric acid and hydrochloric acid have been used for the control of microorganisms and reduction of pH so that the raw material becomes useful for the utilization of feed product. A large number of reports are available on the use of inorganic, organic and mineral acids for the preservation offish and poultry waste for feed. (Edin., 1940; Lisac, 1961; Gildberg and Raa., 1977; Strom *et al.,* 1980; Arnesen, Arsaon and Jonsson., 1981; Jangaard., 1991; Mahendrakar *et al.,* 1991; Stormo., 1993).

Though inorganic acids are inexpensive, they were not convenient because their preservation action comes into effect when pH value was down to about 2.0 (Edin., 1940; Arnesen, Arason and Jonsson., 1981). The feed stuff, therefore has

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to be deacidified before it is fed to the animals (Petersen., 1953). Organic acids though expensive than mineral acids have been preferred because their usage has given stabilization at a higher pH (around 4.0). Thus silages produced with organic acids could be used in feed stuffs without neutralization (Tatterson and Windsor., 1973; Gildberg and Raa., 1977). There were reports on the use of alkalis such as Sodium hydroxide, ammonia and urea for ensiling (Han, 1978; Hober *et al*., 1978, 1980).

Direct usage of lactic acid in chick drinking water and feed has been practiced as described by Giordani *et al.,* 1979; Samanta and Biswas., 1994; Meluzzi *et al,* 1980; Vasilena *et al,* 1992 and Samanta., 1993; in order to safeguard the lactic acid bacterial microflora in chicken. Although acid silage is most common, there are several methods known - the use of nitrate to preserve viscera (Freeman and Hooglad., 1956), the use of ammonia (Ramanathan and Moorjani., 1975; Dagbjartsson *et al.,* 1976). Mixtures of organic and inorganic acids have also been used. Olsson., 1942 and Disney, Tatterson and Olley., 1977, have used mixtures of formic acid with sulphuric acid. In the place of sulphuric acid, hydrochloric acid has been used (Disney and Hoofman., 1976; Disney and Tatterson and Olley., 1977) or phosphoric acid (Jensen and Schmidtsdorff., 1977)

The modern trend is the use of pure and mixed cultures for hastening the fermentation process. Pure cultures of lactobacilli (Lactobacillus acidophilusì have been used in feed (Tibbetts., 1983; Tibbetts *et al*., 1984). They have fermented poultry viscera. Barns., (1987) has used lactic acid bacteria for fermentation of poultry by-product. Hercules and Christine., (1985) have used

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eight different lactobacillus cultures to test their suitability for fish silage making. Ami James (1966) has ensiled Jew fish and silver bellies for animal feeds by fermentation with pure cultures of Lactobacillus plantarum NCIB 6105. Russel *et al.,* (1993) have analyzed the effect of inoculating lactic acid bacteria during fermentation of broiler processing waste. Rabia Zuberi *et al.,* (1993) prepared fish silage by microbial fermentation using Lactobacillus plantarum broth.

A mixed culture fermentation such as the association of yeasts and lactic acid bacteria are very common in wide variety of traditional beverage fermentations and traditional food. This usage of mixed cultures has also been reported in animal feed at U.S.A. (Novel process), but with the use of corn as substrate (Wood., 1981). A yeast mixed culture has proved to be a good resort for improvement of the quality of the biomass, to raise the efficiency of the use of the nutrients or even to promote the growth of the microorganisms incapable of doing so alone in a determined medium. Though this could be carried out with different combination of species (Akakai., 1965; Petersen., 1975; Achremowicz, Kosi Kowski and Masuyama., 1977; Harrison., 1978; De Angelis *et al.,* 1979; Abouzied and Reddy., 1987; Fellows and Worgran., 1987 a,b); Lingren and Pleje., (1983) studied the fermentation of a mixture of 80% herrring offal, 10% mollasses and 10% cereal grain. They inoculated the mixture with Pediococcus acidilactis and Lactobacillus plantarum (2×10^8 LAB /g). The advantage of mixed pure cultures have been reported to be more rapid substrate utilization and removal of product inhibition.

In practice there is also the non-sterile mixed culture process which finds extensive application today. The non-sterile process depends upon selecting the

proper conditions of temperature, pH, nutrients and humidity that will favour the growth of desired organisms and will inhibit many of the unwanted organisms. In some cases, a heavy inoculum of desired culture will outgrow undesirable organisms that may be present. The non-sterile mixed culture process constitute the major parts for waste re-cycling now and in the near future. (Bellamy., 1983).

Nutritive value of animal waste silage

There has been considerable interest in the use of microorganisms to upgrade waste and by-products and provide a source of good quality protein for the animal feed industry. Many microorganisms including bacteria and fungi have been investigated (Wiseman and Cole., 1983). They are particularly useful means of detoxifying troublesome pollutants like waste from slaughter house. In fact the number of substrates capable of being fermented by for example, yeast, has been considerable. (Vananuvat., 1977). A consequence of this is that the nutritive value of the final product may be variable, depending on the type of microorganisms. The upgrading of animal offal produces feed stuffs of good quality which have been referred to as meat meal (Batterham *et al.,* 1980). These feed stuffs varied in quality (Cooke and Pugh., 1980).

Several workers have successfully utilized acid preserved silage obtained from different raw materials in the diet of different animal species. Some reports showed that silage was a good source of protein and its nutritional value has been comparable with that offish meal. (Stormo and Strom., 1978k Skrede., 1981; Strom and Eggum., 1981; Raa and Gildberg., 1982; Jackson, Kerr and Cowrey., 1984; KrogdhaL, 1985).

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The nutritional quality of liquefied fish has been found to be superior to thirty day old conventional silage and nearly equal to that to fish meal. (Stone and Hardy., 1986). The studies in fermented fish silage revealed good nutritional value during long term storage. (Nilson and Rydin., 1963; Kompiang, Yushadi and Creswell., 1980; Raa., 1981; Raa and Gildberg., 1982). In fact, fermented fish silage has been shown to have a significantly better nutritional value for chickens than that of fish silage preserved by acid. (Kompiang, Arifudin and Raa., 1980). The nutritional value of fish silage incorporated in animal rations has been shown to be good (Cameron., 1962; Hillyer *et al,* 1976; Disney, Parr and Morgan., 1978; Austreng., 1982). Fish silage has been realistic alternative of fish meal in utilizing fish waste, surplus fish, low value fish, particularly in tropical countries (Disney., 1979). The nutritional status of ensiled fresh water fish species has been reported by Krishnaswami *et al,* (1965).

The value of animal product in nutrition cannot be disputed since they are important sources of protein and amino acids and may contain other nutrients which are beneficial to the growth of the animal.

Ensiling and autolysis do not alter the amino acid composition significantly. Hassan and Heath., (1987) suggested that fermented fish or fish waste increased the soluble nitrogen content, also increased the level of free amino acids and short chain peptides. There were indication however that the trytophane was unstable in acid condition and it is clear that tryptophane was apparently the first limiting amino acid in the fish silage. (Backhoff., 1976; Kompiang, Arifuden

and Raa 1980). In an animal diet the tryptophane level could be improved by cereal protein. (Johnesen and Skred., 1981; Gildberg and Almas., 1986).

The high content of crude protein is relative to grass silage. Data have been produced by Gildberg and Raa., (1977) for silage made from Cod viscera, and by Wirahadikusumah., (1968) for silages made from mixtures of uneviscerated cod, cereal meal and malt, which showed good protein content. When comparing with fish meal, liquid fish silage is similar in terms of chemical composition with regards to dry matter. This silage when incorporated into the diet at about 10% by weight it gave similar responses in nutrition of pigs (Smith and Adamson., 1976). The nutritional quality of liquefied fish has been found to be superior to 30 day old conventional silage and nearly equal to that fish meal (Stone and Hardy., 1986).

A few reports are available on poultry offal silage. Poultry by-product meal is equal to that offish meal. (Machin *et al,* 1984; Shrivastava and Singh., 1985; Hazarika and Bearukah., 1993). Poultry offal is rich in protein (Potter and Fuller., 1967; Neelakantan ., 1976; Erdos., 1984). The amino acid composition of poultry by-products have been described (Wisemen., 1964; Moran *et al,* 1967 b; Jackson., 1971). It was observed that poultry viscera can be preserved by fermentation process (Ockerman and Liu., 1995).

The use of silage as animal feed has been reported by several workers. The possibility of preserving surplus fish and fish offal in a liquid form for animal feed has been suggested (Sperling *et al,* 1950; Petersen., 1951; Hanson *et al,* 1951; Freeman *et al,* 1956). Its usage as a feed to chick, fish, pig and rat has been

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described. Djajasewaka and Djajadiredja., (1980) showed that fresh water carp also grew well on silage base feeds. Work on Salmons Austreng., (1982) revealed good growth on silage diets. Experiments conducted by Emmanuel and Jeong., (1988) on feeding broiler chicks with herring fish meal at 5% showed superior feed: gain ratio. Rainbow trout fed diets containing silage preserved with propionic acid did not show aversion to feed nor growth depression (Lall., 1991). Silage produced from whole herring, dogfish and cod were efficiently utilized by Atlantic salmon (Lall., 1991). Feeding trial with rainbow trout on diets containing 60% silage preserved with hydrochloric acid observed growth equivalent to that on a diet of fresh fish, similar good results had been reported for Atlantic salmon (Lall., 1991).

LACTIC ACID BACTERIA

Lactic acid bacteria are the most microorganisms involved in ensiling. Lactic acid bacteria are associated with raw materials which are used for preparation of silage. It has been reported that lactic acid bacteria are natural inhabitants in fish (Schroder *et al,* 1980; Knohel., 1981), but they are present in low numbers (Knohel., 1981; Cunnigham and Cox., 1987), while the principle genera of bacteria associated with intestinal tract of health birds are lactobacillus (Shapiro and Sarles., 1949) and respiratory tracts (Simbert *et al.,* 1958).

Genus lactobacillus founded by Beijerinck 1901, 212 [AL]. Described by Otto Kandier and N.Weiss (Peter Sneath *et al.,* 1986). Lactobacillus refers to lacto in Latin lac, lactis milk and bacillus a small rod. The first attempt to classify

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these bacteria was made by Orla-Jensen., (1919). The members of this group are unique having the following properties;

=> all are gram positive

=> cells vary from long and slender, sometimes bent rods to short

=> Pertichous flagella when present but motility uncommon, chain formation common.

=> Asporogenous

=> Metabolism fermentative, obligatively saccharolatic

=> Atleast half of the end product carbon is lactate. Lactate is usually not fermented. Additional products may be acetate, ethanol, carbondioxide, formate or succinate. Volatile acids with more that two carbon atoms are not produced.

=> They are microaerophillic

=> Nitrate reduction highly unusual

=> Gelatin not liquified

=> Catalase and cytochrome negative

=> Pigment production rare

=> Complex nutritional requirements for amino acids, peptides and nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters and fermentable carbohydrates. Nutritional requirements are generally characteristic for each species often for particular strains only.

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Further division is based on cell morphology and type of fermentation; whether it is hetero fermentative where products (carbon dioxide and ethanol) are formed from sugars in,addition to lactic acid particularly, or it is homo fermentative in which case sugars are fermented entirely to lactic acid. Other features employed whether stoichiometric properties of the lactic acid with respect to polarized light and temperature optima for growth. The pioneering work of Orla Jensen (1919) still forms the basis of current taxonomy of the lactic acid bacteria. Since then there have been many studies on the systematic grouping and metabolism of these organisms and much information has been accumulated in their cultural and physiological characterstic (Ruschmann and Kosh., 1930; Allen and Harrison., 1936; Cunnigham and Smith., 1939, 1940; Orla Jensen., 1974; Rogosa *et al.,* 1951; Stirling., 1953; Nilsson., 1956; Gibson *et al,* 1958, 1961; Keddie., 1959; Rogosa and Sharpe., 1959; Langston and Bouman., 1960; Langston and Bouman, 1960; Langston *et al,* 1962; Whittenbury., 1964; Beck., 1972).

Pathogenicity of lactic acid bacteria are generally considered apathogenic apart from dental caries (Rogosa *et al*.,l953). The finding that some rumen lactobacilli decarboxylate indole acetic acid to skatol, a compound know to be responsible for active bovine pulmonary emphysema, the naturally occurring form of the bovine respiratory disease. (Yokoyama and Carlson., 1981), may be a first positive step in elucidating the pathogenicity of lactobacillus.

Habitat of lactobacillus shows that lactobacillus grows under anaerobic conditions to atleast under reduced oxygen tension in all habitats producing ample

carbohydrates, breakdown products of protein and nucleic acids, and vitamins. A mesophilic to slightly thermophilic temperature range is favourable. However strains of some species (e.g: L,virideseens. L.sake. L.curvatus. L.plantarum) grow, although slowly even at low temperture close to freezing point (e.g: refrigerated meat Kitchell and Shaw 1975), Fish (Schroder *et al,* 1980). Lactobacillus are generally aciduric or acidophyllic. They decrease the pH of their substrate by lactic acid formation to below 4.0, thus preventing, or atleast severely delaying growth of virtually all other competitors except other lactic acid bacteria and yeasts. These properties make lactobacilli valuable inhabitants of intestinal tract of man and animals and important contributors for food technology.

Ecological studies on lactobacillus reveal that they can be obtained from plant sources although low in number at all plant surfaces (Keddie., 1959; Mundt and Hammer., 1968; Stirling., 1953; Stirling and Whittenbury; 1963; Whittenbury., 1968; Anderson., 1956) and together with other lactic acid bacteria grow luxuriously in all decaying plant material, especially in decaying fruits. Hence, lactobacilli are important for the production as well as production of fermented vegetable, food and feed for example silage, saurkraut, mixed pickles and beverages e.g: beer, wine, juice. Species chiefly isolated have been L. plantarum. L.brevis. L.cornyiformis. L.casei, L.curvatus. L.sake. L.fermentum (Carr 1975; Sharpe., 1981; Steinkraus., 1983; Kandler., 1984). Counts of LAB usually rise significantly by the time the herbage reaches the farm. This may be because inoculation with microorganisms from farm machinery (Keddie., 1959; Gibson *et al.,* 1961; Henderson *et al,* 1972; Mcdonald., 1976), aided by the

growth of microorganisms in sap liberated during laceration. Greenhill (1964) stated that the release of plant juices was prerequisite to the production of significant amounts of lactic acid resulting in good quality silage. This finding has been confirmed by Gibson *et al.,* (1961) and Ceale *et al.,* (1982). Lactobacilli are observed in milk and dairy products. Milk contains no lactobacillus when it leaves the udder and becomes very easily contaminated with lactobacillus by dust, dairy utensils, etc. It is traditionally used in starters for the production of Swiss cheese and other types of hard cheese e.g: Grana, Gogonzola and Parmesan (Bottazzi *et al,* 1973). Nowadays L.delbrueckii sub.species bulgaricus or sub.species lactis are also used (Biede *et al.,* 1976; Auclair and Accolas., 1983). In all types of cheese with riping periods longer than about 14 days, several misiphyllic bacillus (L,plantarum. L.brevis. L.casei) originating from the milk or the dairy environment reach levels as high as 10 to 10 / g. Cheese. (Sharpe., 1962; Abo Elnaga and Kandler., 1965 a; Van Kerken and Kandler., 1966).

L.delbrueckii well known yogurt flora (Davis., 1975) and L.kefir in Caucasian sour milk kefir. These sour milks are the only known habitats of these two lactobacilli.

Lactobacilli are also know to inhabit: meat, meat products and marinated fish. The most common naturally occurring species found in ripening raw sausages are L.plantarum. L.brevis. L.farciminis. L.alimentarius (Reuter, 1970, 1975) and L.sake and L.curvatus (Kagermuer., 1981; Kagermeier *et al,* 1985).

Lactobacilli harbours in the intestinal tract of man and animals. (Lerche and Reuter, 1962, Mitsouka, 1969) living as commensals intimately associated

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with the mucous surface epithelium. L,salivarus the most typical species of mouth flora, although it is also found in the intestinal tract (Rogosa *et al,* 1953). The most prominent species, probably indigenous to the intestine, is Lactobacillus acidophilus. which is believed to exert a beneficial effect on human and animal health. They are used industrially in the preparation of acidophilis sour milk and pharmaceutical products for restoring normal intestinal flora after disturbance caused by diseases or treatment with antibiotics. Lactobacillus. ruminis and Lactobacillus vitamins are described from bovine rumen. L.ruminis has also been isolated from the human intestine. Many reports revealed prove presence of lactobacillus in chicken intestine (Fuller., 1973) and Turney., (1971), Sarles.

They are also found in sewage and manure. In marine, L·.coryniformis and L.curvatus. neither recorded as intestinal, are frequently found (Albo Elnaga and Handler., 1965 a). L.vaccinostercus has only been found in cow dung (Okada *et al,* 1979). In municipal sewage levels of 10^5 to 10^4 lactobacilli / ml have been found (Weiss *et al,* 1981).

Lactic acid bacteria are known to play a vital role in fermentation for the production of lactic acid which helps in preservation. Michel Woolford (1984) stated that the efficiency of ensilage can be judged according to the relative proportions of acid produced. The greater the ratio of lactic acid to butyric acid the higher the efficiency. Thus, in colloquial terms a silage is considered as good quality if lactic acid predominates or of poor quality if butyric acid is predominant. Thus silages are characterized by low and high pH.

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Unique properties of lactic acid bacteria is the production of lactic acid which is very important in reduction of pH in silage. (Levin., 1944; Stetter., 1945; Fornachon *el al.,* 1949; Wood., 1961; Arul James., 1966; Whittenbury,, 1968; Bryian Jones., 1969; Holzaptel, Hechelmann and Leistner., 1976; Kandler., 1983; Jehanno, Thuault and Bourgeois., 1992) and stabilization of the raw material. Lactic acid bacteria have been known for their antibacterial activity and for inhibition of undesirable microorganisms (Deklerk and Smith., 1967; Upreti and Hinsdill., 1973, 1975; Gandhi and Nambudripad., 1975; Filippov., 1976 a,b; Tagg *et al.,* 1976; Filippov and Rubanenko., 1977; Konisky., 1978; Lindgren and Clevstrom., 1978; Bearfoot and Klaenhammer., 1983; Rolf Andersson., 1986; Rammelsberg and Radlr., 1990); Yann Hechard *et al.,* 1992; Rongguang Yang., 1992; Nettle and Barefoot., 1993; Gino Naclerio *et al,* 1993; Hoover and Steenson., 1993). These two major properties why lactic acid bacteria have since the past century been considered as 'the' organisms for preservation in material as to the type of fermentation. Production of antibiotic substances by lactobacilli has repeatedly been claimed (Dekterk and Coetezee., 1961; Lindgren and Clevstrom., 1978 a,b; Schroden *et al.,* 1980;). However frequently there is no clear distinction between an antibiotic effect and the inhibition effect of lactic acid and / or hydrogen peroxide by the organisms.

There is further evidence as to lactic acid bacteria producing antibacterial proteins because in the search for a food biopreservative, investigations on certain antibacterial proteins (Bacteriocins) from the lactic acid bacteria have been very popular (DaescheL, 1990; Klaenhammer., 1988; Raa., 1992).

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However it has also been recognized that lactic acid bacteria produced inhibitory substances other than organic acids (lactate and acetate) that are antagonistic to other microorganisms. These substance are produced in much smaller amounts and include hydrogen peroxide, diacetyle, bacteriocins and secondary reaction products such as hypothiocyanite generated by the action of lactoperoxidase on hydrogen peroxide and thiocyanite.

Historically the role of lactic acid bacteria has been one of preservation attributed to the production of lactic acid, hydrogen peroxide secondary metabolites including bacteriocins, which have the potential to inhibit a variety of other microorganisms (Daeschel., 1989, 1993). The importance and usefulness of bacteriocins of lactic acid bacteria in food systems have been extensively covered by the following authors. (Hoover and Steenson., 1993; Nettles and Barefoot., 1993 and Salmines and Wright., 1993).

Lactic acid fermentation is traditional method which was originally used for preventing a wide variety of food stuffs from spoilage. The ability of lactic acid bacteria to inhibit spoiling bacteria, including pathogenic strains, is well known and this antagonistic property means the lactic acid fermentation will lend to selection of lactic acid bacterial population, it is well known fact all lactic cultures can to some extent repress the growth of other bacteria. This antagonistic property can be the result of:

- acid production and pH reduction

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- hydrogen peroxide production
- carbondioxide production
- nutrient depletion
- decrease in reduction-oxidation potential
- production of antibiotic like compounds

Although many examples of antibiotics have been described *nisin* is the only one produced on a large scale and used as food preservative in many countries (Baxter *et aL,* 1983).

Report on bacteriocin produced by L.sake was studied on Listeria monocytogenes are potent pathogen in meat and meat products. Results revealed L.sake reduced viable counts of Listerias by about one log cycle indicating therefore it may have some potential as a protective culture in meat products (Schillnger *et aL,* 1991).

The synthesis of anti-bacterial polypeptides by lactic acid bacteria was first noticed in Streptococcus lactis (Rogers., 1928) which produced nisin and in Streptococcus cremoris (Oxford., 1944). which produced diplococcin. The production of anti-bacterial macromolecules have been demonstrated for many lactobacilli (de Klerk *et al,* 1967; Reddy and Shahani., 1971; Upreti and Hinsdill., 1975). These substances seem to be of importance for a rapid dominance of lactic acid bacteria in associative cultures (Collins., 1961). The antagonistic effect of the

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lactic acid bacteria commonly ascribed to an undisassociated organic acids produced in the fermentation process (Sorrells and Speck., 1970).

A macromolecular fraction showing anti-bacterial activity has been found in fish silage. The greater sensitivity of the substances has shown to occur in Streptococcus cremonis. The substance was probably involved in a stabilization of bacterial composition which results in a fermenting flora at the end of the active process (Lindgren and Gunnal., 1978). Important bacteria like Pediococcus> Leuconostoc and Lactobacillus produce an environment which inhibits the growth of most microorganisms causing food stuff spoilage in silage fermentation process (Mossel,, 1971; Hurst., 1973).

Wirahadikusumah., (1971) isolated lactic acid bacteria from fish silage prepared as recommended by Nilson and Ryden (1965). By using gel diffusion technique he was able to show weak antibacterial activity against Pseudomonas aeroginosa and Clostridium botulinum by macromolecular substances.

In silages of grass, cereals, slaughter house waste and fish, the lactic acid bacteria could be stimulated to produce a strong antagonistic environment against spoiling or pathogenic microorganisms (Kreuzer., 1954; Langstone and Bouma., 1960 a,b; Nilsson and Rydin., 1965; Wriahadikumah., 1968; Whittenbery., 1968; Mckie *et al,* 1971). Once again this preservative property brought about ensiling process, which has made animal by-product (the so called waste) into a useful value added, proteinaceous product.

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Importance of silages as feed

Silage is assuming ever increasing importance in the feeding of livestock owing to the escalating cost of feed concentrates throughout the world. Until recently silage has been considered as a material of bad odour, uncomfortable to handle, health hazardous, difficulties of handling a heavy bulk of material at feeding time, and effluent problems. However, the principles of ensilage are now more fully understood and the conditions necessary to obtain a good product are well defined.

Silage has successfully replaced or rather substituted fish meal to a great extent. The cost of fish meal (due to importing) and the differential grades has led to find alternative sources. Reports are also available as to where silage can help replace fish meal (Smith and Adamson., 1976; Machin *et al.,* 1984; Shrivastava and Singh., 1985). All these workers have conducted experiments on the utilization of waste mainly poultry by-products and compared with that of fish meal, or how potential these waste products in comparison to fish meal. All experiments derived the same results though conducted at different times and different ways yet showing the yield of the birds fed on the waste to have no effect on the production adversely. The work of Hazarika and Banwah., (1983) indicated that as many as 100.4 grams (24.7%) to 149.7 grams (26%) of inedible by-products could be obtained from the slaughter of the birds which are generally thrown off by the poultry processing plants.

Livestock require energy protein, minerals and vitamins for their growth. The aim of the conservation process is to preserve as many as of the original

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nutrients as possible, particularly the energy and protein components, since technically it is a relatively easy task to compensate for the deficiencies in minerals and vitamins.

Silage is a very valuable source of proteins and substances which influences the growth of animals and can be used alone or in combination with other forms of feed. From the above citations, it clearly indicates silage can replace fish meal partly because the results in feed : gain ratio and yield from birds showed positive indications of replacement of fish meal by silage without any adverse effects in birds.

By replacing waste, firstly it adds to the profit and reduces the costs of meat as fish meal is very expensive as compared to the throw away non-marketable proteinaceous waste, which by converting to silage would also help reduce environmental pollution. As exposure of animal by-product to environment will lead to pollution, silage making can reduce the BOD and prevent environmental pollution to a great extent. Ensiling has brought out a meaningful way of preserving waste which could be used for feed, minimizing the cost of animal feed.

Alertness to the problem of waste disposal and its utilization has been regularly postulated (Inglett., 1973; Birch *et al.,* 1976; EL Boushy., 1986; Boucque and Fiems., 1988; Boda., 1990; EL Boushy., 1990). The increasing cost and pressures concerned with waste disposal stress the need for reappraisal of utilization of waste. Either directly (as a diet ingredient) or indirectly (upgrading

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by microorganisms) for livestock and poultry feeds. These resulted in efforts to control the disposal of wastes (Rolfe.. 1976).

Λ review conducted by Sahoo,(l993) on the utilization of the poultry processing plant waste, stated in the conclusion that poultry offal meal can be utilized commercially to fight against the rising cost of fish meal in our country and maintain the production performance of poultry.

Naturally fermented silage has been used as an animal feed for experimental mice. Spies and Chambers., (1949), cattle and pigs, Pekzar *et al,* 1977 and Van Wyk *et al,* (1983) and it has been reported that the nutritional value of silage is comparable to that offish meal.

The acceptability and voluntary intake of silage by animals have found to be satisfactory as per the feed : gain ratio. A comparative evolution of fermented fish waste, fermented whole herring and fish meal by Emmanuel and Teong Sim., 1990) showed the highest ratio was associated with chicks fed on fermented herring. Work on replacing animal protein from poultry ratio with animal by-products by Hazarika and Baruah., (1993) proved relatively better results by stressing on the significant differences observed ($P < 0.05$) in gain in body weight, dressing weight and evisceration weight among the experimental groups indicating superiority. As indicated 50% replacement offish meal in poultry meal would not affect the production adversely. Dafwing *et al.,* (1986) noted aerobic fermentation of offal had significant growth stimulation at 8% to 10% preserved slaughter house blood and offal meal by pickling with 3% commercial sulphuric acid producing offal meal comparable with commercial equivalents.

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Slaughter by-products can be divided into primary by-products and secondary by-products (Mann., 1967). The primary products may include hides and skin, feathers bones and also hatchery by-products such as infertile eggs and egg shells; the secondary class of by-products includes a wide range of products manufactured from the primary by-products. These secondary products include blood meal, meat meal, egg albumin, etc.

The quantitative and qualitative potency of raw materials that are suitable for the manufacture of feeds from by-products varies widely between economic classes and regions throughout the world. This is a particular reason why a given definition of offal or of by-product is often vague. The terms by-products and offal are basically used to denote the parts which are not included in the primary product (often used for human consumption). A group of organs such as spleen, brains and lungs are edible parts of poultry in certain developing countries whereas they are considered rather inedible in developed ones.

Particular items derived from FAO statistics: the conversion factor for broiler slaughtery waste was estimated to be 53.8% of the broiler meat which is similar to 34.3% of the total live weight (EL Boushy., 1990; EL Boushy *et al,* 1990).

The benefits derived from the use of feed for these waste are the new, local industry will benefit from recycling waste by-products and benefits from it leads to the following features. That is if a local industry such as the poultry industry is able to transfer all the offals by rendering and produce poultry offal meal (blood, feet, heads, intestines and feathers) a product will be created with high nutritive value (EL Boushy., 1986). This application may lower the present imports of feed stuffs, fertilizers, cereals and Soya, animal products and total agricultural products, that require foreign currency. Value of feed stuffs imported in the year 1989 was 219,203 in developing market. Economics (X 1,000 $) per year. (Reproduced from trade year books, volumes 26,30,35,39,43 published by the FAO 1972-1990).

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INTRODUCTION

Booms of agriculture in India have been the impact on untiring endeavours on technical advancement and rational use of available resources. There are many reasons why the poultry industry in India can be considered as an important source of nutrition, creation of rural employment and fulfilling the nutritional standards of the country. If compared with other animal products, the input output ratio for poultry is favourable. For 1 kg of similar feed broiler type chicken gives 450 gms of protein, swine five 160 gms of protein, beef type cow gives 96 gms of protein and sheep and goat gives 225 gms of protein approximately. The future for poultry looks even brighter if few constraints are removed. Poultry meat has proved to be affordable and widely acceptable meat to the non-vegetarians. Poultry products can also earn foreign exchange for the country through exports. Industry is quite confident that if proper inputs are provided India can earn foreign exchange worth Rs. 200 crores per year, within the next 4 to 5 years.

The broiler industry in India has really expanded in a miraculous way and last few years have been a real year mark for the industry and it is expected that within 2 years, the broiler industry in India would reach to a population level of 500 million. As per the experts the poultry meat products in India has increased by 126% in the last two decades and with this, mechanisation poultry has also increased and is expected to expand in a magnificent way in the next couple of years. Broiler production started as a novelty in the early seventies, the rearing of broiler has made much head way since then from a negligible output of 4 million in 1971 to 30

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million in 1980, and 190 million in 1990. After stagnancy in the early nineties, it is again on upswing and is expected to double itself to 400 million by 1996. As the purchasing power of people increases, broiler production is expected to grow rapidly.

Continued efforts on broiler production may be expected to yield sufficient quantities of chicken flesh along with huge piles of slaughter wastes that is offals, feathers, blood, head and feet whose disposal in ordinary manner may pose threat to the environment. It is likely that a significant number of waste problems will not be immediately amenable to by-product recovery or recycling. Nevertheless, these problems too will require solution. In some cases the way to generate food from waste is to minimize the formation of waste in the first place. There is no ideal solution, only the most appropriate solution for a given situation. There has also been an ardent need to standardize cost as well as time effective us of poultry by-products. Development and wide application of biotechnology offered scope for processing of food products, in analytical procedures and in extraction of protein from live stock wastes.

The concept of producing feed from waste has become of great interest in the last few years as a result of increasingly frequent food shortages and price rises. Direct human food production by fermentation of most waste materials is unlikely at present due to the problem of meeting safety requirements. Feed production is more likely and the study will focus on schemes for fermentation of waste to animal feed ingredients. Economics is the overriding factor in the decision to implement any process. Another significant consideration is the availability of raw material with respect to quantity and distribution in time.

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The economic considerations of high BOD waste disposal schemes leave with the Semitic conundrum of whether something is a waste or a by-product. Obviously, once a waste stream achieves some measure of value it is no longer waste even though the original impetus for processing was the cost of disposal. In fact, the assigned value for a waste is often a question of locale.

Wastes are generated particularly by the agricultural, industrial and municipal segments of the population, including wholesalers and consumers. Now-a-days a confrontation with the challenge of the processing and disposal of these byproducts as a result of modern industrialization is taking place. The utilization of such waste needs urgent investigation because the recycling and reduction of waste can reduce pollution and ameliorate the present investigations therefore must include studies on the management of waste; its technology and subsequent feeding value for livestock. The rapid change in modern animal and poultry farming implies scrutiny in the studies on nutritional evaluation with respect to the target animal, species and a low-technology approach.

The means of waste disposal include waste treatment or waste utilization and in general conversion for use directly into food, feed or upgrading by micro organisms are efforts to control the disposal of wastes (Rolfe, 1976). Wastes can be directly converted into feed. This means the conversion of animal proteins and of minerals into live stock feed for ruminants, pigs and poultry who will in turn provide protein for humans. This application draws attention to the palatability, digestibility and nutrition value of these produces when included in livestock diets. In addition health hazards have to be considered for the reservations regarding the feeding of

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animal wastes are usually based on the potential risks related to several factors. The factors include the nature of the waste biomass, its high bacteriological activity, the accumulation of anti-metabolities, feed additives and other anti-nutritional excretory products derived from the wastes (Muller, 1980). These problems however do also related to conventional feed stuffs. The nutritional as well as the toxicological evaluation of wastes as an animal feed is therefore a most relevant one. Finally if wastes are unsuitable for either of the uses, they may be converted into products through their conversion by means of microorganisms. Both fermentation and ensilage can be used.

The current trend towards animal waste recycling is motivated by both economic and environmental considerations. Since feed costs are about 80% of the total animal production costs (Boucque and Fiems, 1988) the substitution of conventional feed stuffs by processed animal wastes will lead to a significant reduction in the cost of animal feed and the ultimate derived products. The economic potential of the utilization of animal waste as new feed resources is of great importance and a greater efficiency of this utilization is urged for the future on the basis of the total estimated production of poultry (broiler) slaughter wastes in the world and in developed and in developing countries in the present being 23 million tonnes, 13 million tonnes. 8 million tonnes respectively and estimated for 2050 as 55 million tonnes, 30 million tonnes and 15 million tonnes respectively (El Boushy and Van der Peel 1994)

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Birds are slaughtered and dressed m both organized and unorganized sectors together producing about 65,000 tonnes of poultry intestines (about 150g/bird).

Poultry intestine is a foetidious material due to the presence of various microorganisms both spoilage and pathogenic. It is these spoilage organisms which bring about the putrefaction of the intestines. Poultry intestine has a very low sugar content therefore it does not facilitate the growth of lactic acid bacteria or yeast, leading to spoilage of the material. But with the addition of a substrate like a carbohydrate source, the lactic's become dominant over the other microflora. A succession of organisms can be seen from the most predominant coliforms, enterococci which during the course of fermentation have been suppressed by the acid producer lactic acid bacteria. The unique properties of acid and antimicrobial substance production has played the most important role of preservation.

During the course of fermentation several additives have been used to prevent undesirable microorganisms and make conditions favourable for the lactic acid bacteria so that the end product would be microbiologically safe, free from all undesirable substances so that a value added product can be developed.

The additives used in silage enables the lactic acid bacteria to predominate over other organisms such that the material can be preserved against toxic substances which may be produced by the undesirable microorganisms. Antimycotic agent helped reduce mould count though not the yeast directly. Molasses added as a substrate enhanced the sugar content in the silage and directly enabled lactic acid bacteria to proliferate at a faster rate as it was used as the sole

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source of sugar content. This change from an aerobic microbial profile to a micro aerophillic one has preserved the raw material, together with the acid production of the microaerophiles which brings down pH to the desired level of 4.2 and the inhibitory effect of the anti microbial substances. All these properties have led to make silage effective as a value added product, which can be converted to feed for animals, taking into consideration the nutrient value, where protein content is high, rich in amino acids and minerals, free of pathogenic and spoilage microorganisms and their toxins.

Keeping these factors in mind, the present investigation was undertaken to study the microbiological and related changes during the fermentation of poultry intestine.

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OBJECTIVES AND SCOPE OF THE WORK

Poultry intestine is well understood to contain digested and undigested food material. It is due to the presence of the undigested material that poultry intestine gets spoiled very fast, because of the large number of spoilage and pathogenic bacteria in it. Putrefaction takes place in the material if left at ambient temperature, without treating it at appropriate time. This necessitate the development of simple and cost effective methods for conserving poultry intestine.

Fermentation technique is the cheapest and most apt way found for ensiling poultry intestine so that the nutrient value of the raw material is not hampered, further the protein content could be increased by way of the bacterial mass involved in the ensiling process. The interesting feature of poultry intestine is the abundance of the lactic acid bacteria associated with it. This is an advantageous feature for the ensiling of poultry intestine.

There is no literature available on the application of ensiling process for conserving poultry intestine. Large amount of data are available on ensilage of grass, forage, herbs and fish. But microbiological aspects regarding fermentation of poultry intestine are not studied.

I. The main objectives of this study were:

- Control spoilage of poultry intestine waste by fermentation process
- To develop the stabilized fermented poultry silage as a value added product to supplement feed ingredients in the poultry feed.

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II. The programme during investigation of work is detailed below:

- ♦ Quantitative and qualitative determination of microflora associated with poultry viscera. Isolation and identification *of* spoilage and pathogenic microorganisms and lactic acid bacteria.

- ♦ Characterization of lactobacilli isolated:- Biochemical, physiological, growth characteristics and antimicrobial substance detection.

- ♦ Effect of environmental variables such as temperature and additives on fermentation process.

- ♦ Microbial succession during fermentation process

- ♦ The addition of backslop material and resultant changes such as pH, and its effect on pathogenic bacteria.

- ♦ Stabilization of the fermented product and its evaluation for chemical composition and safety.

The results of the study would benefit the poultry processing plants for proper dispossal of intestine.

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Part - II

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Chapter 1

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COMMON MATERIALS AND METHODS

Collection and sampling of poultry intestine

Broiler birds (6 to 8 wk.) of the cobb strain have been selected for procuring the intestines. The birds were reared at the nearby hatcheries and brought to the local market where the birds were slaughtered. Intestines were brought immediately (within lh. of slaughter) to the laboratory for analysis. Intestines were homogenized in a Stephen mill with the addition of molasses and propionic acid for 10 to 15 min. This was distributed into batches of 1kg each in plastic buckets which were covered by thick polypropylene sheets and covered tightly with the bucket lid in order to induce microaerophillic conditions. This was incubated at ambient (25 ± 2 º C) and 37 º C for fermentation process.

Microbial sampling methods

For each batch of homogenized poultry intestine, the sample was thoroughly mixed and, 50g of the sample was placed in to 450ml sterile physiological saline and blended for two minutes in stomacher. This provides 1:10 dilution. This formed the first decimal dilution. Further serial dilutions were made upto 10^9 using 90ml sterile physiological saline. 1ml of the sample was put into petri dishes and pour plated with the differential medias for enumerating E.coli. Coliform. Staphylococcus. Feacal Streptococcus. Yeast and Mold, Lactic acid bacteria (LAB) and Clostridia. The unit for the microbial counts were always represented as log cfu/g.

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Microbial enumeration

The following procedures were followed for analyzing microbial counts.

Escherichia. **coli:** This was determined using Eosine Methylene Blue Agar (APHA, 1984). For each dilutions to be plated 1.0ml of sample suspension was aseptically transferred to duplicate plates. Dilutions of 10^5,10 and 10 in duplicates were estimated for the microbial count to this melting medium cooled to 45 °C was pour plated and allowed to solidify. The plates were inverted and incubated at 37 °C for 24h. Distinct colonies having metallic sheen were enumerated as E.coli.

Coliforms The number of Coliform bacteria were determined by pour plate method in which 1.0ml of each dilutions was transferred into plates in duplicate. To this 10.0ml of Violet Red Bile Agar (APHA, 1984) was added and swirled, in order to obtain and even distribution of counts. Colonies appear deep purplish red. These colonies were enumerated after incubating the inverted plated at 37 °C for 24h.

Staphyîococcus: This was determined using Baird Parker agar (APHA, 1984). For each dilution to be plated, 1.0ml of sample suspension was aseptically transferred to duplicate plates of Baird Parker Agar, and distributed 1.0ml inoculum over the plates. The plates were retained in upright position until inoculum was observed by the medium (about min). The plates should be inverted and incubated at 37 º C for 45 to 48h. Black colonies surrounded by a halo were counted as Staphylococcus aureus. The black colonies without halos were treated as other Staphylococcus species.

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Enterococci: These microorganisms were enumerated by taking desired dilutions in (10^5,10^6 and 10^7), each dilution of 1.0ml was transferred to petri plates in duplicate. To this KF.Streptococcal Agar (APHA, 1984) melting medium cooled to 45 º C was poured at 10.0ml quantities. The agar was allowed to stand for l0min till it solidified then incubated at an inverted position at 37 º C for 48h. Due to the addition of 1.0ml of 1% Triphenyl tetrazonium chloride solution, colonies attaining a deep red colour was enumerated as Streptococcus faecalis.

Yeast and Mold: Yeast and Mold were examined on Potato Dextrose Agar acidified (APHA, 1984). Colonies that appeared were counted and accounted for yeast and Mold, no differentiation was made of the two species. Each dilution of 1.0ml was transferred into plates in duplicate. To this melting Potato Dextrose Agar of 45° C was added to give the medium at pH of 3.5 ± 0.1 immediately before 15 to 20 ml quantity of 10% tartaric acid was poured into the plates. The plates were allowed to stand for l0min for absorption of inoculum into the medium, and when solidified plates were inverted and incubated at 25 °C for 72h, as excessive growth develops on the plates.

Lactic acid bacteria: Lactic acid bacteria were enumerated in pour plates of deMan, Rogosa and Sharpe (MRS) agar (APHA, 1984). After each dilution, 1.0ml was transferred into petri plate in duplicate, MRS agar was transferred at 10ml quantities and gently swirled thoroughly in order obtain good distribution of colonies. When MRS agar solidified this was over laid by agar medium containing 12g agar and 1.0g

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natamycin per litre. Since lactic acid bacteria are micro aerophillic in nature, this no

Clostridia: Clostridia enumeration was determined by the Most Probable Number (MPN) technique. Presence of clostridia in poultry intestine are of low number. Thus an enrichment procedure employing minced liver broth (laboratory manual for Food Canners and Processors., (1968) was employed. Three serial dilutions were inoculated into 15 tubes containing 15 to 20ml of liver broth medium overlaid with agar medium; and incubated at 37 °C for 48h. The positive tubes showed turbidity and gas production was noticed by breaks on the overlaid agar. The numbers of microorganisms in the original sample was extrapolated from the number of tubes containing microbial growth by the use of standard MPN table. This method provides a statistical determination of viable cells and MPN results. The MPN technique has been used to detect many microorganisms in water, several foods including broiler carcass (APHA, 1984).

Preparation of backslop material (BSM)

The poultry intestine was blended with molasses and propionic acid and allowed to ferment at 37 º C and 25 ± 2 º C (ambient) separately till the desired pH 4.2 was reached. The fermented material was considered stable, which contained large number of lactic acid bacteria (10^9 to 10^{10} /g) and free from pathogenic and spoilage microorganisms. This fermented material is referred to as Backslop material (BSM).

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was used as a culture to inoculate the fresh batch of poultry intestine containing molasses and propionic acid.

MEDIA USED IN THE PRESENT STUDY **Tryptone Yeast Extract Glucose Agar** (Total plate count agar-APHA, 1980)

Ingredients	Quantity
Tryptone	5 grams
Yeast Extract	2.5 gram
Glucose	1.0 gram
Agar	15 gram
Distilled water	1,000 ml

Dissolve ingredients in water to boil. Dispense into tubes or flasks and autoclave 50 min at 121ºC. The final reaction should be pH 7.0±0.1.

Volatile Red Bile Agar (VRBA) (APHA,1984)

Ingredients	Quantity
Yeast extract	3.0 grams
Peptone	7.0 grams
Sodium Chloride	5.0 grams
Bile salts	1.5 grams
Lactose	10. grams
Neutral Red	0.03 gram
Crystal violet	0.002 gram
Agar	15 gram
Distilled water	1,000 ml

Suspend ingredients in water and allow to stand for a few minutes. Mix thoroughly and adjust to pH 7.4±0.2. Heat with agitation and boil for 2 min. Do not

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sterilize. Prior to use, cool to 45 °C and use as plating medium. After solidification add a cover layer above the agar of approximately 3 to 4 ml to prevent surface growth and spreading colonies.

Eosine Methylene Blue Agar (Levine, APHA, 1984).

Ingredients	Quantity
Peptone	10 grams
Lactose	10 grams
Di-potassium phosphate	2 grams
Eosine	0.4 gram
Methylene Blue	0.065 gram
Agar	15 grams
Distilled water	1,000 ml

Mix ingredients in distilled water. Boiled to dissolve phosphate and agar in 1,000 ml water. Dispensed in 100 or 200 ml portions and autoclave 15 min at 121 °C Cooled to 45 º C to 50 º C, swirled content and pour into plates. Final pH should be 7.0 ±0.1.

Indole test (APHA, 1984)

a. Tryptone broth:- Add 10 grams of tryptone per 1,000 ml of distilled water. Dispensed in 5 ml portions in test tubes. Sterilized by autoclaving at 121 º C for fifteen minutes.

c. Kovac's Reagent:- Dissolved 5 grams para-Dimethylaminobenzaldehyde (Merck, Germany) in 75 ml isoamyl (or normal amyl) alcohol, and added 25ml concentrated hydrochloric acid. The reagent should be yellow. The amyl alcohol solution should have the pH value below 6.0.

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a. MR-VP(APHA,1984)

Ingredient	Quantity
Peptone	7 grams
Glucose	5 grams
Di-potassium phosphate	5 grams
Distilled Water	1,000 ml

Dissolve ingredients in distilled water, autoclave for 15 min. At 121 °C to a final pH of 6.9±0.2.

b. Methyl Red Indicator

Ingredient	Quantity
Methyl Red	0.10g
Alcohol, 95% (Ethanol)	300.0ml

Dissolve Methyl Red in 300 ml of Alcohol and make up to 500ml with distilled water. Incubate test cultures 5days at 30 °C. Alternatively incubate at 37 °C for 48h. Add 5 or 6 drops of reagent to cutures. Do not perform test on cultures incubated less than 48h. If equivocal results are obtained, repeat tests on cultures incubated 4 or 5 days. Duplicate test should be incubated at 22 to 25 °C.

c. Voges-Proskauer Test

Solution A - dissolve 5g of urified alpha naphthol in 100 ml ethyl alcohol (absolute). This solution was prepared fresh each day.

Solution B - 40g of potassium hydroxide is dissolved in 100 ml of distilled water.

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Simmon's Citrate Test **media:** (APHA, 1984).

Ingredients	Quantity
Magnesium Sulphate ($MgSo_4.7H_2O$)	0.2 gram
Ammonium dihydrogen phosphate ($NH_4H_2Po_4$)	1 gram
Dipotasium phosphate	1 gram
Sodium citrate	2 grams
Sodium chloride	5 grams
Bromothymol thimol blue	0.08 gram
Agar	15 grams
Distilled water	1,000 ml

Tube for long slants and sterilize at 121 °C for 15 min.

Baird Parker Agar (APHA, 1984)

Ingredients	Quantity
Tryptone	10 grams
Beef extract	5 grams
Yeast extract	1 gram
Lithium chloride	5 grams
Glycine	5 grams
Agar	20 grams
Distilled water	1,000 ml

Dissolve ingredients in water and the pH adjusted to 7.2 before dispensing in 90ml amounts and autoclaved at 121 º C for 15 min.

To the molten basal medium at 45 º C to 50 º C were successively added warmed (45° C to 50 º C) filtered solutions of the following: 20% glycine to a final concentration of 1.2%, 1.0% potassium tellurite to 0.01%; 20% sodium pyruvate to 1% and concentrated egg yolk emulsion to 5%.

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Potato Dextrose Agar (acidified) (APHA, 1984).

Ingredients	Quantity
Infusion from white potatoes	200 ml
Dextrose	20 grams
Agar	15 grams
Distilled water	1,000 ml

Heat to boiling to dissolve ingredients; distribute into flasks and autoclave for 15 min at 121 °C. When used as plating medium and yeasts and Molds, melt in flowing steam or boiling water, cooled and acidified to pH 3.5 ± 0.1 with 10% sterile tartaric acid . Mixed thoroughly and poured into petri dishes.

Pseudomonas Isolation Agar. (King *et al,* 1954)

Ingredients	Quantity
Peptone	20 grams
Magnesium chloride	1.4 grams
Potassium sulphate	10 grams
Agar	15 grams
Irgasan	0.025 gram

Suspend 45 grams in 1000ml of distilled water containing 10ml glycerol. Boiled to dissolved the medium completely and sterlize by autoclaving at 15 Lb pressure for 15 min.

K. F. **streptococcal** Agar (APHA, 1984)

Ingredients	Quantity
Proteose peptone	10 grams
Yeast extract	10 grams
Sodium chloride	5 grams
Sodium glycero phosphate	10 grams
Maltose	20 grams
Lactose	1 gram
Sodium Azide	0.4 gram
Bromo Cresol purple	0.015 gram
Agar	20 grams
Distilled water	1,000 ml

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Suspend 76.4 g in distilled water. Boil to dissolve the medium completely. Continue heating for an additional 5 min. Dispense in 100ml quantities. Do not autoclave. Cool to 50 °C and aseptically add 1ml of 1% TTC (Triphenyl Tetrazolium chloride) solution to each 100ml of sterile medium. Mix thoroughly and use for making pour plates. Final pH 7.2 ± 0.2.

Brilliant Green Lactose Bile Agar (APHA, 1984)

Ingredients	Quantity
Peptone	8.25 gram
Lactose	1.9 gram
Oxgall	0.00295 gram
Sodium Sulphite	0.025 gram
Ferric Chloride	0.0295 gram
Mono Pottasium Phosphate	0.0153 gram
Erioglaucine	0.0649 gram
Basic fuchsin	0.776 gram
Brilliant green	0.0000295 gram
Agar	10.15 gram
Distilled water	1,000 ml

Dissolve ingredients in distilled water by bringing to a boil. Dispense into tubes or flasks and autoclave for 15 min. at 121 °C. Final reaction should be approximately pH 6.9. The medium is sensitive to light and should be stored in the dark.

Selenite-Cystine Broth (APHA, 1984)

Ingredients	Quantity
Tryptone	5 grams
Lactose	4 grams
Disodium phosphate	10 grams
Sodium selenite	4 grams
Cystine	0.01 gram
Distilled water	1,000 ml

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Dissolve ingredients in distilled water by heating frequent agitation. Do not autoclave. Heat 10 min in flowing steam. Final pH 7.0 ± 0.2. Medium is not sterile. Use same day as prepared.

Bismuth sulphite Agar (APHA, 1976)

Ingredients	Quantity
Peptone	10 grams
Beef extract	5 grams
Dextrose	5 grams
Disodium phosphate	4 grams
Ferrous sulphate	0.3 grams
Bismuth ammonium citrate	1.85 grams
Sodium sulphite	6.15 grams
Brilliant green	0.025 grams
Agar	10 grams
Distilled water	1,000 ml

Dissolve ingredients in distilled water by boiling approximately for 1 min. Adjusted to pH 7.7 ± 0.2. cooled to 45 °C to 50 °C, suspending precipitate with gentle agitation and pour plates without sterilizing medium. Let plates dry with cover partially open.

Brilliant Green Agar (APHA, 1976)

Ingredients	Quantity
Yeast Extract	30 grams
Ploy peptone	10 grams
Sodium Chloride	5 grams
Lactose	10 grams
Phenol Red	0.08 grams
Sucrose	10 Grams
Brilliant green (0.25% solution)	5 ml
Agar	20 grams
Distilled water	1,000 ml

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Dissolve ingredients by boiling in distilled water. Autoclaved at 121 °C for 12 min. Cool to 45 °C to 50 °C and pour 20ml portions in petri dishes. Let dry for 2h with covers partially open and then close plates. Final pH 6.9 ± 0.2.

Dccarboxylase test media (APHA, 1984)

Basal media for use with lysine, arginine, ornithine, Moeller method (1954, 1955)

Ingredients	Quantity
Peptone	5 grams
Beef extract	5 grams
Bromocresol purple (`1.6 %)	0.625 ml
Cresol Red 0.2 %	2.5 ml
Glucose	0.5 gram
Pyridoxal	5 milli gram
Distilled water	1,000 ml

The basal medium is divided into two equal portions, one of which is tubed without the addition of any amino acid. These tubes of basal medium are used for control purposes. To the other 2% DL-Lysine dihydrocloride, since the microorganisms apparently are active against the L-forms only. The amino acid medium may be tubed in 3 or 4 ml amount in small (13 X 100 ml) screw capped tubes and sterlized at 121 °C for 10 min.

Inoculation: Inoculate lightly from a young agar slant culture. After inoculation, add a layer (about 10mm in thickness) of sterile mineral (parrafin) oil to each tubes including the control. A control tube always should be inoculated with each culture under investigation. Incubate at 37 °C, examine daily for 4 days.

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Positive reactions are indicated by alkalinization of the medium with the colour change from yellow violet. Weakly positive reactions may be bluish grey.

Triple Sugar Iron Agar (APHA, 1984)

Ingredients	Quantity
Polypeptone	20 grams
Lactose	10 grams
Sucrose	10 grams
Glucose	1 gram
Sodium chloride	5 grams
Ferrous ammonium sulphate $6H_2O$	0.2 gram
Sodium thiosulphate	0.2 gram
Phenol Red	0.025 gram
Agar	13 grams
Distilled water	1,000 ml

Add ingredients to distilled water and bring to boil. Distribute in tubes, using enough medium to obtain a deep butt. Autoclave 15 min at 121 °C. Remove from autoclave and slant to obtain a deep butt. Final reaction should be approximately pH 7.4±0.1.

Urease Test (APHA, 1984)

Urea Broth

Ingredients	Quantity
Yeast Extract	0.1 gram
Monobasic potassium phosphate	0.091 gram
Dibasic Sodium phosphate	0.095 gram
Urea	20 grams
Phenol Red	0.01 gram
Distilled water	1000 ml

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Mix ingredients in the distilled water. This medium is filter-sterlized and tubed in sterile tubes in 3ml amounts. The basal medium (without urea) may be prepared in 100ml of distilled water and sterlized at 121 °C for 15 min. After cooling 100ml of 20% sterile urea solution are added and the medium dispensed in sterile tubes in 30ml amounts.

a. Transfer a small amount of growth from the presumptive positive TSI agar culture to urea broth with a sterile needle. Include an uninoculated tube of this broth as a negative control, since occasionally, uninoculated tubes of urea broth will turn positive (purple red colour). Incubate at 24 ± 2 h at 35 °C. -

b. Discard all cultures giving a positive test (purple red colour)

c. Retain for further testing all cultures that give a negative test (no change in orange colour of medium).

Liver Broth (Laboratory Manual for Food Canners and Producers, 1968)

Ingredients	Quantity
Fresh beef liver	500 grams
Tryptone	10 grams
Soluble starch	1 gram
Dipotassium phosphate	1 gram
Distilled water	1,000 ml

Remove fat from fresh beef liver grind, mix with 1,000 ml of distilled water and boil slowly for lh. Adjust the pH to 7.6 and remove the liver particles by straining through cheese cloth. Make the volume of the broth back to 1,000ml with distilled water and add the tryptone, Dipotassium phosphate and soluble starch, and refilter. Dispense 15ml of the broth into tubes and add liver particles previously remove to a depth of 1 inch in each tube. Autoclave 20 min at 15 Lbs pressure.

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Gram stain Hucker (APHA, 1984)

Hucker Crystal Violet

Ingredients	Quantity
Solution A:	
Crystal Violet (85% dye Content)	2 grams
Ethyl Alcohol (95%)	20 ml
Solution B:	
Ammonium Oxalate monohydrate	0.2 gram
Distilled water	20.0 ml

a. Mix equal parts of solution A & B (sometimes it is found that the crystal violet is so concentrated that gram negative organisms do not properly decolourlize.. To aviod this difficulty the crystal violet solution may be diluted as much as 10 fold prior to mixing with equal parts of solution B).

b. Lugol’s solution, Gram's modification: Dissolve 1 gram iodine crystals and 2 grams potassium iodide in 300 ml distilled water.

c. Counter Stain: Dissolve 2.5 grams safranine dye in 100 ml 95% ethyl alcohol. Add 10ml of the alcohol solution of safranine to 100ml distilled water.

d. Ethyl alcohol 95%.

MRS Agar (APHA, 1984)

Ingredients	Quantity
Proteose	10 grams
Beef Extract	10 grams
Yeast Extract	5 grams
Dextrose	20 grams
Tween 80	1.0ml
Dipotassium phosphate	2 grams
Sodium acetate trihydrate	5 grams
Triammonium citrate	2 grams
Magnesium Sulphate $7H_2O$	0.2 gram
Manganese Sulphate $4H_2O$	0.05 gram
Agar	12 grams
Distilled water	1,000 ml

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Suspend ingredients in distilled water. Boil to dissolve completely. Dispense into 5 ml amounts into 13 X 100 mm test tubes and flasks. Sterilize by autoclaving at 15 Lbs pressure for 15 min. Final pl·l (at 25 °C) 6.5 ± 0.2.

Isolation and identification of E.coli (APHA, 1984)

50 grams of the sample was placed into 450ml of sterile physiological saline and blended for 2 min. This provided a 1:10 dilution. Desired dilutions were put into 15ml brilliant green lactose broth. Tubes were incubated at 37 °C for 24h. This was the initial enrichment media. Further isolation was followed using the differential test.

Differential Tests:

The differentiation of the Coliform group into Escherichia coli aerobacter aerogenous and Escherichia ferundii (or intermediate) species were carried out on the basis of the results of 4 tests (indole, Methyl Red, Vogas-Proskauer and Sodium citrate). Often referred to collectively as the "IMVIC" tests.

Tests	Pattern
Indole	+
Methyl Red	+
Vogas-Proskauer	—
Sodium citrate	—

Microorganisms from tubes showing gas formation were streaked on Eosine Methylene blue agar. E.coli grew with the dark center and have a greenish metallic sheen. Suspect E.coli colonies were subjected to "IMVIC" reactions and confirmation

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made with indole test, Vogas-Proskauer test, Methyl Red test and Civics test. The suspected colonies were further identified by serotyping to confirm for E,coli. The final identification and strain differentiation was carried out by serological methods at the National Center for Serotyping E.coli and Salmonella situated at the CRI, Kasauli (Himachal Pradesh).

Isolation of Salmonella and its Identification (APHA, 1984)

Ten batches of homogenized poultry intestine was taken for analysis. Each consisted of 1kg material. Samples of 25g was put into 225ml selenite cystine broth as enrichment media for 48h at 37 °C. A loopful was aseptically withdrawn from the selenite system broth and streaked on to one or more selective, differential media. Bismuth sulphite agar (BSA) and Brilliant green agar (BGA) were used. Incubation was carried out at 37 °C for 24h. Suspected colonies (transparent pink to deep fuschia colonies on the red portions of the plates in the case BGA., and green or black shining colonies surrounded by a halo in the case of BSA) were picked and inoculated into triple sugar iron agar tubes, making a stab and a streak. After 24h incubation at 37 C, the tubes were checked for typical Salmonella reaction: acid / yellow butt; alkaline / pink slant; gas and hydrogen sulphide (black deposits).

Biochemical reactions:

Suspected colonies grown on selective solid media were purified and further classified by biochemical reactions and finely verified by serological identification.

Bacto purple broth base was used for the preparation of carbohydrate broth used in fermentation studies. The concentration of the carbohydrate employed for

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testing the fermentation reaction of bacteria was 0.5%. To bacto purple broth base (Difco, 1953) the selective carbohydrate was added in the desired quantity and when the solution was complete the medium was distributed in fermentation tubes. The tubes were sterilized by autoclave at 15 Lbs pressure for 15 min. Fermentation tests with dulcitol, lactose and sucrose were carried out. Biochemical tests for ureas, lysine decarboxylase, production of indole, determination of the Voges - Proskauer, Methyl red and citrate (Simmons) utilization. The final identification and strain differentiation was carried by serological methods at the National Center for Serotyping E.coli and Salmonella situated at CRI, Kasuli (Himachal Pradesh).

Biochemical reactions of Salmonella

Test / Substrate	Positive	Negative	Salmonella reaction
(TSI) H_2S	Blackening	No Blackening	+
Urease	Purple Red colour	No colour change	–
Lysine decarboxylase broth	Purple colour	Yellow colour	+
Bacto purple dulcitol broth	Yellow colour gas	No gas, no colour change	+
Bacto purple lactose broth	Yellow colour gas	No gas, no colour change	–
Bacto purple sucrose broth	Yellow colour gas	No gas, no colour change	–
Indole Test	Deep red colour at surface	Yellow colour at surface	–
Voges-Proskauer test	Pink to red colour	No colour change	–
Methyl red Test	Diffuse red colour	Diffuse yellow colour	+
Simmon's citrate	growth; blue colour	No growth; no Colour change	V

+, 90% positive in 1 to 2 days; -, 90% negative in 1 to 2 days and; V, variable

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Isolation and Identification of Lactobacilii

Preparation of Inoculum: Poultry intestine was homogenized in a Stephen mill and was sampled in MRS broth. After incubation at 37 °C for 72h, culture were -plated on MRS agar. Suspected lactobacilli colonies from MRS agar were maintained in MRS broth. Standard procedures were followed for further identification of lactobacilli (Sharpe, 1926; Bergy's Manual of Systematic bacteriology, 8th edition 1986).

Identification of the Isolates: Identification was based on the following characteristics: Catalase activity, gram reaction, morphology, pigmentation, gas production from glucose, acid production from glucose, fructose, galactose, sucrose, mannose, maltose, trehalose, xylose, arabinose, raffinose, manitol, growth at 37 °C and 45ºC.

Fermentation of Sugars: Acid production from carbohydrate was carried out by the following method. Inocula of cultures were inoculated into test substrate, using washed microorganisms, which must be from vigorously growing strains. Therefore suspected colonies were subcultured twice in the basal medium. The basal medium recommended is MRS broth with glucose and meat extract omitted and an indicator added-0.5% (w/v) chlorophenol red is the indicator generally used in basal medium. Filter-sterilized solutions of the test carbohydrates are added to final concentration of 1.0%. tests are incubated at optimum growth temperature at 37 °C and results recorded after seven days.

Biochemical Test: The biochemical reactions carried out where the hydrolysis of arginine, nitrate reduction.

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Proximate Analysis of poultry intestine: Proximate composition o† the sample was estimated according to AOAC (1990). To estimate the protein content of the sample, total nitrogen content was estimated initially by using Kjeldhal method in Tecetor digestion and distillation unit (Kjeltee system, Sweden). Nitrogen value was multiplied by 6.25 to obtain protein content.

Estimation of fat: This was carried out by Soxlet method using diethyl ether (60 to 80 %) as solvent, in tecetor extract unit (Soxtec system, Sweden).

Ash Estimation: Ash content in the sample was estimated by charring the samples in silica crucible and heating in a muffle furnace at 450 °C to 500 °C to constant weight.

Moisture Estimation: Moisture content of the sample was done by placing the known weighed sample in weighed petri dish in the oven (102 °C) until constant weight is reached.

Temperature maintenance: All biochemical tests conducted for the isolation of pathogenic, spoilage organisms and lactic acid bacteria when temperature treatments were specific i.e. 37 °C, 45 °C and 4 °C were incubated in BOD incubator and thermostat regulated incubators.

Titration and pH: pH was recorded using pH meter, Copenhagen, Denmark. This was preffered over titrable acidity, expressed as lactic acid or acetic acid may be used as an indirect measure of bacterial growth in brines and liquid foods. However, since the acid available to be neutralised with standard alkali depends on the buffering capacity of the liquid, pH is a more accurate indicator. (APHA, 1984).

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Statistics: All experiments were conducted six times (Six replicates) in order to get correct statistical evaluations.

Following statistical methods were used:

• ANOVA - Multiple range and multiple F-test (Duncan, 1955) was performed to analyze significant variations.

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Chapter 2

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QUALITATIVE AND QUANTITATIVE DETERMINATION OF MICROFLORA ASSOCIATED WITH POULTRY VISCERA PRIOR TO. ENSILING. ISOLATION OF SPOILAGE AND PATHOGENIC MICROORGANISMS AND LACTIC ACID BACTERIA.

INTRODUCTION

The interesting feature in the bacterial colonization of the intestinal tract of chicks is its association which takes place soon after hatching when the young chicks ingest feed (Ochi,l964). Pathogenic and spoilage bacteria along with the useful bacteria begin their life cycle in the developing intestinal tract of chick. Thus the intestine forms a niche in its own for these bacteria to grow and survive followed by succession. The beneficial bacteria protect the flora of the chicken intestine while the pathogenic microorganisms could be deleterious to the health of the host or is carried to the next host on consumption. Consumer awareness of hygenie quality of poultry meat has led to the establishment of modern poultry processing plants in the country. It has been observed that large quantities of waste generated during poultry processing is not utilised. Thus it has become a source of pollution resulting in health hazards. Further, this waste material will become a source of animal feed since it is rich in proteins. Therefore, it is essential to conserve this material for better utilisation.

Intestine is a major waste that comes from slaughter of broiler chicken. It is highly perishable, and its perishability is largely dependant on the presence of micro organisms assoicated with the intestine. (Salanitro et al, 1978; Barnes *et al,* 1980 a;

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Sarra et al., 1985). Within few hours after hatching, various bacteria including faecal streptococci, enterobacteria and clostridia may be found randomly scattered through the alimentary tract, but within a few days the lactobacilli became established (Barnes, 1979). An assoication between lactobacilli and the epithelial lining of the chicken crop is established within a few days after hatching and persists throughout the life of the chicken (Fuller 1973; Fuller and Brookes 1974). Lactobacilli are the only group of organisms reported as generally present in numbers exceeding 10,000 per gram of intestinal content (Barnes *et al,* 1972). Barnes *et al,* (1972) have also reported that throughout the period investigated lactobacillus are the only group of organisms generally present in the small intestine in numbers exceeding 10^4 /g. According to Ochi *et al,* (1964) lactobacilli are the only organisms normally present in the duodenum and small intestines at levels significantly above 10^3 /g in birds from 2 to 6 wk of age.

It is planned to develop simple techonologies for conserving this waste. In this connection a knowledge on the prevelance of important microorganisms in the poultry intestine prior to ensiling would help in developing techniques to conserve this valuable material in a proper way for further utilization.

Micorbial ecology differs from envrionment to environment and it also depends on climate, feeding and management practices. Data are available on intestinal flora of broiler chicken raised in temperate climate (Cunningham, 1987). There are no studies on the microbial profiles of intestine of broiler poultry raised under tropical conditions.

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The objective of this chapter is aimed at quantifying the microbial counts and isolating specific groups of microorganisms associated with intestine of broiler poultry raised under tropical condiitons.

MATERIALS AND METHODS

Poultry Waste: Poultry intestine has been selected for the study.

Collection of intestines and sampling

Intestines procured were of broiler birds (6 to 8 wk) which were brought from various farms around the locality to the local market, where the birds were slaughtered. Intestines were brought immediately (within 1 hr of slaughter) to the laboratory for analysis. A total of 36 intestines were examined in 6 batches so as to provide the statistical analysis, each batch consisting of 6 intestines. Intestines were collected and analysed over a period of 5 months. Intestines (consisting of cecae, deodenum, ileum, small and large intestine) were taken for the enumeration of pathogenic and spoilage microorganisms using suitable media. From each batch, three intestines were tested along with intestinal contents (unwashed samples) and other three were examined without the intestinal contents (washed samples). 50 grams of unwashed intestine was blended with 450 ml of sterile physiological saline in a Stomacher for 2 min and then used for making serial dilutions. For washing, the intestines were first slit length wise and then cut into 5 cm long pieces to facilitate easy handling. The pieces were transfered into a 3 1 conical flask containing 1 1 oí sterile physiological saline. All this was carried out under aseptic conditions. The flask was thoroughly shaken by placing on a mechanical shaker so that intestinal

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contents may be washed out. The water was drained out completely. This was repeated thrice. The material was then minced. Twenty five grams of intestine was blended with 225 ml of sterile physiological saline in a stomacher for 2 min. Serial dilutions were made up to 10^{-9} for enumeration of microorganisms.

Enumeration of microorganisms

Poultry intestine has been analysed for total plate counts, coliforms, E.coli. yeasts and molds, lactic acid bacteria (LAB), faecal Streptococcus. Staphylococcus aureus and clostridia as per standard procedures mentioned in the chapter 1.

Isolation and Identification of Escherichia coli. Salmonella and Pseudomonas

Escherichia. coli

Seven batches each consisting of 1 kg poultry intestine were analysed for the presence of E.coli. The sample was prepared by blending 50g of poultry intestine in 450ml of sterile physiological saline. One ml of the sample was transferred to a tube containing sterile 10 ml of brilliant green lactose broth (BGLB). The tubes were incubated at 37 °C for 24 hours. Tubes showing gas production are suspected as coliform positive. A loopful quantity was transferred to eosin methylene blue agar plates. The plates were incubated at 37 °C for 24 hours. Plates showing metallic sheen were suspected as E. Coli.The isolates were purified subjected to biochemcial tests. (Bergy's manual of Systematic Bacteriology, 1986). 100 E.coli isolates were

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serotyped at the National Centre for serotyping E.coli and Salmonella, at the Central Research Institute (CRI), Kasauli, Himachal Pradesh.

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Salmonella

Six batches of poultry intestine each batch weighing 1 kg were tested for the presence of Salmonella. The usual ratio of sample size and its pre-enrichment broth volume has been 1:10 selenite cystine broth was used as enrichment broth. The intestine was transferred to the selenite cystine broth and incubated at 37 °C for 24 hours. After enrichment it was streaked onto selective media - Bismuth sulphate Agar (BSA) and Brilliant Green Agar (BGA). The plates were incubated at 37 °C for 24 hours. Suspected colonies (transparent pink to deep fuschia colonies on the red portion of the plate in the case of BGA and green black shining colonies surrounded by a halo in the case of BSA) were picked and stabbed into tripple sugar iron agar (TSI) tubes. After incubating at 37 °C for 24 hours, the tubes were checked for typical salmonella reaction - acid/yellow butt; alkaline/pink slant, gas & hydrogen sulphide (black deposits). Suspected colonies from TSI and BSI were purified and subjected to biochemical reactions (APHA, 1984). Cultures identified as Salmonella on the basis of biochemical tests were sent for serological identification to National Centre for serotyping E.coli and Salmonella, Central Research Institute (CRI) Kasauli, Himachal Pradesh, India.

Pseudomonas

Five batches each consisting of 1 kg poultry intestine were analysed for the presence of pseudomonas. A quantity of 50g intestine was blended in 450ml of

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physiological saline and then serial dilutions were made and used for the isolation of Pseudomonas as per the procedure described by Shewan *et al,* (1960).

Lactobacilli

50 g Poultry intestine was sampled in 450 ml MRS broth. After incubation, cultures were plated on MRS agar. Suspected Lactobacilli colonies from MRS agar were maintained in MRS broth. Standard procedures were followed for further identification of lactobacilli (Bergy's Manual of systematic Bacteriology, 8th edition, 1986) Detailed procedures have been described in Chapter 3.

Statistical Analysis

Experimental data (count of microorganisms) obtained from washed and unwashed poultry intestine, were subjected to ANOVA. Significant difference between means within the sample were tested by Duncans multiple range test (Duncan, 1955) and between washed and unwashed samples by t-ratio test.

Results

Microbial profile of washed and unwashed poultry intestine

The quantitative determination of the microbial profile of poultry intestine is presented in Table 6. The microbial profile consists of the common organisms, having in it both the pathogenic and spoilage microorganisms. One of the most beneficial microorganisms determined in the studies was lactic and bacteria. (LAB). The spoilage microorganisms listed are yeast and molds, coliforms and the

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presence of pseudomonas. The pathogenic microorganisms constitute E.Coli. Staphylococcus. faecal Streptococcus and Clostridia.

From Table 6 it can be seen that coliforms, E. coli. Staphylococcus and Yeast and Molds were 10^6 /g while faecal Streptococcus, clostridia and lactic acid bacteria were 10^7 /g of poultry intestine. The total plate count was 10^7 /g of poultry intestine.

The quantitative studies, revealed that poultry intestine (washed and unwashed) showed high levels of microbial counts. It was observed washing did not prove to be effective in removing microbial populations except that a slight decrease in coliform number as compared to the t-Ratio evaluated by the Duncan's multiple range test. There was no significant decrease in counts in washed and unwashed samples therefore further processing of poultry intestine was carried out with unwashed samples. The Table 6 shows mean of same column followed by different letters differ significantly according to Duncan's multiple range test ($P < 0.05$). Thus pretreatment of poultry intestine did not prove economical. The higher microbial counts recorded in the study provided significant information on the possibility of rapid spoilage of poultry intestine if it is held at ambient temperatures.

Isolates from poultry' intestine

Eseherichia. coli

Predominated among Coliforms in the present study. Serotypes of E.coli detected in the intestine are shown in Table 2. There were 38 serotypes observed in the present study. 7% of serotypes belonged to 062 and 8% represented 064

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followed by 0166, 0116 and 0134. The findings revealed that E.coli serotypes 08, 09,011 were of the order of 1.08, 2.17 & 3.26% These organisms are potent pathogens to poultry because

- ⇒ They are not found on the host body but in the birds. They are persistent
- ⇒ to drug resistance (Hindon *et al,* 1982; Nakamura *et al,* 1982; Secoh *et al,* 1983; Ojeniyii 1985; Mehrotva *et al,* 1985) Break the immune status
- ⇒ (Kot 1983)
- ⇒ Pre disposes to secondary infection and enhances the pathogenicity of other diseases. Thus poultry could become carrier of dreadful diseases.

Salmonella

Species of Salmonella isolated from poultry intestine are presented in Table 3. Salmonella cerro. Salmonella gallinarium. Salmonella typhimurium., Salmonella enteritidis and Salmonella virchow were isolated from poultry intestine. The presence of Salmonella necessitates careful handling of poultry intestine. Salmonella typhimurium was recorded in highest number among the other species isolated having 25% while the other species ranged between 2 and 5%.

During isolation of Salmonella from 6 batches all batches showed the presence of Salmonella. Here again from the 6 batches tested for Salmonella 4 batches were positive for S. typhimurium indicating its dominance in poultry intestine. While S. enteritidis was present in 2 batches, while the rest of the Salmonella species S. cerro. S. gallinarium and S. virchow were present in only one batch.

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Pscudomonas

These are the organisms mainly involved in spoilage of poultry meat. The strains of Pseudomonas isolated in the study are presented in Table 4. six species of Pseudomonas were isolated from poultry intestine of which Pseudomonas cepacia predominated (65%). The other species of Pseudomonas were Pseudomonas maltophila (51%), Ps. pseudomalleli (8%). Ps. stutzeri (1%), Ps* diminuta (3%) and Ps. mallei (1%).

Lactobacilli

It is interesting to note that lactic acid bacteria (LAB) were found in large numbers in the intestinal microflora recorded in the study. Further the present study revealed that lactobacilli were the most predominant in poultry intestine. The different species of lactobacilli isolated are presented in Table 5. These were L. salivarius. L. brevis. L. acidophilus. L. plantarum. L. fermenti and L. jensenni. Among these isolates Lactobacillus plantarum was predominant and recorded 10%.

Proximate Composition of Intestine

Data on moisture, ether extract, protein and ash are presented in Table 1.

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DISCUSSION

The waste arising from animal tissue is mainly made up of proteins and lipids and a low level of carbohydrate and provide nutrients for the growth of microorganisms.

The protein content as given in the present results is 75.46% which is indeed high enough for utilising in feed preparation. The fat content in chicken viscera in the present study is 7.87% which is high enough to provide energy in feed for feeding of chicks. According to a report (Anomyous, 1989) poultry waste contained about 38.5 - 66.8% crude protein, 5.9 - 19.9% fat. This well confirms that poultry viscera does have high protein content and fat content at moderate level.

As reported by Sahoo (1993) the poultry by-product meal consists of the ground dry rendered clean parts of the carcass of slaughtered poultry such as head, feet, undeveloped eggs and intestines exclusive of feathers. It should contain less than 16% ash. Here in the study only poultry intestine was evaluated and found to contain an ash content of about 1.7%

The results of the present study on the enumeration and isolation of bacteria from the intestine of chicken substantiate the chicken microflora prevelent under tropical conditions. Work of Blair (1977) suggested that the digestive tract contents were removed and then washing was done before processing into feed. But the present study reveals that the washing of intestines did not prove more advantageous over unwashed poultry intestine. Bacterial counts ranging from 5.0 to 6.0 log/g were recorded in the small intestine of chicken (Fuller and Turney, 1971). It has been reported that aerobic counts from the intestinal tract of chicks varied from 1.6 x 10^4 to 1.77 x 10^9 and aerobic counts were in the range of 1.5 x 10^4 to 7.9 x 10^{10}

(Salinitro *et al,* 1978). The study of Barnes *et al,* 1972, on the intestinal flora of *the* chicken in the period of 2 to 6 wk of age indicated that the total numbers of bacteria in the duodenum and small intestine were very small when compared with the population in the cecum (10^{11}/g). Small intestine showed high number of coli-aerogenes (upto log 8.3/g) and these organisms were not found in the duodenum and small intestine. The counts of faecal Streptococci are below $10^{5/}$g majority of the samples from the small intestine. These authors have further observed that throughout the period, the lactobacilli were the only organisms present in the small intestine in numbers exceeding 10^{4}/g. These results are in agreement with the finding of the present study.

E.coli is a potent pathogen in poultry. It is a typical coliform organism commonly seen in the intestinal tract and is present in large numbers in the faecal samples of animals. In healthy birds the coliform content of the intestine is less than 1%. Approximately 100 serotypes are known, but serotypes 01, 02 and 078 are common pathogens to avaian species. In India serogroups 08, 09, 018, 088 and Oil were isolated by Gupta *et al,* in 1965 as pathogens to avain species (Panda *et al,* 1988) However 08, 011 were recovered in the present studies but 0166,064, 0116, 062, 0134 were the most predominant in poultry intestine. The presence of coliform bacteria in the intestine and ceca at high levels have been reported by Victor Stanley *et al,* (1992); since their studies involved in the reduction of these roganisms by use of $MgS0_4$. Shapiro and Sarles (1949) indicated that E.coli predominated among Coliforms.

The presence of Staphylococci in poultry was also reported, S.aureus being predominant (Devriese, 1980). In the present study the isolation of S.aureus from poultry intestine has been significant from the point of pathogenicity of the organism to human beings.

Salmonella infections of poultry are world wide in their distribution. The infections are readily spread to domestic poultry from various natural environmental sources and also through the consumption of contaminated feed. (Galton *et al,* 1964; Vonten *et al,* 1974; Borland 1975). Salmonella organisms inhabit the intestines and were detected in cecal contents of poultry. (Brownell *et al,* 1969; Fanelli *et al,* 1971; Labout *et al,* 1983). The presence of Solmonella in the gut on the skin and among the feathers will cause contamination of carcasses among subsequent slaughter and processing (Cliford *et al,* 1990). These findings are in agreement with the results of the present study.

A small percentage of Pseudomonas were also found in the intestinal tract. This organism would contribute for the faster spoilage of the intestines. The present investigation showed the following predominant species of Pseudomonas. Ps.maltophila. Ps.cepacia. Ps.pseudomalleli. Ps.Stutzeri. Ps.diminuta. Ps.mallei.

A number of reports are present as to fungal flora of the respiratory tracts of fowls. (Mundt, 1963; Thompson and Fabian, 1932; Baker *et al,* 1934, Kiss and Kelentey, 1960; Jordon, 1954) In the present study yeast and moulds were found in large numbers which may play an important role during fermentation.

The principle genera of bacteria associated with the intestinal tract of healthy birds are Lactobacillus (Shapiro & Sarles 1949, Fuller. 1973, Gilliiand *et*

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al., 1975). The present study also showed that the Lactobacilli have established symbiotic relationship with the chicken intestine and might help to regulate the intestinal microflora. The unique position of Lactobacillus in fermentation process is known. The presence of these in large numbers of anaerobes or microphilic organisms in the intestine suggests that these species may be involved in hither to unrecognised function in the organ. They may also play a vital role in controlling populations of facultatively anaerobic and pathogenic bacteria in the intestine, such a function has been suggested for the lactobacillary flora in the intestine of chicks. This suggestion as reported by Fuller *et al,* (1974) has been valued as lactobacillus , the main producer of lactic acid which is used as preservative has found value in various products. Thus it can be viewed that Lactobacilli are of an advantage in the further processing and preservation of poultry intestine.

Conclusion

The study revealed microbial levels and specific groups of microorganisms associated with intestine of poultry raised under tropical conditions. It is interesting to note that the presence of large number of LAB recorded in the present study is an added advantage for silage making under natural conditions.

The process of washing poultry intestine did not show any specific advantage over unwashed poultry intestine.

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Table 1

Proximate composition of poultry intestine

PARAMETERS	IN PERCENTAGE
Moisture	75.76
Ether extract	7.87
Protein	12.44
Ash	1.70

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Table 2

Serotypes of E.coli from poultry intestines

Sl No.	Serotype	% obtained from Suspected isolates	SL NO.	Serotype	% obtained from Suspected isolates
1	0166	5.43	20	0154	2.17
2	0144	1.08	21	062	7.65
3	Rough	4.35	22	027	1.08
4	064	8.69	23	03	2.17
5	057	1.08	24	0146	1.08
6	0106	2.17	25	0147	2.17
7	039	2.17	26	0132	1.08
8	084	1.08	27	0159	1.08
9	UT	7.65	28	0134	5.43
10	042	2.17	29	0169	1.08
11	054	1.08	30	0116	4.35
12	016	1.08	31	09	2.17
13	020	3.26	32	0129	1.08
14	0116	5.53	33	021	1.08
15	0103	2.17	34	011	3.26
16	0100	4.35	35	0130	1.08
17	0101	1.08	36	045	1.08
18	0157	1.08	37	0100	3.267
19	081	1.08	38	08	1.081

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Table 3

Serotypes **of salmonella from poultry intestines**

Sl. No.	Serotype	% obtained from suspected Salmonella isolates	No. of * batches showing positive for Salmonella
1	S. typhimurium **	25.0	4
2	S. cerro	2.0	1
3	S. gallinarium	2.0	1
4	S. enteritidis	5.0	2
5	S. virchow	5.0	1

* Total No. of batches = 6 **
Most predominant species

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Table 4

Pseudomonas isolates from poultry intestines

Sl.No.	Species	% obtained from suspected Salmonella isolates
1	Ps. maltophila	5.00
2	Ps. cepacia	65.00
3	Ps. pseudomalleli	8.00
4	Ps. stutzeri	1.00
5	Ps. diminuta	3.00
6	Ps. mallei	1.00

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Table 5

Lactobacilli isolates from poultry intestines

Sl.No.	Lactobacillus Species	% obtained Lactic acid bacteria
1	L. Salivarius	1.66
2	L. brevis	1.66
3	L. acidophilus	6.66
4	L. plantarum	10.00
5	L. fermenti	1.66
6	L. jensenni	1.66

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Table6

Micorbial counts of **poultry intestine**

Treatment Variables	Treatment Means		t-Ratio
	Washed Sample	Unwashed Sample	
Coliforms	6.805 c,d	7.876 a,b	3.0045
E.Coli	6.570 c,d	7.685 a,b,c	1.9566
Staphylococcus	6.898 b,c,d	6.443	1.2198
Fecal streptococcus	7.278 a,b,c	7.366 b,c	0.1920
Clostridia	7.200 a,b,c	7.221 c	1.0034
Yeast and Mould	6.163 d	7.731 a,b,c	2.0299
Lactic acid bacteria	7.893 a	8.151 a	0.8155
Total plate count	7.750 a,b	7.943 a,b	0.5713

Mean of same column followed by different letters differ significantly according to Duncan's multiple range test ($P<.05$)

⇒ n = 6
⇒ varies significantly betweeen samples according to student `t' test.

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Chapter 3

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CHARACTERIZATION OF LACTOBACILLI ISOLATED: BIOCHEMICAL, PHYSIOLOGICAL, GROWTH AND ANTIMICROBIAL ACTIVITY

INTRODUCTION

The Lactic acid bacteria (LAB) are represented by the lactococci, streptococci, lactobacilli, pediococci and the leuconostoc. Poultry intestine as examined qualitatively and quantitatively in chapter 2 revealed the presence large number of Lactic acid bacteria. Lactobacillus among LAB not only are involved in the preservation of certain foods but are responsible for unique identity and sensory attributes unattainable by other food processing methods. Lactobacillus plays an important role in fermentation process of foods, wastes and feed materials. This is because of production of lactic acid, antimicrobial substances and flavour compounds. Understanding the properties of lactobacilli specific to poultry intestine would help in fermentation process.

Therefore, a study has been conducted on the isolation and characterziation of lactobacilli associated with poultry intestine. The study also determined the growth behaviour and production of antimicrobial compound of predominant lactobacilli..

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria

Sampling procedure: Fresh poultry intestine was procured from the near by market place (Devaraja's market) where chicken were brought from near by hatcheries

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in and around Mysore city. Intestines from the slaughtered chicken were procured and brought to the laboratory within lh period.

The poultry intestines tested were of five batches containing two kg samples each. The material was blended in a Stephen mill, for 10 min and samples of 10 g was homogenised in sterile peptone water (1:5 dilution) for 2 min in a Stomacher. Further 10 fold dilutions were then prepared (Mara *et al,* 1991)

Lactic acid bacteria were enumerated as mentioned chapter 2. Colonies were isolated and purified on MRS agar and maintained in MRS broth. The confirmation and grouping of Lactic acid bacteria are based on the biochemical and physiological characterization. The identification methods have been conducted as per data reported mainly by Bergy's manual (1986) and data taken from Rogosa and Sharpe (1959) which provide differentiated characteristics of lactobacilli.

Morphological tests

Twelve colonies isolated from each of six batches were subcultured twice to attain a pure culture and were further tested. Cultures were examined for gram reaction, morphology motility and catalase production. The gram positive, catalase negative, non-motile rods and cocci were examined further.

Biochemical Tests

Gas production from glucose was determined by the method of Gibson and Abdel- Malek (1945) using modified MRS agar overlayed with a plug made from

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petroleum jelly and paraffin wax (1:1). The triammonium citrate of the MRS medium was replaced by ammonium sulphate since some homofermentative lactobacüi evolved gas from citrate in the presence of glucose (Gasser, 1964). Tubes were incubated for 7 days and observed daily.

Deamination of arginine was determined by the method of Niven et al (1942) also used by Carr (1970) and Jayne-Williams (1975), which consisted of 0.5% yeast extract, 0.5% tryptone, 0.2% K_2HP0_4, 0.05% glucose and 0.3% arginine-HCl. After 48h of incubation the NH_3 production was detected by the appearance of a strong orange colour upon addition of one drop of Nessler's reagent.

The media for nitrate reduction was the same as described by Carr (1970), viz, 1% yeast extract, 1% glucose, 0.1% $KN0_3$, pH 4.8. To detect nitrate, 0.8% sulfanilic acid were added in equal amounts. The formation of a red colour was positive for the presence of nitrate, but if the test was negative, zinc powder was added to confirm that nitrate was intact or nitrate was metabolized.

Test tubes with litmus milk (Difco) were incubated and observed during 48, 72 and 86h at 37 º C. A change in colour to light rose indicated acid condition in the media. The presence of a milk clot was also observed.

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PHYSIOLOGICAL TESTS

Procedures **for testing special characters**

The MRS medium of de Man, Rogosa and Sharpe (APHA, 1984) is used for general cultivation of strains, determination of growth temperatures, and for the carbohydrate fermentation methods was followed as indicated in Bergy's manual (1986).

Carbohydrate fermentation : MRS broth without meat extract and glucose with 0.5% (w/v) chlorophenol red is generally used as basal medium. Filter sterilized solutions of the test carbohydrate are added to a final concentration of 1%. Tests are incubated at the optimum growth temperatures and results recorded upto 7 days. In a few cases, for example some strains of L.delbrueckii the addition of 0.2% meat extract broadens the pattern of fermented carbohydrates some what and the fermentation of glucose is distinctly improved. For strains which will not grow reasonably in MRS broth, the optimal growth medium should be used as basal medium. Acid production from different Carbohydrates : Fructose, Galactose, Glucose, Lactose, Maltose, Mannitol, Raffinose, Sucrose, Trehalose and Xylose were tested.

Cultural conditions : To observe growth at 37 °C and 45 °C cultures were cultivated in MRS broth with 2 ml of 1.6% bromocresol purple per litre of media were inoculated in duplicate. One was incubated at 37 °C for 7 days and the other at 45°C for 7 days.

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Maintenance procedures : For short term preservation cultures were inoculated into MRS medium, incubated until growth became visible, stored at 4 - 7 °C and transferred monthly.

Estimation **of growth**

Culture growth was monitored by measuring the absorbance of cell suspension at 550 nm in a spectrophotometer (Shimadzu UV 160 A), by visible cell count by plating suitable dilutions on MRS agar and incubating at 37 º C for 48 -72h or by determination of the dry weight of cells. For determining the dry weight of cells 50.0ml of cultures was centrifuged at 8000 rpm for 10 min and the cells were washed twice with distilled water. The cell pellets taken in pre-weighed glass tubes were dried to constant weight at 80 º C and the weights were determined.

Enumeration of lactic count and preparation of culture filtrate

Glucose medium in requisite quantities (20 ml) were inoculated with individual test cultures of Lactic acid bacteria at a level of 10 cfu/ml and incubated at 37 °C for requisite period. The cultures prior to inoculation were subcultured twice in the same growth medium used for experimental trials. Incubated broth samples were centrifuged at 6000 rpm for 20 min, at ambient temperature, the resulting supernatants in 3 ml quantities were lyophilized. The lyophilized material was disolved in 0.5 ml of sterile phosphate buffer and the same was used to assay for antibacterial activity.

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Prior to preparing the culture filtrate, appropriate dilutions of the incubated broth was pour-plated using MRS agar. Inoculated plates were incubated at 37°C for 24 - 48h, following which colonies apearing in the plates were counted and expressed as colonyforming units per gram (cfu/g). Simultaneously, pH of the incubated broth was recorded in a digital pH meter.

Preparation of phosphate buffer

0.02M phosphate buffer was prepared by mixing 3.56 g/1 of Na_2HP0_4. $2H_20$ and 9.0 g/1 of NAcl. This was adjusted to pH 7.4 with 0.1 N Hcl.

Seeding **of indicator organisms**

25 ml aliquots of nutrient agar was taken in duplicate and sterilized. On cooling one was inoculated with 1.0 ml of E.coli at 10 4 -10 5 cfu/g, priorly grown in nutrient broth for 24 h at 30 º C while similarly S.typhimurium also grown in nutrient broth for 24 h at 30 °C was inoculated at 1.0 ml directly into 25 ml of nutrient agar both indicator organisms used were isolated from poultry intestine. This was pour-plated and allowed to set and then kept incubated at 4 °C . These plates were used within 48h for antibacterial assay.

Test organism **and substrate:** The test organisms were L. fermenti and L. plantarum. both isolated from poultry intestine. These were grown in MRS broth for 36h prior to use in antagonism assays at 37 °C The culture prior to inoculation was twice submitted in MRS broth. Following incubation, the culture broth was

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centrifuged and the culture filtrate prepared. This included the agar diffusion assay. (Schillinger and Lucke 1989 a).

Test **for inhibitory activity**

The inhibitory activity of culture filtrates of lactobacilli against the indicator bacteria was determined by agar diffusion assay method. An appropriate number of wells (2 Nos.) each 5 mm in diameter were made in the agar nutrient agar and inoculated with individual indicator bacterial cultures at 1% level. To each well, was added 65µl of the culture filtrate of Lactic acid bacteria. The assay for each culture was carried out in duplicate. The plates were pre-incubated at 6 °C for 3h for the diffusion of test material into the agar, followed by incubation at 37 °C for 2lh. After incubation, the plates were examined for zones of inhibition around the wells. An inhibition zone diameter was recorded.

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RESULTS

The average total lactobacilli count of viable organisms on MRS agar, from the six batches plated during the 5 month period obtained from fresh poultry intestine was 10 cfu/g. 70% of the organisms present on these plates were catalase negative. 130 isolates were taken at random from the MRS agar plates. Of these, 30 were catalase positive and were not studied further. 100 isolates were studied for their characteristics.

Colonies of some of these isolates showed characteristic rough edges on the agar plates after growth. While most were small 2-5 m.m and a few colonies 6 - 7 mm with entire margins convex, smooth glistening and opaque without pigment. The growth in MRS broth generally was throughout the liquid, but the cells settle soon after growth ceases. Growth of few with very fast settlement could be observed within 18h. While others showed turbidity of liquid. The sediment was smooth and homogenous nearly 1-2 mm in height. Within 24 h no profound odour could be smelt, but on further incubation gave a strong smell.

The cultures after inoculation in MRS broth and incubated at 37 º C grew well. When kept at 4 º C none of the cultures showed turbidity showing failure to grow. But at 45 º C all cultures showed turbidity showing the presence of growth.

Morphological properties of the catalase negative isolates

These gram positive strains on microscopical morphological evaluation as revealed from Table 1, batch 1, 2 and 3 possessed 40 bacilli isolates and 10 cocci

isolates while batch 4 and 5 showed only 20 bacilli isolates and 30 were of the coccoid andcocco bacilli type isolates.

Physiological and Biochemcial properties

Most of the gram positive, catalase negative microorganisms were glucose positive revealed in Table 2. Acid production in glucose was evidenced by a change in chlorophenol red to yellow within 24 h and was left for 5 days to confirm any change. Many of the isolates gave positive to glucose, sucrose, lactose and fructose sugars except xylose which gave a clue that the species may belong to L.plantarum. L. fermenti. L. acidophilus. The bacilli were separated from cocci and further tested separately for their physiological and bio-chemical reactions.

The cultures were tested for the fermentation of glucose, sucrose, lactose and fructose. These cultures utilized these sugars rarely. By testing these cultures with other sugars: raffinose, melibiose, sorbitol and mannitol, a clear differentiation was observed between species. The present studies revealed that out of 100 LAB tested, 60 were Lactobacilli, 20 were Enterococci, 10 were Pediococci and the remaining 10 were Leuconostoc. The results from Table 3 reveals 8 species of lactobacilli have been obtained from poultry intestine. L. Plantarum isolated from poultry intestine was microphotographed. Plates 1 showed the morphological feature of L. plantarum.

The cocobacilli, coccoid natured isolates were tested for the genus level only. These cocci isolates belonged to streptococcus group while pediococus and

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leuconostocs were also obtained out of the total cocci obtained from batches 1,2,3.4 and 5.

On the basis of utilization of carbohydrate tests" are shown in Table 2, it was noticied that L,plantarum and L.acidophilus were the most common as they were observed in all the 5 batches. Among the lactobacilli isolates tested L.plantarum was nearly 35 in number, while L.acidophilus was about 15 in numbers while the remaining 10 belonged to L.brevis (3). L.fermenti (2). L.salivarus (1). L.murinus (11, L.jensenni (2). L.reuterri (2).

From Table 3 the results revealed both homofermentative and heterofermentative species of lactobacilli are present in poultry intestine.

These lactobacilli belong to the three main groups. Thermobacterium, streptobacterium and betabacterium by their end products of carbohydrate fermentation. L. plantarum. L.murinus belonging to streptobacteria and L. fermenti to Betabacterium while L.acidophilus and L.Salivarius to Thermobacterium group.

Table 4 gives in detail the Biochemical and physiological characters of the species of L.plantarum which is most predominantly found in fresh poultry intestine.

Growth **of Lactobacilli plantarum**

Data on the growth of L.plantarum was recorded as it accounted for the largest number in poultry intestine among other lactobacilli. The growth of L.plantarum could not be studied in its original habitat from where it was isolated as the material

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was a mixture of various and numerous micro organisms. Therefore the growth behaviour of L.plantarum was estimated in MRS broth the best liquid medium in which cultivation is easily facilitated at 37 °C.

Growth of L.platarum is given in Fig 1. Maximum growth was attained at 18 h and then onwards, it was stationary up to 48 h. From 48 h, the growth began to decrease gradually. The log phase was from 6 h to 18 h this shows the organism has **a** fast growth. This reveals why acid production is obtained within 24 h, being observed by colour change of chlorophenol red to yellow as seen in Table 2. The inoculated culture was maintained at 37 °C owing to the fact that 37 °C was favourable for the growth of L.plantarum. For the calculation of doubling time growth during the exponential phase (that is between 6 h and 12 h) was taken. The doubling time of L.plantarum was calculated and is found to be 4 h and 2 minutes.

Screening of Lactobacilli for antibacterial activity

From plate 2 and 3, it is clear that L.plantarum and L.fermenti inhibited E.coli and S,typhimurum. Clear inhibitory zones were found around the wells. It is observed that the antigonistic substance had diffused to 5-5.5 mm around the well preventing the growth of E.coli and S.typhimurium. Beyond 5.5. around the edges of the agar wells it is noticed that E.coli and S.typhimurium grew luxuriously indicating the spectrum of activity of the antimicrobial compound. Agar well diffusion assay can be used to determine the inhibitory potential of the lactobacilli against gram negative feed borne pathogens. The results are given in Table 5 as zone sizes measured as the distances

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from the border of the test spot to the edge of the clear zone. Inhibitory zones of the antimicrobial substances of L,plantarum were slightly broader than that of L.fermenti. The agar diffusion well assay method showed that the natural isolates of lactobacillus species from poultry intestine (L,plantarum and L,fermenti) possessed good antibacterial activity.

The properties of acid production, fast growth rate and production of anti-microbial substance reveal a promising foundation for using lactobacilli for the further studies of ensiling poultry intestine.

DISCUSSION

Bacterial colonization of the intestinal tract of chicks takes place soon after hatching when the young animals ingest food (ochi *et al,* 1964; Salanitro *et al,* 1974). During the first 2 to 4 days, streptococci colonize the small intestine and cecum. The work of Ochi *et al,* (1964), indicates that after the first week, the composition of the flora stabilizes and the lactobacilli predominate in the small intestine with small number of streptococci. The present study reveals that the largest composition oí Lactic acid bacteria were of the lactobacilli. The results of this study in the isolation of Lactic acid bacteria from the intestinal tract of chicken (8 week old) partly confirms the work of other investigators related to LAB in particular lactobacilli. Barnes *et al,* (1972) and Smith (1965) also observed that lactobacilli were the predominant organisms (a population ranging from 10^5 to 10^8 /g) of intestine.

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Lactobacilli which have been found in large numbers from chick cecae or faecal material in other studies (Ochi *et al,* 1964 and Smith, 1965; Huhtanen *et al,* l965; Barnes *et al,* 1972; Mead and Adams, 1975; were isolated as minor components of the cecal flora. LAB have been isolated from various parts of the intestinal tract like pdenum, ileum, cecum, small intestine (Salanitro *et al,* 1978; Ochi *et al,* 1964; Smith, l965).

The lactobacilli isolated in the present study were both of the komofermentative and heterofermentative type. The characterization and species of lactobacilli from chicken intestine has not been reported so far.

In this present study many of the rods (genus lactobacilli) followed a pattern of carbohydrate ferementation which they fermented: glucose, sucrose, lactose, fructose. maltose, mannitol. These findings are similar in one way with that reported by Beverley *et al,* (1982) for example, fermentation of these compounds (lactose, glucose, maltose or mannitol) is considered typical of L.plantarum and is also common for streptobacteria in general (Sharpe, 1979) The names of these subgenera are not valid according to Bergy's manual (Breed *et al,* 1957). They are at present recognised as a useful subdivision of the genus lactobacillus (Sharpe, 1979).

In the present study few isolates of Leuconostoc have also been identified on their ability to ferment trehalose. Rogosa *et al*.,(1951) and Mabbitt and Zienlinska, (1956), they reported that Leuconostoc and L.brevis are likely to be isolated together and may not be readily distinguished morphologically, as they can all occur as coccobacilli

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B(Percy and Sharpe, 1960), however they can be differentiated by their ability to ferment Itehalose, but not to hydrolyse arginine, while L,brevis does not ferment trehalose and lydrolyses arginine..

A large number of reports are available on the antibacterial activity of Lactic acid bacteria against pathogenic bacteria or characterisation of the compound produced by Lactic acid bacteria which are popular (Daeshel, 1989, 1990, 1993, Klaenhammer, 1988; Ray, 1992; Hoover and Steenson 1993; Nettles and Barefoot, 1993; and Salmines and Wright, 1993. These bacteria produce an environment which inhibits the growth of most organisms causing food stuff spoilage (Mossel, 1971; Hurst, 1973).

The antagonistic effect of the Lactic acid bacteria are primarily ascribed to undissociated organic acids produced in the fermentation processes (Sorrells and speck, 1970). However, other mechanisms may be involved. For instance, some LAB lacking a catalase system can produce toxic levels of hydrogen peroxide (Dahiya and speck, 1968; Prince and Lee, 1970).

Bacilli produce many antibiotics although clostridia have not been reported to produce antibiotics (review by Hurst, 1969). Almost all LAB studied can inhibit I other microbes with which they are associated. In consequence they frequently become the dominant microflora. These organisms are capable of causing inhibition by virtue of showing acid production, H_20_2 and antibiotics. Earlier reports on the antagonistic effect of LAB against Cl.botulinum was ascribed by Benjamin *et al,* (1956), chiefly to lactobacilli. Sadine (1963) also reports on an antibiotic like effect from L.acidophillus

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which Tramer, (1966) believes was due to pH. Dahiya and speck, (1968) and Price and Lee, (1978) confirm the strong inhibitory properties of peroxide produced by lactobacilli on a variety of gram positive and gram negative organisms. In the present study it could be revealed that the antibacterial compound produced by Lactobacilli plantarum and L,fermenti inhibited both gram negative E.coli and S.typhimurium.

An interesting fact that lactobacilli isolated from poultry intestine could easily counteract against the pathogenic E.coli and S.typhimurium isolated from poultry intestine, Showing the preservative capacity of lactobacilli towards the poultry intestine.

From the present study it can be seen lactobacilli from poultry intestine had inhibitory influence on S.typhimurium and E.coli. Results reported by Mara Lucia *et al.* (1991) where in L.plantarum culture inoculation was active only towards S.aureus. Using the agar spot method L.plantarum strain were found to produce inhibition zones only against S.aureus and not S.typhimurium. But the positive results obtained by the agar diffussion method in the present study, that the unassociative growth approach of S.typhimurium and E.coli indicate they are sensitive to the antagonistic activity ascribed to lactobacilli in poultry intestine. So far no reports are produced as to the antibacterial activity of lactobacilli isolated from poultry intestine and its inhibitory activity against microflora from its own environment and the nature of the compound. Thus the present investigation provided additional information with regard to the antibacterial activity of lactobacilli isolated from poultry intestine. But a review

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published by Juven *et al,* (1991) shows the antagonistic effect of lactobacilli to control intestinal colonization by human entero pathogens in live poultry, where the workers live prepared probiotics of LAB which has been beneficial in treating certain gastrointestinal disorders (Sandine *et al,* 1972)

From the present study it can be concluded that the predominance of lactobacilli, it's ability to double in 4 h, and produce acid within 24h along with the iroduction of anti bacterial compound, could improve the nutritive value of poultry intestine as feed and mainly help conserve this large quantity of protein in poultry intestine making it useful.

Conclusions

The study revealed the predominance of lactobacilli in poultry intestine. L.plantarum and L.acidophilus dominated LAB group. Lactobacilli demonstrated homo and heterofermentative character. Lactobacilli are potent producers of acid and antibacterial compounds. Thus, native lactobacilli can play a vital role in ensiling poulty intestine.

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Table-1

Properties **of** lactobacilli **isolated from poultry intestine**

Group number (batches)	1	2	3	4	5
Number of isolates tested	10	10	20	10	10
Growth on MRS agar at 37 °C	10	10	20	10	10
at 45 °C	10	10	20	10	10
NH3 from arginine	1	4	2	7	7
Gas from glucose	8	8	17	12	13

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Ttable 2

lization of substrates by Iactobacil!i isolated from poultry intestine

Group number	1	2	3	4	5
Number of Isolates tested	12	12	16	10	10
Fermentation : Arabinose	8	8	10	ND	HD
Glucose	8	8	14	10	10
Lactose	7	7	16	10	10
Mannitol	6	6	10	6	8
Salicin	8	8	11	7	8
Sucrose	5	6	21	10	11
Xylose	5	7	6	3	0
Ramnose	ND	ND	ND	0	0

Numbers indicates: Species +ve 0
indicates: all negative ND
indicates: not done

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Table 3

Itrcentage of Lactic acid bacteria isoalted from poultry intestine

Sl.No.	A. LACTOBACILLI	%	B. OTHERS	%
1	L.plantarum	35	Streptococcus sps	20
2	L.acidophilus	15	Pediococcus sps	10
3	L.brevis	3	Leuconostoc sps	10
4	L.fermenti	2		
5	L.salivarius	1		
6	L.murinus	1		
7	L.jensenni	1		
8	L.reuteri	2		

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Table 4

kmical characteristic of the strain L.plantarum

Sl.No.	Character	Response
1	Gram strain	Gram positive
2	Micro scopic appearance	Single rods, Sometimes in short chains of 3-4 with rods having rounded ends
3	Catalase	Negative
4	Citrate Utilization	Positive
5	Arginine hydrolysis	Negative
6	Nitrate reduction	Negative
7	Growth at 15 °C	Positive
8	Growth at 45 °C	Positive
9	Carbohydrate Fermentation:	
	a. Galactose	Positive
	b. Glucose	Positive
	c. Lactose	Positive
	d. Maltose	Positive
	e. Mannitol	Positive
	f. Raffinose	Positive
	g. Sucrose	Positive
	h. Xylose	Positive

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Plate 1

L.plantarum ISOLATED FROM POULTRY INTESTINE

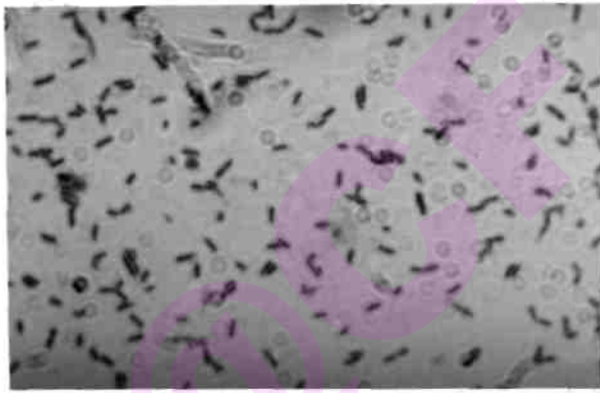

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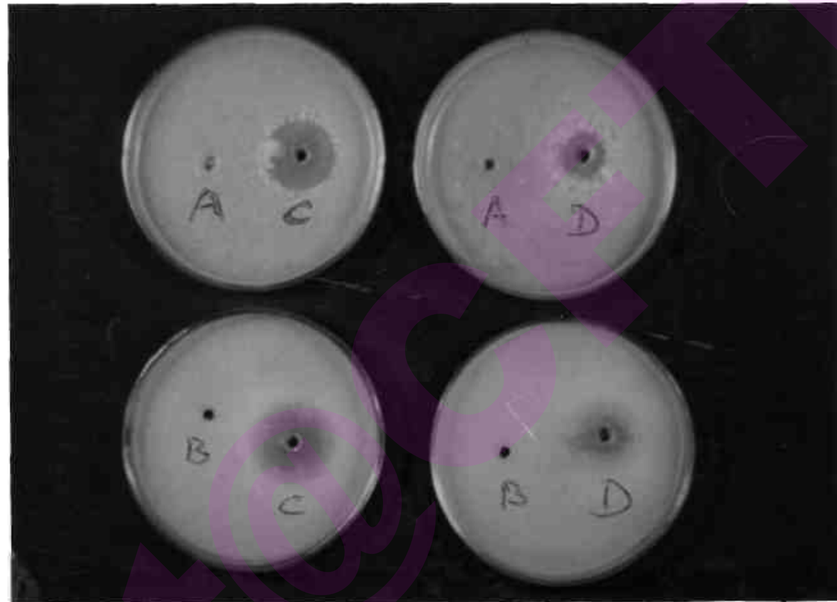
Plate 2

INHIBITORY EFFECT OF SUBSTANCE PRODUCED BY

L.plantarum on E.coii and S.typhimurium

and

L.fermenti on E.coii and S.typhimurium



Control

A: L.plantarum B:

B. L.fermenti

C: H.coli

D: S.typhimurium

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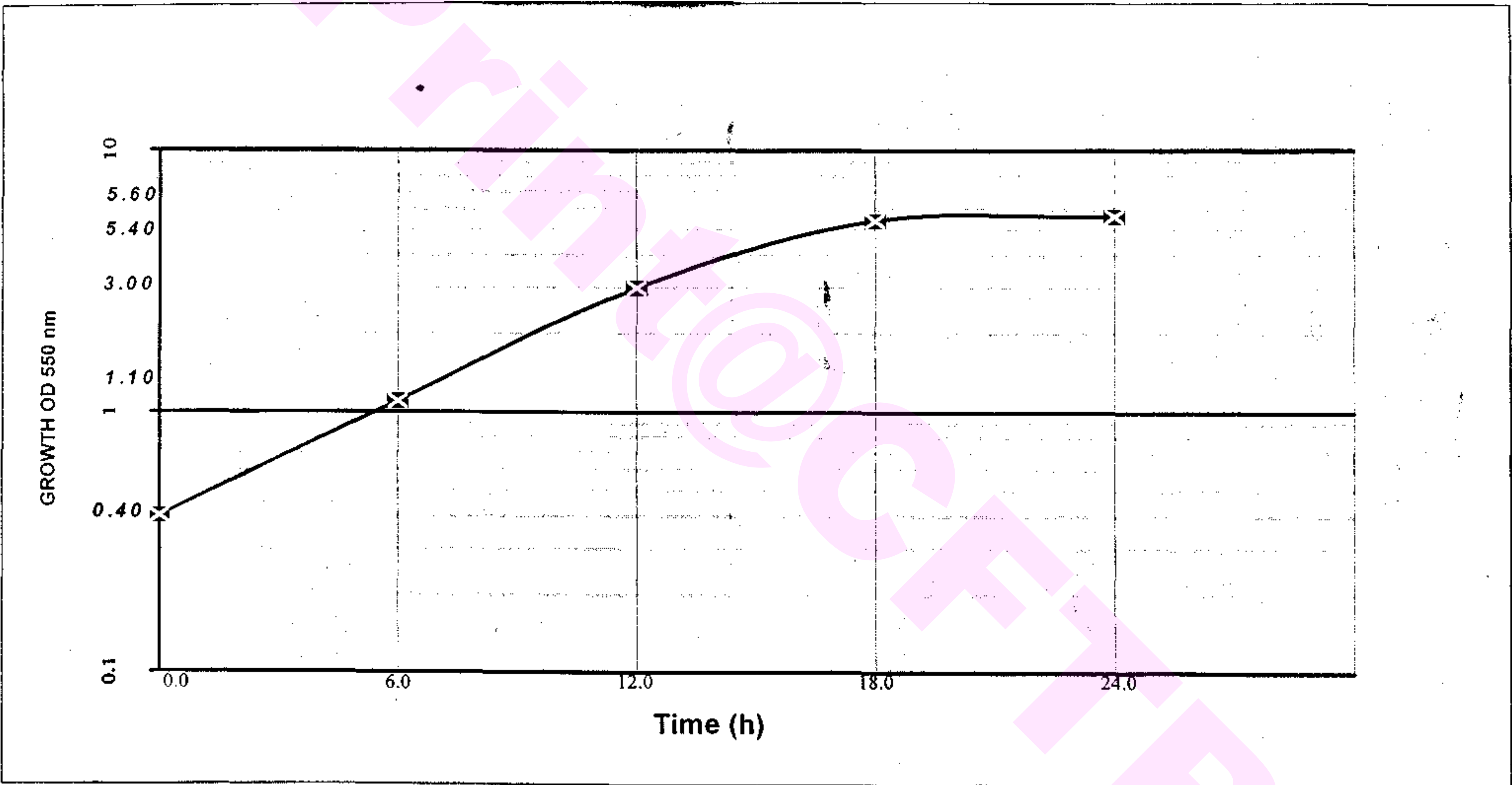

Figure 1:Growth of L. plantarum isolated from poultry intestine

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Chapter 4

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Plate 1

Fresh Poultry Intestine

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Plate 2

Addition of molasses to homogenized poultry intestine

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Plate 3

Offal homogenized in Stephen universal machine

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Plate 4

Fermentation process

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OPTIMIZATION OF MOLASSES AND THE EFFECT OF ADDITIVES ON THE ENSILAGE OF POULTRY INTESTINE.

INTRODUCTION

The quest into the selection of poultry intestine is obviously due to its availability, low price, it's chemical composition (particularly rich in protein) and the requirement of an animal protein source in animal feeds. Recycling of these materials would provide a source of good quality animal protein. The composition of the raw material also varies according to processing method and season. As such wastes are highly perishable, it must be treated immediately and made into a stable product. Due to scattered nature of operation and small volume of production, the selection of technology will depend very much on the logistics. In recent years much of the interest is revived in ensiling techniques in treatment of such waste material. The ensiling through bacterial fermentation appears to be a more promising method over other methods using enzyme digestion technique.

There is a need to look for suitable additives which would retain the nutritive value of the raw material, and conserve the material for better use.

Additives are of 6 major categories : Preservatives, nutritional additives, flavouring agents, colouring agents, texturizing agents and miscellaneous additives.

Chemicals like benzoic acid, propionic acid, sorbic acid, common salt, have also been used for the purpose of preservation (Baird Parker, 1980; Chichester, *et al,* 1968; Kao *et al.,* 1978; Njagiand Gopalan, 1980). These preservatives are used to prevent or retard both chemical and biological deterioration of foods. The primary

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additives used to prevent biological deterioration are the antimicrobials. It is estimated that with the use of antioxidants, the shelf-life of many food products have increased substantially by 200% (Branen, 1975). A combination of preservatives to control both chemical and biological deterioration can further extend the shelf-life. The spoilage problem need to be first identified in a particular system and then the possible preservation system must be evaluated through model studies.

Poultry intestine contains no carbohydrate/sugar; thus to achieve a successful process of ensilage, the addition of molasses has been preferred as a source of carbohydrate and it is easily available at relatively cheap rates. Molasses comes under the group of additives or potential additives called fermentation stimulants which operates by encouraging rather than suppressing the silage fermentation. The addition of molasses as carbohydrate source would enable the naturally occuring lactic acid bacteria to poliferate and promote the process of fermentation of intestine.

The objectives of the study in this chapter was to optimize the levels of molasses and salt and to study the effect antimycotic agents for acheiving fermentation ensiling of poultry intestine.

Materials **and methods**

Chemicals utilized for this study were Sorbic acid, Propionic acid, Benzoic acid and Common salt. Molasses was procured from Mandya Sugar Manufacturing Industry (local). The black strap molasses a heavy, viscous liquid separated from the final local grade massecuite was used as a carbohydrate source.

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Composition of molasses

Determination of reducing sugar (total) was followed according to the methods described by Indian Standard Specification For Cane Molasses 15:1162 - 1958. Appendix D [Table I, item (iii)].

PROCEDURE : Preparation of Solution :

Weigh accurately about 12.5 gms of molasses and transfer to a 250 ml volumetric flask. Add 25 ml of the lead acetate solution. Make up to volume, mix and filter. To 100 ml of the clear filtrate in a 500 ml volumetric flask, add 10 ml of the sodium phosphate potassium oxalate mixture. Make up to volume with water, shake and filter. Reject the first few drops of the filtrate and use the clear filtrate for preparation of invert solution preparation of invert solution : to 100 ml of the filtrate, in a 250 ml volumetric flask, add 25 ml of water, and 10 ml of concentrated hydrochloric acid. Heat on a water-bath to 70 °C regulate heat in such a way that the temperature is maintained at 70°C. Place the flask in a water-bath, insert a thermometer and heat with constant agitation until the thermometer in the flask indicates 67 °C, leave the flask in the water-bath for exactly 5 minutes, during which time the temperature should gradually rise to about 69.5 °C. Plunge the flask at once into water at 20 C. When the contents have cooled to about 35 °C, remove the thermometer from the flask, rinse it and add 10 ml of 6 N sodium hydroxide solution

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for neutralization of acid, leave the flask in the bath at 20 °C for about 30 minutes and then make up exactly to volume with water. Mix the solution well.

Standard **method of titration**

Pipette 10 ml of Fehlings Solution into 300 ml conical flask and run in from the burette almost the whole of the prepared solution rquired to effect reduction of all the copper so that, if possible not more than one millilitre shall be required later to complete the titration. Gently boil the contents of the flask for 2 minutes. At the end of 2 minutes of boiling, add without interrupting boiling, one millilitre of methylene blue indicator solution. While the contents of the flask continue to boil, begin to add the solution (one or two drops at a time), from the burette till the blue colour of the indicator just disappears. The titration should be completed within one minute, so that the contents of the flask boil altogether for 3 minutes without interruption.

Estimation of protein, fat and moisture

The detailed description is given in Chapter 1 (Material and Methods).

SILAGE PREPARATION

Experimental design:

a. Addtion of molasses at different levels:- The poultry intestine procured from the local market was from broiler birds (6 to 9 wk.) of the cob strain. Intestines of 6 kg were minced in a Stephen mill. 3 kg homogenate was equally distributed into (3) 1 kg lots (placed in 3 kg buckets). Molasses was added at 8%, 10% and 12% level to the 1 kg lots. This was used for microbial analysis. The remaining 3 kgs was

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distributed into 250 gram lots (placed in 500ml beakers). Molasses was added at 5% to 15% level to the 250 gram lots. This was used to determine the pH.

b. **Addition of molasses and salt:-** Likewise 3 kg poultry intestine homogenate consisting of 10% molasses was distributed into (3) 1 kg lots to which 0.5%, 1% and 2% levels of salt was added for the analysis of microbial profile and pH determination.

c. **Addition of molasses and antimycotic agents:-** Similarly to poultry intestine homogenate consisting of 10% molasses, antimycotic agents **i.e** sorbic acid, benzioc acid and propionic acid at 3 different levels (0.1%, 0.3% and 0.5%) was added into (3) 1 kg lots (placed in 3 kg buckets), for the analysis of microbial profile and pH determination.

In the above cases poultry intestine homogenate with 10% molasses on addition of additives was mixed thoroughly for even distribution and covered with LDPE covers and the lids of the buckets were fastened tightly in order to develop a microaerophilic conditions and left for incubation at ambient (26 ± 2 °C) for ensiling.

pH **determination**

pH was measured at intervals of 0, 6, 12, 18, 24, 48, 72, 96, 120 and l44h, (luring fermentation on ensiling of poultry intestine. The experiment was carried out in 6 replicate batches.

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Microbial analysis of molasses

Microbial analysis was done by placing 50 gm of sample into 450 ml sterile physiological saline and blending it in a stomacher for 2 minutes. This formed the lirst decimal dilution. Further, serial dilutions were made upto 10 using aliquots containing 90 ml sterile physiological saline. TPC, Coliforms, E.coli. Siaphylococcus, Enterococci, Yeast and Mold, Lactic acid bacteria were estimated as per the procedures of APHA, 1984.

Microbial analysis in salt containing samples (0.5%, 1.0% and 2.0%) were analysed by similar method as described for molasses, but only Yeast and Molds were analysed in antimycotic containing samples.

Shelf-life of poultry intestine silage

Poultry intestine silage was prepared by homogenising 6 Kg poultry intestine, 10% molasses and 0.5% propionic acid in a Stephen Mill for 5 minutes to 7 minutes and incubated in 5 Kg capacity plastic buckets in duplicate. This was covered over by LDPE and the bucket lid tightly placed to create a microaerophilic condition. Samples were withdrawn every 15 days for 90 days to check the microbial load, whether there was an increase in fungi, (the main spoilage microorganism) 50 gram w sample was taken from the buckets and placed in 450 ml of serile physiological saline, and serially diluted, further dilutions were made upto 10 . pH was also recorded simultaneously.

Protein content was also analysed to check any decrease in the nutritive value. Protein was analysed by Kjeldhal Method.

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Statistical Analysis

Experimental data (count of microorganisms) obtained from washed and unwashed poultry intestine, were subjected to ANOVA. Significant difference between means within the sample were tested by Duncans multiple range test (Duncan, 1955).

RESULTS

Optimization of Molasses:

The reducing sugar in molasses is 46.3 % (Table 7). It is a good source of carbohydrate suitable for poultry intestine fermentation as this offal lacks in carbohydrates. This sugar in molasses would stimulate the growth of large numbers of Lactic acid bacteria present in poultry intestine. Moisture in molasses is 30% which together with 70% moisture in poultry intestine totally forms a semi soild mass but later on liquifies at higher temperature (at 37 °C). This would facilitate easy mixing with feed ingredients at the final stage of feed production. But this moisture both in poultry intestine and molasses has shown to have no effect on pH or microbial profile of poultry intestine silage as depicted in Table 4 and Fig. 1. Molasses does not contribute to the protein value or fat as it is as low as 0.3%. It is also free from other microorganisms. If it contains Lactic acid bacteria the results from Table 1 would prove that 5% molasses is sufficent to decrease pH by 24h and lead to stabilize poultry intestine silage. But it can be noticed from table 4 at 24h the pH reaches 4.3 close to desired pH (4.2) but remains at pH 4.3 till about 48h, and later decreases producing an

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off odour in poultry intestine silage. At 144h pH raised to 4.8. This justifies the absence of Lactic acid bacteria in molasses. But the addition of 8% molasses refrained poultry intestine from spoilage. pH 4.2 reached in 48h with addtion of 8% molasses. But on addition of 10% pH decreased to 4.2 in 24h which is acceptable, because slower the pH decrease, the lesser would be the nutritive value of silage. When molasses was used at 10% and 15% levels, pH decreased to desirable level in l8h to 24h. But product stability and inhibition of spoilage bacteria with addition of 10% molasses is more economical than of 14% and 15%.

Fig. 1 shows effect of 8%, 10%, 12% molasses on the microbial profile of poultry intestine silage. Here 8% inhibited Enterococci and E.coli at 96h. While with 12% molasses the E.coli was inhibited at 72h, Enterococci and Coliform at 96h. In samples containing 8% molasses, Coliform counts was 3 log / gram. While Staphylococcus, Yeast and Mold still persisted. Lactic acid bacteria did not decrease but remained at 10^9 to 10^{10} counts. Table 1 shows addition of 12% molasses did not show any additional beneficial effect over 10% molasses. 10% molasses showed significant difference over 10% among all microorganisms except Enterococci and TPC irrespective of the hours. Therefore 10% molasses was added for further studies as it was found to be optimum. It is evident statistically that molasses at 10% was adequate to attain stabilized silage within 24h.

Effect of **defferent levels of antimycotic agent on pH and microbial profile**

As depicted in Table 6 addtion of sorbic acid, benzoic acid and propionic acid had no adverse effect on the pH of poultry intestine silage. Desirable pH of 4.2 was

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attained in 24h irrespective of the antimycotic agent used. The poultry intestine silage remained stabilize as noted from pH results. This was mainly due to the presence of l0% molasses added as substrate along with antimycotic agent. But Yeast and Mold was effected according to the diffent antimycotic agents as well as the different percentages. From Fig.2 and Table 2 it is seen that 0.5% benzoic acid exerted no pronounced effect on the Yeast and Mold counts. (2 log decrease from Oh) at l44h. While 0.5% sorbic acid decreased counts from 96h and the counts at l44h where nearly half the initial counts (7.65 to 4.87). So also propionic acid (8.82 to 5.16). This shows better capacity of sorbic acid and propionic acid over benzioc acid in decreasing levels of Yeast and Mold during ensiling of poultry intestine, where superscript Ά' is present for both propionic acid and sorbic acid do not differ significantly but it is significant over benzoic acid.

Effect of **Salt on pH and microbial profile of poultry intestine during fermentation.**

Results on the effect of different levels of salt are given in Table 2, effect of Salt on pH is given in Table 5. 1 % and 2 % salt showed better performance over 0.5% irrespective of hours, similiarly irrespective of percentage of salts microorganisms decreased towards l44h except Lactic acid bacteria. pH decrease to 4.2 was in 24h at 0.5%, 1% and 2%(in Table 3b,c). Higher levels of salt in (1% and 2%) decreased the volume rise due to gas production by Coliforms and spoilage microorganisms during fermentation. But addition of even 0.5% of salt to silage on wet basis would be increased to a 20% level of salt on a dry basis, which is not palatable as feed for livestock. Therefore there was no addition of salt during preparation of poultry intestine silage in the present study. Further work was carried

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out by addition of 10% molasses, 0.5% propionic acid during fermentation of poultry intestine silage.

Production of Stabilized silage

Molasses at 10% and antimycotic agent at 5% was added to homogenized poultry intestine material and allowed to ferment to obtain a stabilzed product in 24h, with a desirable pH of 4.2 and free from pathogenic and spoilage microorganisms. Study conducted on shelf life of fermented poultry intestine silage revealed that though the original count of Lactic acid bacteria reduced to half (log 10 to log 5.76) and Yeast and Mold were not detected. Protein content remained the same. No pathogenic microorganisms were detected. pH 3.9 or below was conducive in retaining good conditions in poultry intestine silage upto 90 days. It was also interesting to note no development of fungi around the lid of container or on the upper layers of the poultry intestine silage.

DISCUSSION

Molasses as **fermentation stimulant**

Molasses, which is a by-product of sugar refining, contains about 50% (by weight) of sucrose. Molasses used in the present study contained 46.3% of reducing sugar. As a relatively cheap source of readily fermentable substrate molasses has received considerable attention as an additive for improving the reserves of sugar in silage. Traditionally it was applied to forage at about 4 Kg / tonne fresh weight following dilution with an equal weight of water, in order to facilitate the distribution of an otherwise viscous material. (Woolford, 1984). But this problem was not faced

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during the study, as poultry intestine contained a high moisture content of 70% as indicated in Table 2 of Chapter 2. Thus during the process of fermentation, moisture from the poultry intestine was liberated, making it a semi solid material.

Examination of data relating to experiments on the use of molasses in silage indicates that their effect on fermentation are sometimes difficult to interpret (Woolford, 1984). But one point is clear; whether the carbohydrate is in the form of mono or dissacharides is of no consequence since lactic acid bacteria can utilize these with relative easy as demonstrated by Rydin and Colleagues (1956). This substantiates why molasses is preferred in the study, as poultry intestine is a source of abundant native Lactic acid bacteria which cannot start fermentation without the presence of Sugar. With the addition of a carbohydrate or sugar source of molasses Lactic acid bacteria receive a source of substrate to thrive on poultry intestine and prevent it from putrifying. Generally, sugar addition resulted in an accelerated growth of Lactic acid bacteria, a more rapid development of acid condition, and the early elimination of coliform bacteria as depicted in Fig.2.

Number of reports are available on the use of molasses in different silages. Herbage silage (Catchpoole, 1966; Greenhill, 1964; Weeth and Rosenberg, 1954, Ross and Rondo, 1958; Damron and Harms, 1968). Molasses has been effectively used as a feed ingredient for all types of livestock for over 100 years. Molasses may be successfully used to replace a portion of the cereal grain in diets for broilers, layers and turkeys. Many of these early studies have been reviewed by Scott (1953) and Ewing(l963).

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The present study shows 10% molasses was adequate to achieve the desirable pH of 4.2 in 24h and reduce the Coliforms within 24h. Coliform are referred to as the yardstick of the silage. Therefore, its reduction is of great significance in bringing a faster development of stable silage. An early reduction in pH is beneficial because it helps protect the nutritive value in poultry intestine and prevents poultry intestine from putrifaction by the spoilage and pathogenic organisms.

Work of Devuyst and co-workers (1975) considered that atlelast 10 g Kg - 1 is required to reduce proteolysis. One of the earliest reports on the use of molasses for young chicks was by Winter (1929). He reported that cane molasses could be used to replace cereal grains, Kg for Kg, at levels upto 10% for growing chicks. Other reports suggest either a lower or higher quantity of molasses should be used. With regard to ensilage of com, grass and herbage (Budzier, 1967; Catchpoole, 1966; Greenhill, 1964; Devuyst *et al,* 1975; Nevens *et al,* 1936), while there is scarce information on the use of different percentages of molasses in animal silages.

Rosenberg (1954) in his work isonitrogenous diets, chicks fed upto 46% of a molasses - bagasse or molasses wheat bran mixture (5:1 ratio of molasses) grew as well as chicks fed on control diet. Rosenberg (1955 a, b) indicated that cane final molasses could be effectively used at upto 34.5% of the diet. Growth rates were almost identical to those of chicks fed on the control diet. However, in 8 week feeding trials, Upp (1937), observed inferior growth higher feed intake, and lower feed efficiency with diets containing 5 to 15% molasses in place of equal amounts of corn. He recommended that no more than 5 to 7% molasses be used in chick diets. Ott *et al,* (1942a) fed female chicks to 24 weeks and males to 12 weeks of age on

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lets upto 6% molasses and concluded that growth rates were equal, but total feed intake was increased significantly by the inclusion of molasses in the diet. Soldevila et a*l,* (1970) incorporated molasses into broiler diets at levels upto 20%. Molasses was added at the expense of corn although the diets were said to be isocaloric and isonitrogenous. Winter (1929) reported that cane molasses could be used to replace cereal grains Kg for Kg, in levels upto 10% of the diet. Ott *et al,* (1942b) fed 2 generations of laying hens diets containing from 0 to 6% cane molasses. They reported that performance was not significantly influenced by the presence of molasses in the diet. Walker and Ross, (1960) conducted taste panel studies with eggs from hens fed upto 30% molasses. The panel could not differentiate between eggs of hens fed on molasses or 15% molasses, but eggs from hens fed on 20% and 30% had lower scores. However, none of the eggs tested were considered to be undesirable or objectionable. The work of Twiddy *et al,* (1987) showed spoilage bacteria increased, more rapidly when rice was added a substrate.

From the above experiments cited, it could be confirmed that molasses, though itwas added at 5% level or 46% level had no effect on the chickens fed with the diets. This shows molasses could be added at any level, mostly above 10%. But, in the present study, it was seen from both pH and microbial analysis 10% and above gave the similar results while 9% and below indicated slow decrease in pH only after 48h while inhibition of microorganisms was not evident; this does not lead to development of a good silage. This shows that molasses at 10% level is sufficient enough to produce a stable silage. In spite of reports of similar or variable losses being sustained in molasses and untreated silages (Murdoch *et al,* 1955; Wittwer *et al,* 1955;

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Anderson and Jackson. 1970; Mc Donald and Purves, 1956) the mass of evidence lies in favour of it being an effective treatment in terms of promoting a lactic fermentation, assisting the decline in pH, discouraging a clostridial fermentation and proteolysis, and generally reducing losses of organic matter (Archibald, *et al,* 1960; Mc Donald *et al,* 1965; Carpintero *et ai,* 1969; Mc Carrick, 1969; Zetter, 1960; Podkowka, 1971; Podkowka and Pauli, 1973). In terms of nutritive value of the resultant silages, molasses has brought a mixed response insofar as digestaibility, intake and animal production are concerned (Kendall *et al,* 1955; Murdoch, 1956; Ohyama and Inoue, 1968; Bratzler *et al,* 1956).

Selection **of propionic acid as antimycotic agent for ensilage of poultry intestine**

The principle of acid preservation of fish was explored by Edin (1940). Unlike mineral acids, organic acids have specific antimicrobial properties. For example, propionic acid is regarded as being distinctly antifungal (Gross and Beck, 1970; Daniel *et al.,* 1970), and it does have some action against endospore forming bacteria, Woolford (1975). At pH 6.0 spore forming bacteria especially rope bacteria (Bacillus subtilis), are primarily affected. As pH decreases to 5 or 4, Yeasts and Molds become susceptible to inhibition, although not to the same extent as bacteria (Woolford, 1975 b).

The antifungal properties of propionic acid are well known and made use of in the preservation of grain. From the results it is noted that fungi were absent, but Yeast were predominant to the end of fermentation. It has been reported that 1.5 to 6.0 gram/litre propionic acid is inhibitory to Yeasts in pure culture (Gross and Beck, 1970; Beck, 1975). Viewing the Shelf-life of poultry intestine silage upto 90 days,

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prepared from 10% molasses and 0.5% propionic acid, the results revealed only presence of Yeast and lactic acid bacteria during storage period. There was no formation of fungal growth on the sides of the bucket or above the silage mass as when 0.1% or 0.3% propionic acid was used. This indicates, safe and stable poultry intestine silage, free from pathogenic and spoilage microorganisms. At pH 5.0 and below propionic acid has proved to have a strong inhibitory effect both on Molds and spore bearing bacteria (Woolford, 1975). The antimicrobial activity of weak organic acids is usually enhanced when pH is lowered. This is probably because the undissociated molecule can freely pass the plasma membranes of the microbes, whereas the uptake of the charged anion is restricted. It may therefore be advantageous to preserve with a mixture of a rather weak acid with potent antimicrobial activity than a stronger acid. At pH 4.5, most of the propionic acid (pka 4.87), exists in the undissociated state. (Woolford, 1975).

Propionic acid tended to restrict fermentation in silage and afforded good protection against aerobic deterioration when applied.at levels upto 20g Kg^{-1} fresh weight (Daniel *et al,* 1970; Britt *et al,* 1975; Mann and Mc Donald, 1976; Mc Donald and Henderson, 1974; Woolford and Cook, 1980). Although propionic acid was observed to encourage the growth of lactic acid organisms (Papendick and Singh-Verma, 1972), the opposite has been found in so far as the total viable organisms are concerned (Mann and Mc Donald, 1976). The latter outcome is more consistent with the trend toward a restriction of fermentation resulting from its use in silage. Similar results have also been observed in the present study. As reported by Huber and Soejono, (1976), propionic acid was more effective in increasing intake. In

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view of these factors propionic acid was prefered over other organic acids like benzoic acid and sorbic acid, because these additives reduced the acceptability of the silage to animals, as well as its relatively high cost, its potential as silage conservant seems very limited. (Woolford. 1984). Yet another point to substantiate the use of propionic acid in poultry intestine silage is, it's regulatory status. Propionic acid (21 CFR 182.3081) is approved as GRAS substances for miscellaneous and general purpose usage. The acceptable daily intake is not limited under limitations (mg/Kg body weight) unconditionally. For sorbic acid the regulatory status was considered GRAS (21 FR 182.3089). The maximum concentration of sorbic acid is set 0.2% in cheese, 0.1% in wines. While in silages as reported by Shearer and Cordukes (1962), they observed no benefits from the use of sorbic acid upto 5g kg^{-1} with grass/legume silage. The latter workers maintained that the negative response from the use of the acid may have been due to insufficient amount being applied to the forage. Since the acid was applied to only the top silage. With increase in quantity it reduced the acceptability of the silage to the animals. While for benzoic acid the regulatory status also regards it generally a safe preservative (code of Federal Regulations, 1977, Title 21, Section 184.1021) upto a maximum permitted level of 0.1%. Owing to these factors described above, propionic acid was used as preservative in poultry intestine silage preparation.

Spoilage of fermented food products is mainly caused by the activity of Yeast that are known to assimilate Lactic acid (Beck, 1978). However this phenomenon does not occur during storage in anaerobic atmosphere. But during aerobic storage the degradation is initiated by the Yeast and continued by proteolytic bacteria and

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Molds (Beck. 1978; Hurst and Collins, 1979; Mc Donald, 1981). Thus fungistatic effect of butyric acid and propionic is well established (Beck, 1978). The effect of propionic acid on Yeast is however limited (Mc Donald, 1981). But however in anaerobic micoaerophilic silages like that poultry intestine silage the activity of Yeast on the assimilation of Lactic acid leading to spoilage of the product doest not happen. Even though the propionic acid has an limiting effect on Yeast, its association with Lactic acid bacteria as described in chapter 5 explains its beneficial effect when associated with Lactic acid bacteria.

Conclusion

It was found that 10% molasses was sufficient and effective as carbohydrate substrate. 0.5 % propionic was prefered over benzoic acid and sorbic acid in inhibiting Yeast and Mold. And (0.5% to 2%) salts though found to decrease volume raise caused by coliforms, addition of which even at 0.5% would cause an increase in salt on a dry basis in feed which unpalatable to livestock.

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Table 1.1

Microbial profile of poultry intestine with different level of molasses

during **fermentation** .

TPC Log cfu/g

Hours / % Molasses	0	6	12	18	24	48	72	96	120	144	**
5	8.74	8.58	9.0	8.61	8.62	9.39	9.78	9.59	9.57	8.79	9.06^{x}
8	9.20	9.57	9.34	9.39	9.61	9.18	8.92	8.04	7.34	7.24	8.78^{x}
10	8.81	9.29	8.94	9.05	8.43	7.83	8.86	8.21	7.69	7.51	8.46^{x}
12	8.30	8.96	9.34	8.72	8.88	8.55	8.72	8.69	8.36	8.06	8.65^{x}
*	8.76^{cd}	9.1^{d}	9.16^{d}	8.94^{cd}	8.88^{cd}	8.73^{cd}	9.07^{d}	8.63^{cd}	8.24^{abc}	7.9^{a}	

SE= 0.3117, df = 195, SEm = 0.4546

Table 1.2

Microbial profile of poultry intestine with different level of molasses

(luring **fermentation** .

Coliform Log cfu/g

Hours / % Molasses	0	6	12	18	24	48	72	96	120	144	**
5	8.43	8.74	8.61	8.52	8.42	8.54	8.46	8.26	8.54	8.45	8.4z
8	7.36	8.17	7.76	7.79	7.04	6.94	5.27	4.88	3.75	3.73	6.56^{y}
10	7.72	8.03	7.71	7.65	6.22	7.70	0	0	0	0	4.3^{w}
12	7.63	7.77	7.76	7.53	7.47	6.64	4.95	4.38	0	0	5.41^{x}
*	7.78^{de}	8.17^{e}	7.96^{de}	7.87^{de}	7.28^{cd}	6.96^{c}	4.67^{b}	4.38^{b}	3.07^{a}	3.04^{a}	

SE= 0.4101, df = 195, SEm = 0. 261438

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Table 1.3

Microbial profile of poultry intestine with different level of molasses during fermentation .

E.coli Log cfu/g

Hours / % Molasses	0	6							120	144	**
5	8.19	7.88	7.88	7.67	7.14	7.37	7.37	6.08	0	0	5.95^{z}
8	7.78	7.42	7.26	7.36	6.5	4.26	4.13	0	0	0	4.47xy
10	7.53	7.92	7.48	7.18	5.94	4.50	0	0	0	0	4.05^{x}
12	8.15	7.71	7.53	7.66	7.2	6.23	4.0	0	0	0	4.85^{y}
*	7.91^{f}	7.73^{f}	7.54^{f}	7.46^{f}	7.69^{e}	5.59^{d}	3.87^{c}	1.52^{b}	0^{a}	0^{a}	

SE= 0.1898, df = 195, SEm = 0. 17785

Table 1.4

Microbial profile of poultry intestine with different level of molasses during fermentation .

Staphylococcus Log cfu/g

Hours / % Molasses	0	6	12	18	24	48	72	96	120	144	**
5	8.55	7.13	7.26	7.32	7.38	7.45	7.75	7.91	7.57	7.46	7.58^{z}
8	6.2	5.04	4.98	5.28	4.91	4.91	4.82	4.6	4.78	4.53	5.00^{y}
10	5.57	5.21	5.2	5.09	4.90	4.91	4.78	4.90	4.93	4.70	5.02^{y}
12	4.1	4.28	4.20	4.17	4.28	4.32	4.31	4.21	4.28	4.32	3.82^{x}
*	6.10^{b}	5.41^{a}	5.41^{a}	5.46^{a}	5.36^{a}	5.39^{a}	5.41^{a}	5.40^{a}	5.41^{a}	5.25^{a}	

SE= 0.1776, df = 195, SEm = 0.17204

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Table **1.5**

M¡crobial profile of poultry intestine with different level of molasses

during **fermentation**

Enterococei Log cfu/g

Hours / % Molasses	0	6	12	18	24	48	72	96	120	144	**
5	8.89	9.55	9.25	9.84	9.56	9.63	9.85	9.81	9.62	8.54	9.45^{y}
8	8.09	8.07	8.24	8.41	7.97	6.93	6.07	0	0	0	5.37^{x}
10	8.36	8.39	7.97	8.32	7.73	5.93	4.9	0	0	0	5.16^{x}
12	7.99	8.39	9.04	8.62	7.63	7.34	4.16	0	0	0	5.31^{x}
*	8.89^{d}	8.6^{d}	8.62^{d}	8.79^{d}	8.22^{c}	7.45^{c}	6.24^{b}	0^{a}	0^{a}	0^{a}	

SE= 0.5417. df = 195, SEm = 0.30047

Table 1.6

Microbial profile of poultry intestine with different level of molasses

during **fermentation** .

Yeast and Mold Log cfu/g

Hours / % Molasses	0	6	12	18	24	48	72	96	120	144	**
5	9.69	9.45	9.83	9.55	9.52	9.83	9.83	9.83	9.35	9.71	9.65^{z}
8	8.26	8.46	8.21	8.14	7.39	7.87	7.18	6.18	6.48	6.43	7.46^{x}
10	8.33	8.43	7.87	7.29	7.64	8.15	7.40	6.65	7.45	7.37	7.75^{xy}
12	8.56	8.45	8.38	8.30	8.37	8.26	8.16	8.44	7.98	7.54	8.24^{y}
*	8.71^{b}	8.69^{b}	8.57^{b}	8.57^{b}	8.23^{ab}	8.31^{ab}	8.14^{ab}	7.77^{a}	7.81^{a}	7.76^{a}	

SE= 0.2380, df = 195, SEm = 0.19916

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Table 1.7

Microbial profile of poultry intestine with different level of molasses

during **fermentation** .

Lactic acid bacteria Log cfu/g

Hours / % Molasses	0	6	12	18	24	48	72	96	120	144	**
5	9.20	9.43	9.76	9.92	10.24	10.05	9.93	9.73	9.86	9.93	9.80^{z}
8	9.56	9.87	9.90	9.96	9.96	9.41	9.19	9.64	9.25	9.32	9.60^{xy}
10	8.6	9.92	9.79	10.15	9.98	9.58	10.12	10.00	9.38	9.76	9.72^{y}
12	9.09	9.36	9.46	9.80	9.85	9.86	9.69	10.01	9.54	9.26	9.59^{x}
*	9.11^{a}	9.64^{ab}	9.72^{b}	9.92^{b}	10.0^{b}	9.72^{b}	9.73^{b}	9.84^{b}	9.50^{ab}	9.56^{ab}	

SE= 0.2053, df = 195, SEm = 0.18497

Legends:

Mean Rows

** **Mean Column**

SEm Standard Error of mean

df Degrees of freedom

Row Means is bearing different superscript a,b,c,d,e,f differ significantly

Column Means is bearing different superscript x,y,z differ significantly

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Table 2.1

Microbial profile of poultry intestine with different level of Salt during fermentation.

TPC **Log cfu/g**

Hours / % Salt	0	6	12	18	24	48	72	144	**
0.5	8.45	9.13	9.23	8.63	8.75	8.05	7.0	7.91	8.39[x]
1.0	8.48	8.91	8.55	8.43	8.55	7.52	7.70	8.05	8.27[x]
2.0	9.02	9.42	9.55	9.53	8.90	7.93	7.98	7.67	8.75[x]
*	8.65[b]	9.15[c]	9.11[c]	8.86[bc]	8.73[bc]	7.83[a]	7.56[a]	7.87[a]	

SE= 0.1902, df = 115, SEm = 0.17804

able 2.2

Microbial profile of poultry intestine with different level of Salt during fermentation.

Coliform **Log cfu/g**

Hours / % Salt	0	6	12	18	24	48	72	144	**
0.5	7.65	7.48	7.28	7.27	7.0	6.11	0	0	5.34[x]
1.0	7.83	7.39	7.38	7.0	6.8	5.41	0	0	5.22[x]
2.0	7.91	8.2	7.76	7.49	5.64	2.92	0	0	4.99[x]
*	7.79[d]	7.69[d]	7.47[d]	7.25[d]	6.48[c]	4.8[b]	0[a]	0[a]	

SE= 0.2336, df = 115, SEm = 0.19731

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Table 2.3

Microbial **profile of poultry intestine with different level of Salt during fcrmentation.**

E.coli **Log cfu/g**

Hours / % Salt	0	6	12	18	24	48	72	144	**
0.5	7.58	7.72	7.73	7.43	7.01	0	0	0	4.68x
1.0	8.01	7.75	7.75	7.55	6.87	5.58	0	0	5.43x
2.0	8.26	8.28	7.87	7.46	5.36	7.07	0	0	5.03xy
*	7.95^{e}	7.91^{de}	7.78^{de}	7.48^{d}	6.41^{c}	2.8^{b}	0^{a}	0^{a}	

SE= 0.1282, df = 115, SEm = 0.14617

Table 2.4

Microbial **profile of poultry intestine with different level of Salt during fermentation.**

Staphylococcus **Log cfu/g**

Hours / % Salt	0	6	12	18	24	48	72	144	**
0.5	6.10	6.15	5.81	5.50	5.5	5.43	4.66	4.66	5.47^{y}
1.0	6.12	6.06	5.90	5.3	5.4	5.04	4.55	4.72	5.38^{x}
2.0	4.47	4.17	4.46	4.44	4.45	4.54	3.52	3.06	4.13^{x}
*	5.56^{b}	5.46^{ab}	5.39^{ab}	5.08^{ab}	5.11^{ab}	5.0^{ab}	4.24^{ab}	4.14^{a}	

SE= 1.0065, df = 115, SEm = 0.40957

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Table 2.5

Microbial **profile of poultry intestine with different level of Salt during** fermentation.

Enterococci **Log cfu/g**

Hours / % Salt	0	6	12	18	24	48	72	144	**
0.5	7.49	6.9	6.38	6.21	4.88	3.47	0	0	4.41^{x}
1.0	7.43	7.35	7.34	6.63	5.29	3.57	0	0	4.70^{x}
2.0	8.57	8.98	9.08	8.81	8.58	7.74	3.58	0	6.91^{y}
*	7.83^{d}	7.74^{d}	7.6^{d}	7.2^{d}	6.25^{d}	4.92^{b}	1.19^{a}	0^{a}	

SE= 0.4226, df = 115, SEm = 0.2653

Table 2.6

Microbial profile of poultry intestine with different level of Salt during fermentation.

Yeast and Mold **Log cfu/g**

Hours / % Salt	0	6	12	18	24	48	72	144	**
0.5	8.2	8.73	7.78	8.90	8.26	8.55	7.58	7.39	8.17^{y}
1.0	8.61	8.63	8.71	8.8	8.18	8.31	7.71	8.21	8.39^{y}
2.0	8.52	8.58	8.05	7.88	7.77	6.22	5.85	6.34	7.40^{x}
*	8.44^{de}	8.64^{e}	8.18^{cde}	8.52^{de}	8.07^{cd}	7.69^{bc}	7.04^{a}	7.31^{ab}	

SE= 0.1835, df = 115, SEm = 0.17488

Table 2.7

Microbial **profile of poultry intestine with different level of Salt during** fermentation.

Lactic acid bacteria **Log cfu/g**

Hours / % Salt	0	6	12	18	24	48	72	144	**
0.5	9.07	9.52	9.85	10.0	10.04	10.21	10.30	9.68	9.83^{x}
1.0	9.15	9.59	9.75	10.14	10.07	10.02	10.33	9.64	9.83^{x}
2.0	9.2	9.64	9.9	10.02	10.03	10.13	9.96	9.86	9.84^{x}
*	9.14^{a}	9.58^{b}	9.8^{c}	10.05^{d}	10.04^{d}	10.12^{d}	10.19^{d}	9.72^{bc}	

SE= 0.0217, df = 115, SEm = 0.06013

Legends:

Mean Rows

Mean Column

SEm Standard Error of mean

df Degrees of freedom

Row Means is bearing different superscript a,b,c,d,e differ significantly

Column Means is bearing different superscript x,y,z differ significantly

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Table3.1

Yeast **and Mold counts of** poultry **intestine with different levels of Antimycotic Lents during fermentation.**

0.1 % **Log cfu/g**

Hours / % / AA	0	24	48	72	96	120	144	**
Sorbic acid	8.82	8.49	7.82	6.58	5.57	5.35	5.07	6.81^{xy}
Propionic acid	8.53	7.30	6.99	5.91	5.96	5.46	5.43	6.51^{x}
Benzoic acid	8.29	7.50	7.85	7.55	7.13	6.82	6.48	7.37^{y}
*	8.54^{d}	7.76^{c}	7.55^{c}	6.68^{b}	6.22^{ab}	5.87 a	5.66 a	

SE= 0.3618, df = 100, SEm = 0.24556

Table3.2

Yeast and Mold counts of poultry intestine with different levels of Antimycotic agents during fermentation.

0.3 % **Log cfu/g**

Hours / % / AA	0	24	48	72	96	120	144	**
Sorbic acid	8.19	8.4	7.42	6.32	5.54	4.84	4.57	6.46^{x}
Propionic acid	8.47	7.46	6.08	5.9	5.12	5.26	5.21	6.21^{x}
Benzoic acid	8.12	7.99	7.70	7.82	7.51	7.13	6.38	7.52^{y}
*	8.20^{d}	7.95^{d}	7.06^{c}	6.68^{bc}	6.05^{ab}	5.74 a	7.38 a	

SE= 0.4137, df = 100, SEm = 0.26258

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Table33

Yeast and Mold counts of poultry intestine with different levels of Antimycotic agents **during fermentation.**

0.5 % Log **cfu/g**

Hours / % AA	0	24	48	72	96	120	144	**
Sorbic acid	7.65	7.13	6.05	5.92	4.73	4.01	4.87	5.76^{x}
Propionic acid	8.32	6.86	6.19	5.59	5.35	4.83	5.16	6.04^{x}
Benzoic acid	8.07	7.76	8.03	7.78	7.19	7.23	6.53	7.51^{y}
*	8.01^{d}	7.25^{c}	6.75^{bc}	6.43^{b}	5.75^{a}	5.35^{a}	5.18^{a}	

SE= 0.2427, df = 100, SEm = 0.20112

Legends:

* **Mean Rows**

** **Mean Column**

SEm Standard Error of mean

df Degrees of freedom

Row Means is bearing different superscript a,b,c,d,e differ significantly

Column Means is bearing different superscript x,y,z differ significantly AA

Antimycotic agent

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Table 4

Effects of **different levels of molasses on pH during fermentation of poultry intestine**

n = 6

Hours / % Molasses	0	6	12	18	24	48	72	96	120	144
5	5.3	5.3	4.82	4.55	4.3	4.3	4.4	4.45	4.6	4.8
6	5.25	5.25	4.82	4.55	4.3	4.3	4.4	4.45	4.55	4.7
7	5.2	5.2	4.82	4.5	4.3	4.3	4.4	4.45	4.55	4.5
8	5.2	5.2	4.7	4.5	4.35	4.2	4.1	4.1	4.1	4.1
9	5.15	5.15	4.7	4.5	4.35	4.1	4.4	4.4	4.4	4.4
10	5.0	5.0	4.5	4.4	4.2	4.05	4.4	3.9	3.8	3.8
11	5.05	5.5	4.5	4.4	4.2	4.0	4.0	3.9	3.8	3.8
12	5.0	5.0	4.5	4.4	4.2	4.0	4.0	3.9	3.8	3.8
13	5.0	5.0	4.45	4.4	4.15	3.95	3.9	3.9	3.8	3.8
14	5.0	4.95	4.45	4.3	4.15	3.95	3.9	3.9	3.85	3.85
15	4.95	4.9	4.4	4.3	4.1	3.9	3.9	3.9	3.85	3.85

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Table 5

Effects of **different levels of salt on pH during fermentation of poultry intestine**

n = 6

% Salt \ Hours	0	6	12	18	24	48	72	96	120	144
0.5	5.2	4.9	4.7	4.6	4.2	3.95	3.95	3.9	3.9	3.9
1.0	5.1	5.0	4.3	4.5	4.1	3.9	3.9	3.9	3.9	3.8
2.0	5.0	4.9	4.2	4.1	4.0	3.9	3.9	3.8	3.8	3.8

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Table 6

Effects of Antimycotic agents on **pH during fermentation of poultry intestine**

n = 6

Hours / % AA	0	24	48	72	96	120	144
0.1 %							
Sorbic acid	5.05	4.2	4.2	4.1	3.9	3.9	3.9
Propionic acid	5.0	4.2	4.0	4.0	3.9	3.9	3.9
Benzoic acid	5.1	4.2	4.1	4.1	3.9	3.9	3.9
0.3 %							
Sorbic acid	5.0	4.1	4.0	3.9	3.9	3.9	3.9
Propionic acid	4.95	4.1	4.0	4.0	3.9	3.9	3.9
Benzoic acid	5.0	4.2	4.1	4.0	3.9	3.9	3.9
0.5 %							
Sorbic acid	5.0	4.2	4.0	3.9	3.9	3.9	3.9
Propionic acid	4.9	4.1	4.0	3.9	3.9	3.9	3.9
Benzoic acid	4.95	4.1	4.0	4.0	3.9	3.9	3.9

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Table 7

(Composition of molasses

n = 3

Proximate Composition	Percentage
Reducing Sugar	46.3
Moisture	30.0
Protein	0.3
Fat	0.3
Microorganisms	Log cfu /g
E.coli	Nil
Lactic acid bacteria	Nil
Yeast	10^3

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Table 8

'Shelf-life **of fermented stabilized poultry intestine silage**

n = 6

Days / Silage Characers	*** 0	15	30	45	60	75	90
Pathogens log cfu /g							
E.coli	0	0	0	0	0	0	0
Coliform	0	0	0	0	0	0	0
Staphylococcus	0	0	0	0	0	0	0
Enterococci	0	0	0	0	0	0	0
Fungi	0	0	0	0	0	0	0
Salmonella	0	0	0	0	0	0	0
LAB	10.0	7.3	6.88	6.53	5.96	5.83	5.76
Yeast	5.86	8.14	4.95	4.0	3.0	0	0
Chemical composition (%)							
Protein	12.4	12.8	12.0	12.7	12.0	11.9	11.7

*** - Stabilized 24h silage

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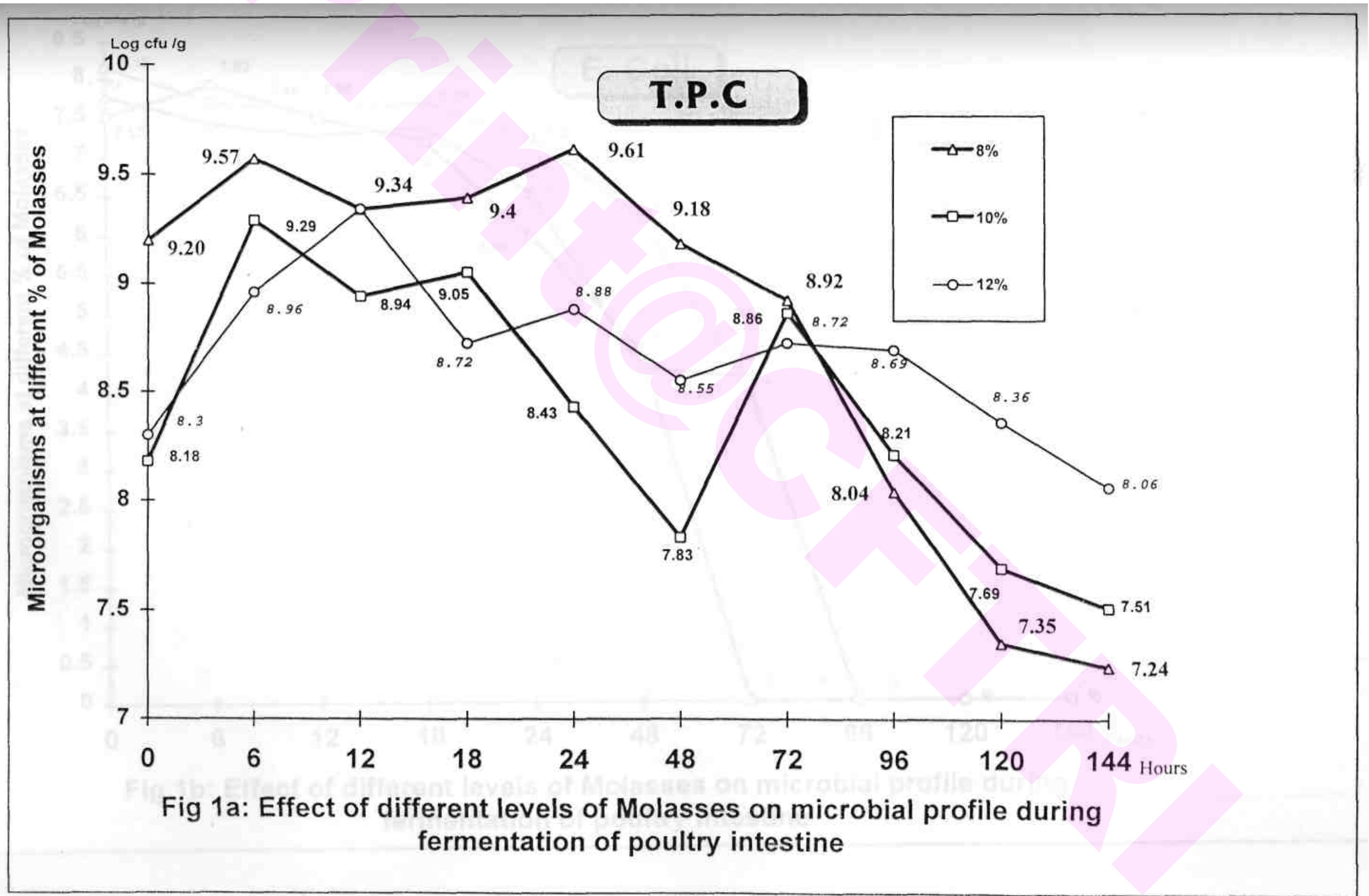

Fig 1a: Effect of different levels of Molasses on microbial profile during fermentation of poultry intestine

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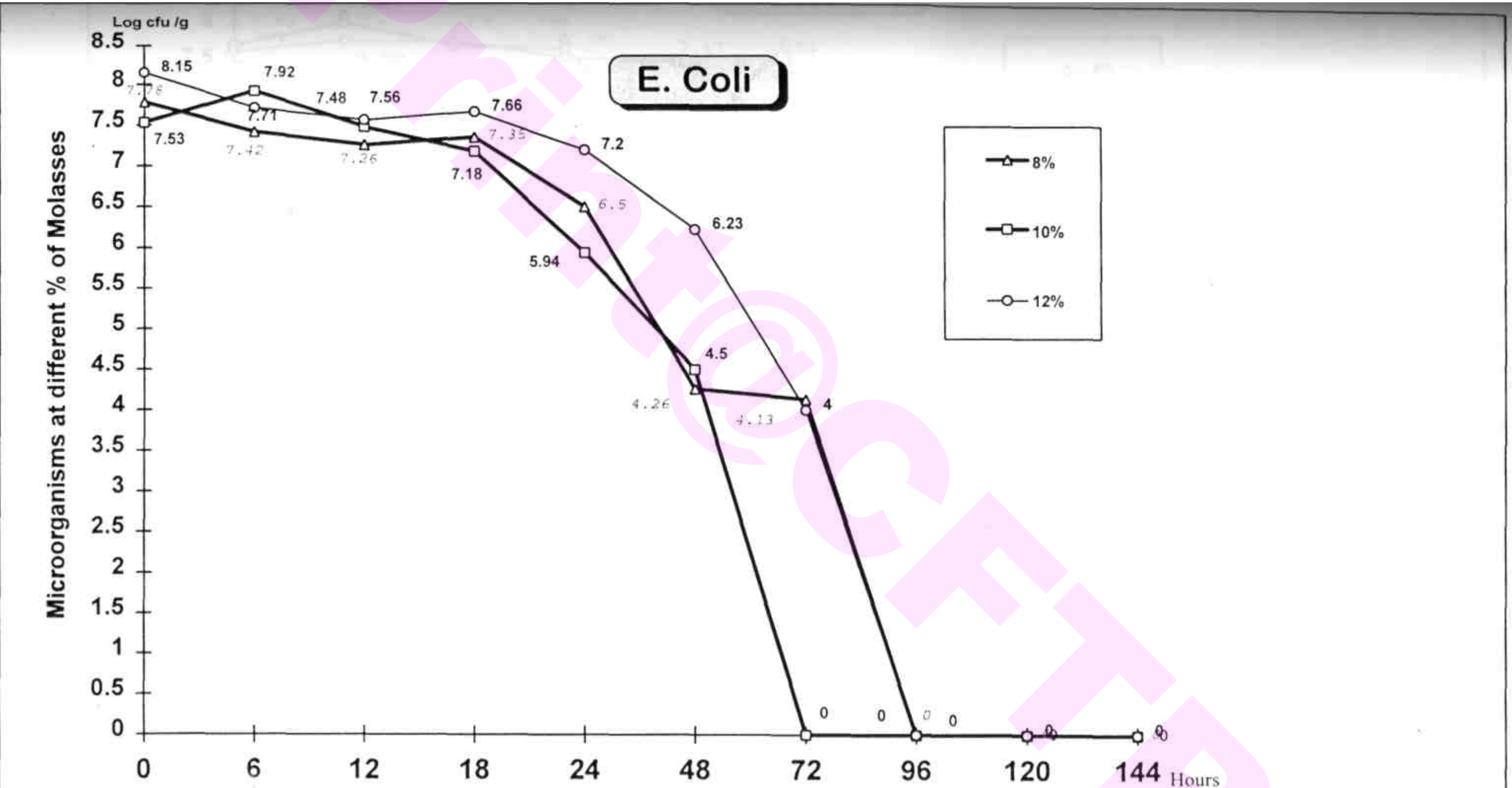

Fig 1b: Effect of different levels of Molasses on microbial profile during fermentation of poultry intestine

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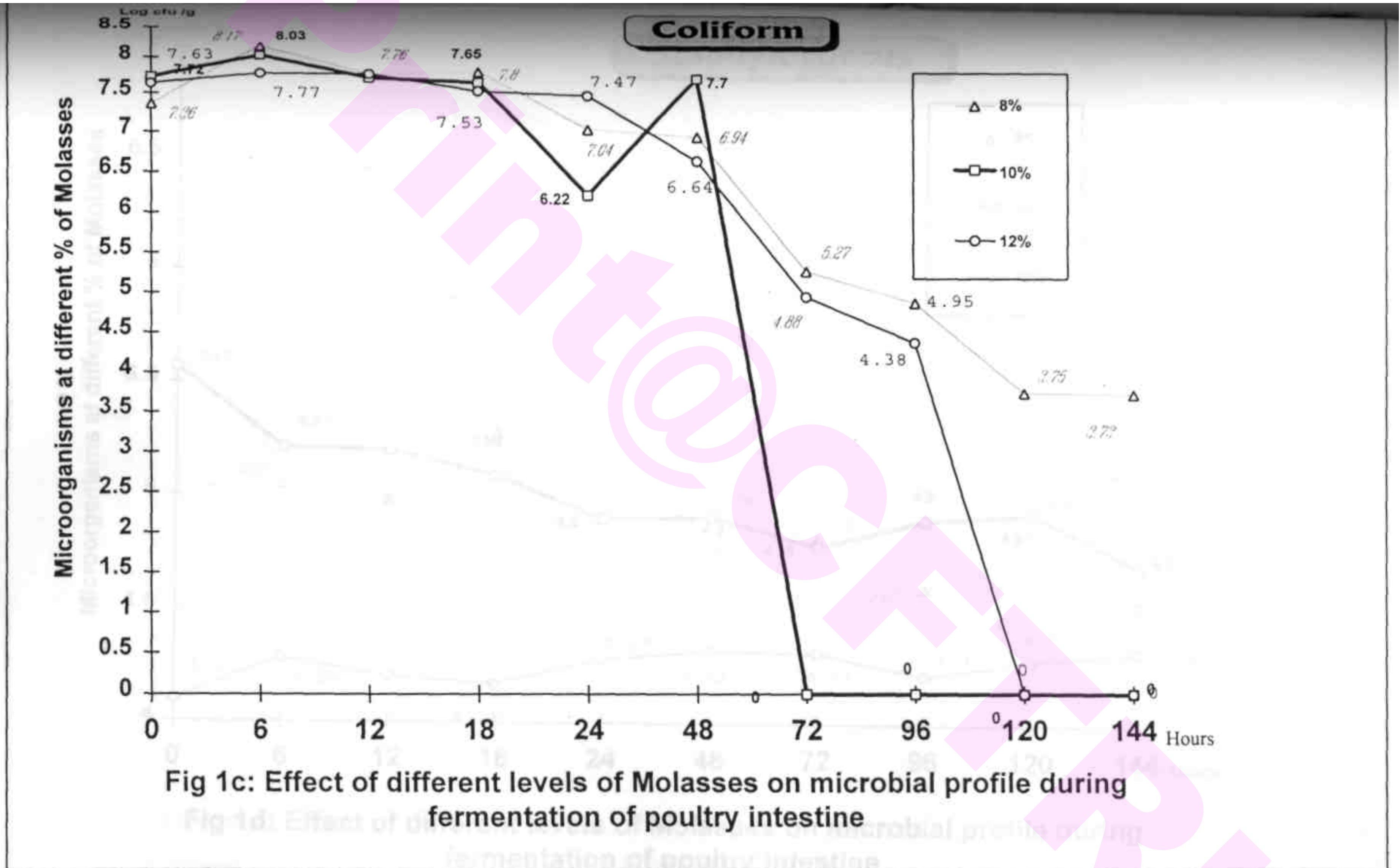

Fig 1c: Effect of different levels of Molasses on microbial profile during fermentation of poultry intestine

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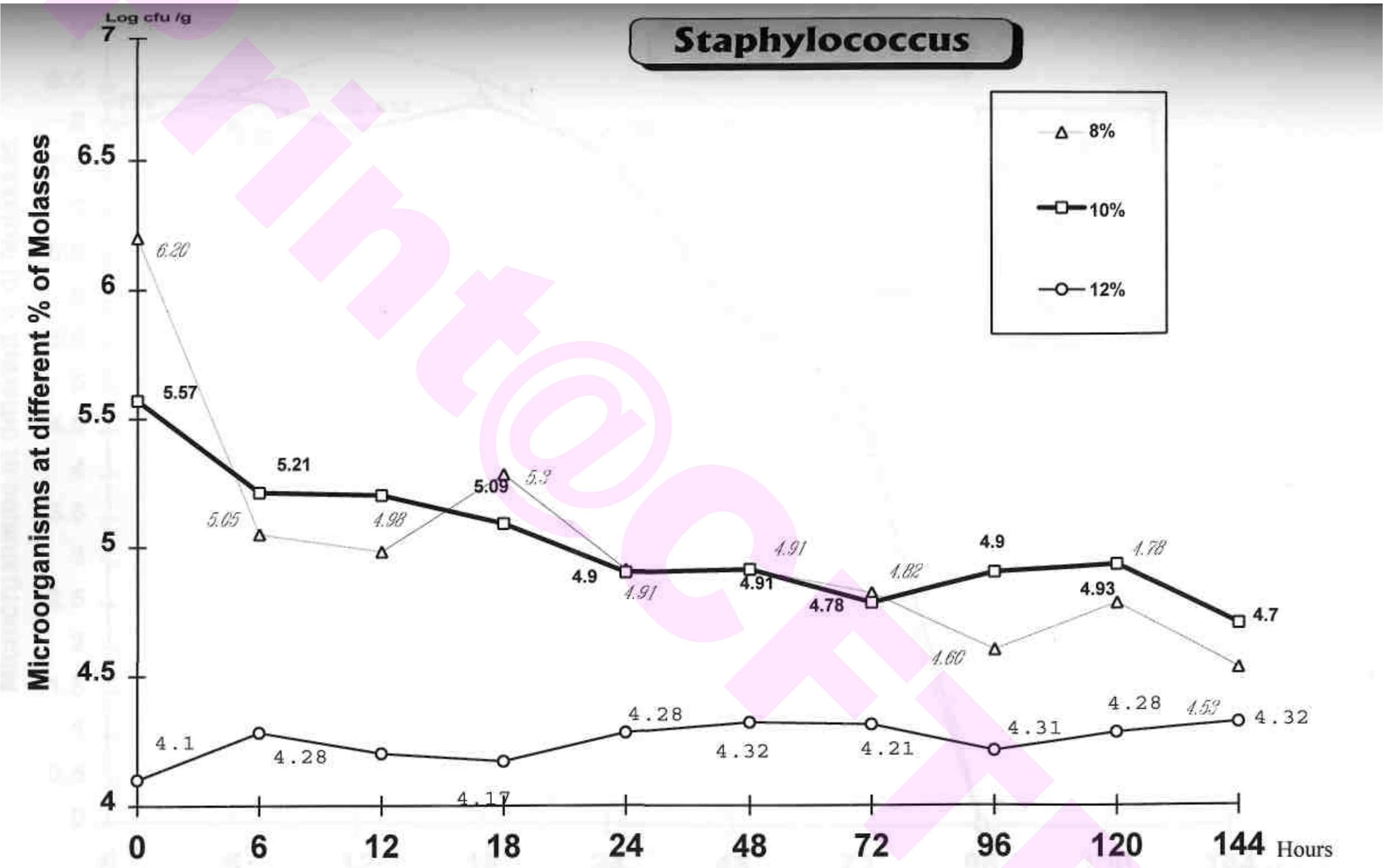

Fig 1d: Effect of different levels of Molasses on microbial profile during fermentation of poultry intestine

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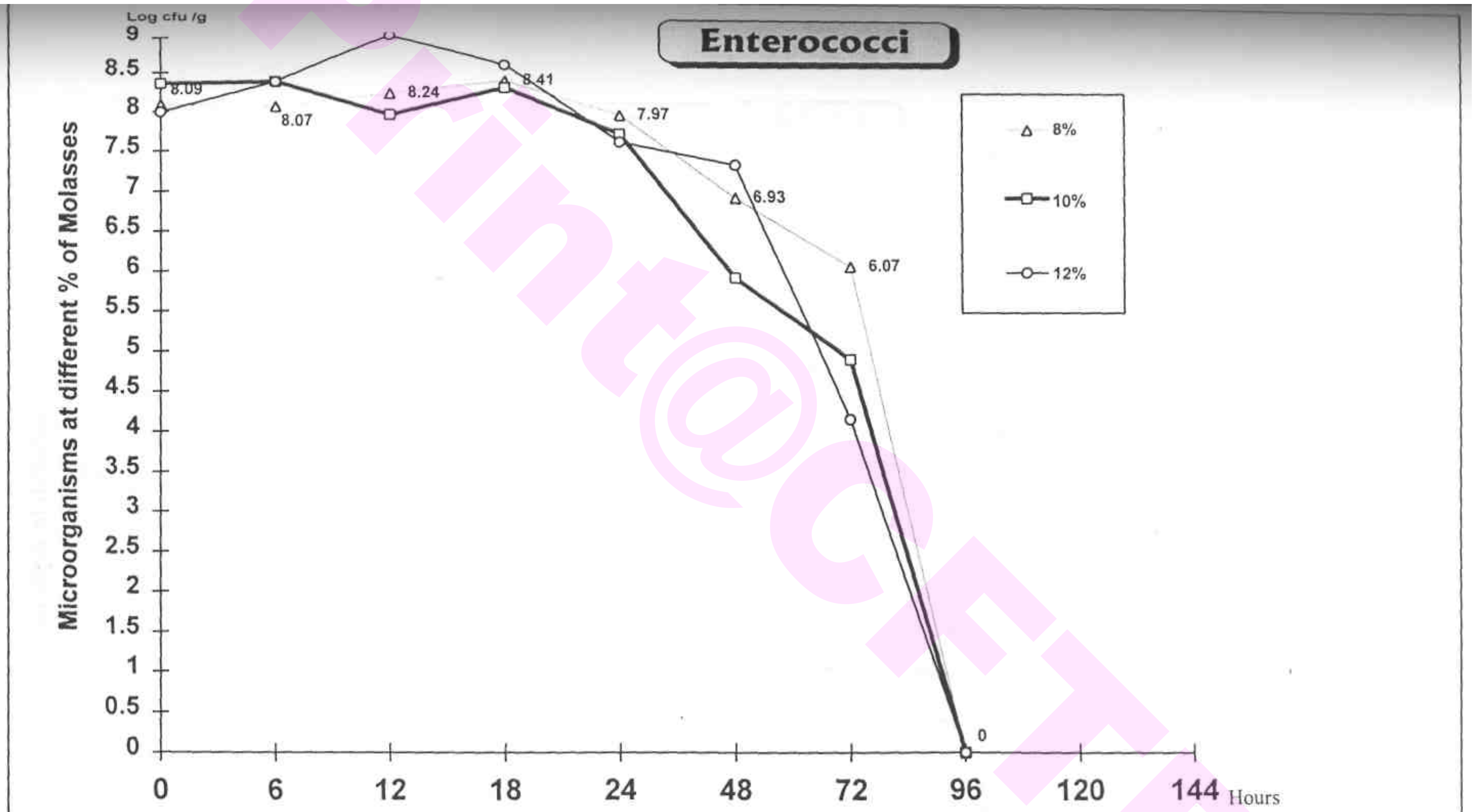

Fig 1e: Effect of different levels of Molasses on microbial profile during fermentation of poultry intestine

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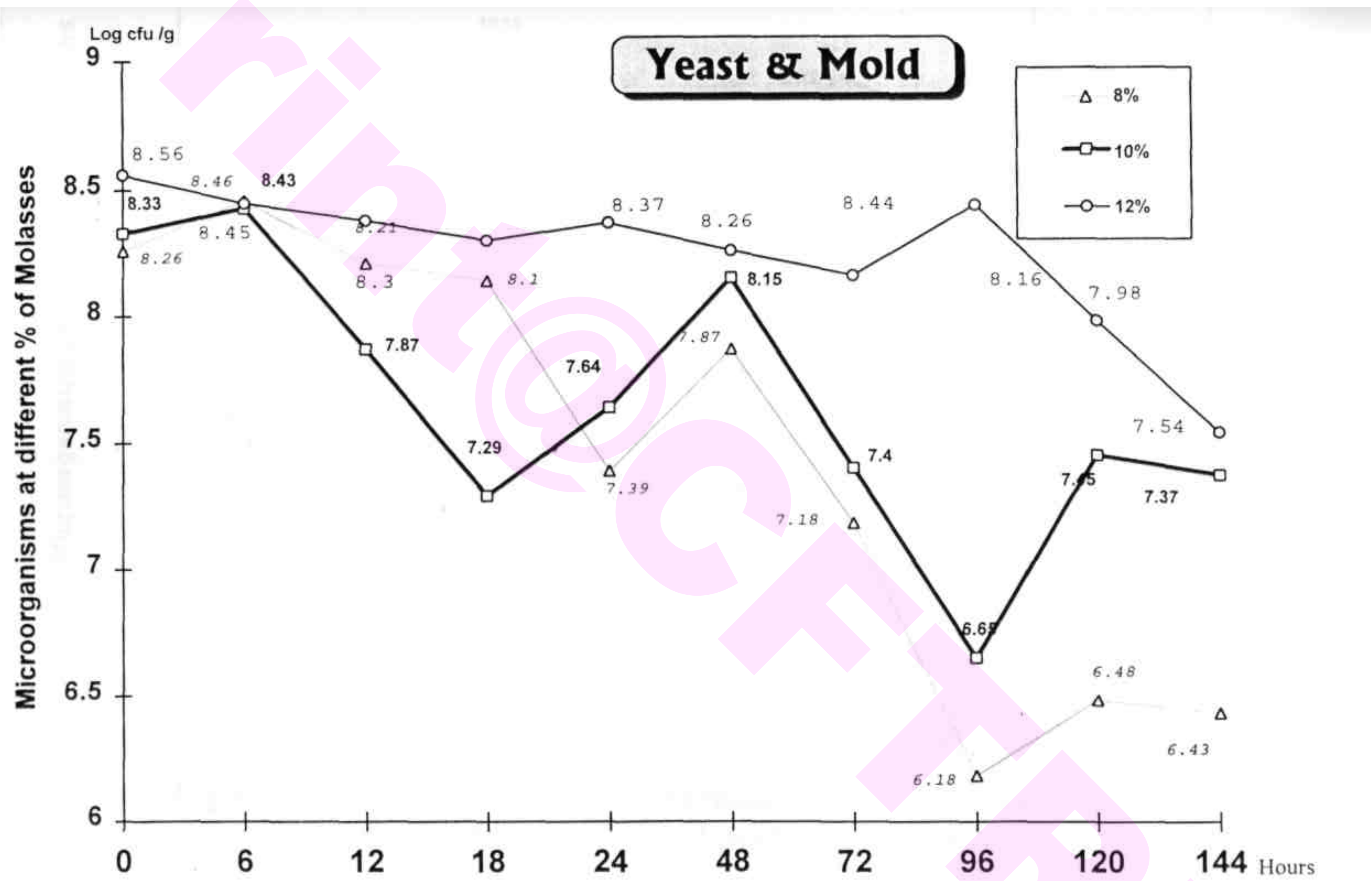

Fig 1f: Effect of different levels of Molasses on microbial profile during fermentation of poultry intestine

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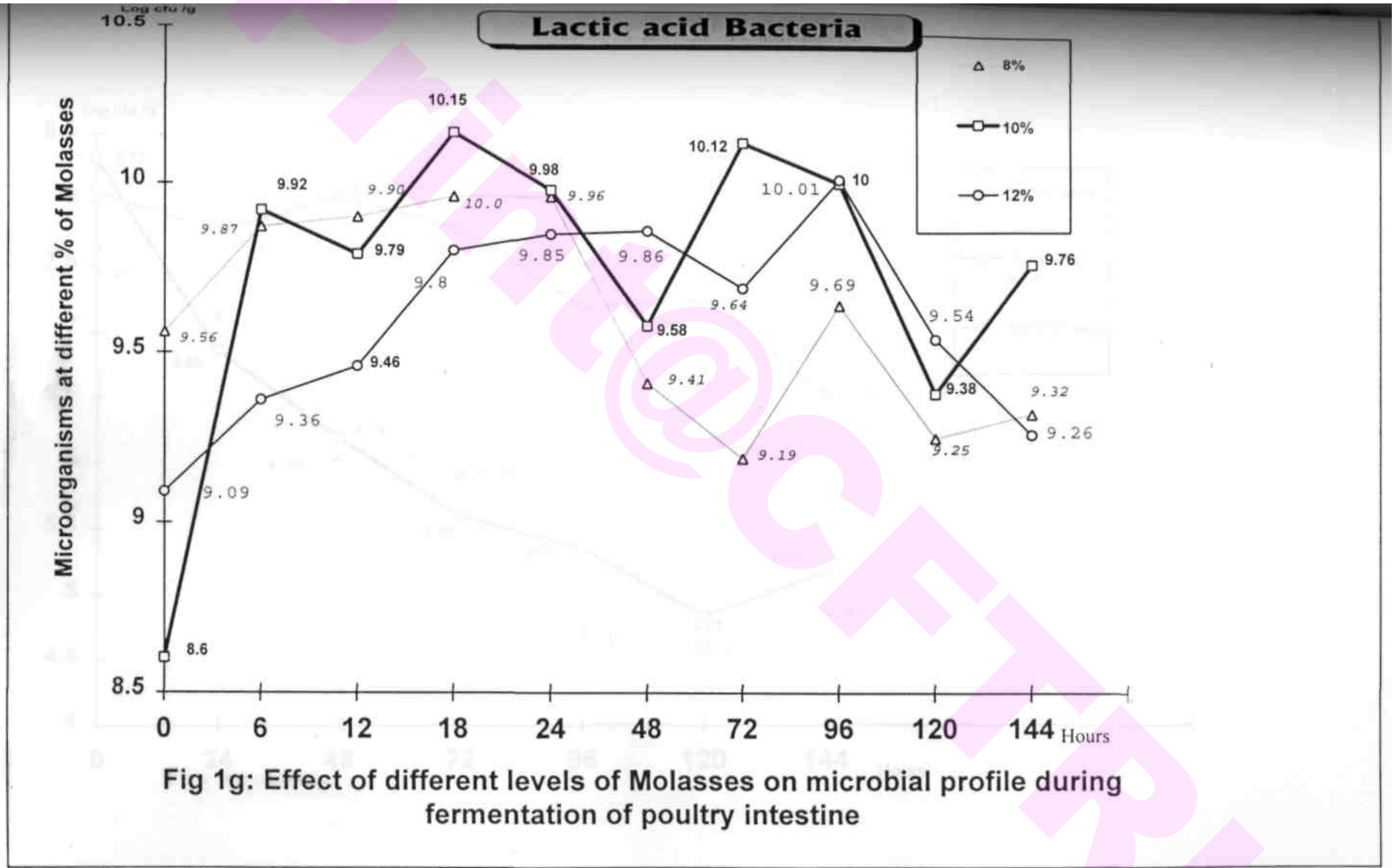

Fig 1g: Effect of different levels of Molasses on microbial profile during fermentation of poultry intestine

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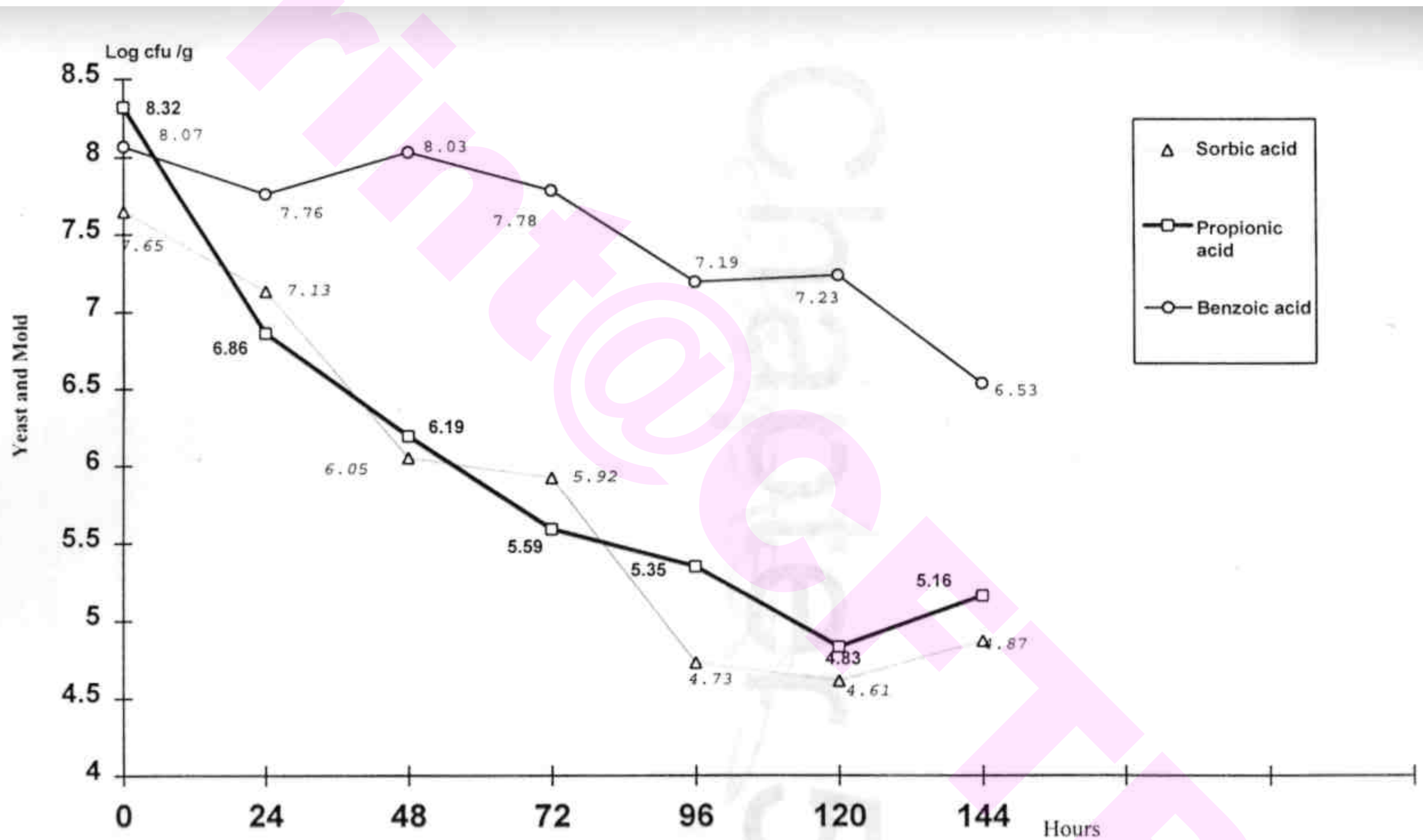

Fig 2: Effect of 0.5% antomycotic agents on Yeast and Mold during fermentation of poultry intestine

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Chapter 5

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MICROBIAL SUCCESSION DURING FERMENTATION OF POULTRY INTESTINE

INTRODUCTION

The use of animal waste, primarily as a nitrogen source in the feeding or refeeding of livestock has been reviewed by Anthony (1971) and Smith (1973). Direct acidification or making conditions conducive to a desirable fermentation have been practised. Lactic fermentation of foods of plant or animal origin is a widely accepted method of preservation. It imparts desirable sensory and nutritional properties to the fermented product (Cooke *et al,* 1987). In silage-making by purely biological processes the basis of positive conservation of the organic material is a lactic acid fermentation. It sets in rapidly and is so intense that it extinguishes all oilier, undesirable microbial or enzymatic activities. Adequate quantities of carbohydrates fermentable by Lactic acid bacteria are required for a successful lactic fermentation. In many types of feed and food stuffs, the lack of such carbohydrates often limits the production of an efficient lactic acid fermentation. Slaughterhouse waste is a good example of such a material. Lactic acid fermentation may be of great importance particularly for materials infected with pathogens and spoilage microorganisms.

Each ecosystem is populated with specific microorganisms. Environment does not remain constant, and changes in the environment bring about changes in the microbial population. The growth and metabolism of organisms themselvels may change the environment dramatically. In some environments the

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changing conditions bring about a highly ordered and predictable succession of organisms. The most dominant microorganisms reveals a particular pattern of growth. The competition for survival of microorganisms within a given results in characteristic microflora generally termed as succession. Previous investigations showed that the Lactic acid bacteria dominated in poultry intestine (Shapiro and Sarles 1949, Shapiro *et al,* 1949, Fuller, 1973, Peter and Sneath, 1986). No studies of the microflora associated and responsible for the fermentation of poultry intestine have been reported.

The present study describes the bacterial association and their succession (changes in microflora), the characteristics of different species and different LAB isolated during fermentation of poultry intestine. The bacterial association (LAB and Yeast) were tested for their ability to produce a stabilised poultry intestine silage product. In-vitro studies on the survival of Salmonella during ensiling of poultry intestine has also been described to justify that the ensilage process would eliminate Salmonella commonly present in poultry intestine, and make the product free from Salmonella and provide safety to the product.

MATERIALS AND METHODS

Collection of poultry intestine and sampling

Intestines were procured from broiler birds (6 to 8 wk), of cobb strain. The birds were reared at the nearby hatcheries and brought to the local market, where the birds were slaughtered. Intestines were brought immediately (within lh of slaughter)

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to the laboratory for analysis. Intestine consisted of the duodenum, ileum, small and large intestines. Intestines were used along with the contents.

Ensilage process

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3 Kg poultry intestine procurred from the local market were homogenized with 10%(w/w) molasses and 0.5% (v/w) propionic acid. Molasses was added as a source of energy for microorganisms while propionic acid served as antimycotic agent. The fermentation experiments were carried out into two plastic buckets in two batches. Each bucket were filled with 1 kg of material and covered with polypropelene sheets and the buckets were covered tightly with lid to create microaerophillic conditions. One bucket was incubated at ambient (26+ 2 °C) and the other at 37 °C for fermentation to follow.

Microbiological analysis

Total plate counts, coliforms, E.coli. Staphylococcus, Enterococcus, Lactic Acid bacteria and Yeast and Mold count were estimated as per the methods mentioned chapter 3. Isolation and identification of LAB were also carried out.

Isolation studies **on the survival of Salmonella durign fermentation process**

Salmonella typhimurium was selected as test organisms. The organisms was grown in sterile trypticase soy broth for 24h at 37ºC and the broth was centrifuged at 8000g/20 min.; the cells suspended in sterile physilogical saline and then used for isolation studies. Salmonella typhimurium was inoculated to fresh homogenized poultry intestine in such a way that 1 g of homogenate contained 10^5 salmonella organisms. The homogenate was transferred in to 500ml beaker and sealed **with**

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aluminium toil to create microaerophilic conditions. This homogeneate was incubated seperately at 25 ± 2 °C and at 37 °C and sampled under sterile conditions at periodic intervals (0.6,12,18,24,48 h) inorder to test the survival of S. typhimurium. **The** measurements of pH of the inoculated poultry intestine was recorded with the Radio meter pH meter (Copenhagen. Denmark).

Enumeration of Salmonella (APHA, 1984)

Salmonella was enumerated by the three tube MPN procedure (APHA, 1984) which consisted of an enrichment step in selenite cystine broth, identification on triple sugar iron agar slants (APHA, 1984). In addition nonselective enrichment steps incubation in lactose broth for 36h, 37 °C, was used prior to inoculation into selenite cystin broth for samples taken after process. The MPN method of enumeration is used frequently to determine the number of specific type of microorganisms in a sample. The ratio of sample volume to medium volume is basically one part of sample to 10 parts of medium should ne maintained. This 1 ml aliquots are used, they are dispersed into 10ml of broth. For determination of total count using MPN tubes are incubated at 35 °C for 48h. Interpretations are as per the MPN tables (APHA, 1984).

pH was measured and recorded with the help of Radio meter pH meter (Copenhagen. Denmark).

Stastistical **Analysis**

Experimental data (count of microorganisms) obtained from washed and unwashed poultry intestine, were subjected to ANOVA. Significant difference

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between means within the sample were tested by Duncans multiple range test iDuncan. 1955) and between washed and unwashed samples by t-ratio test.

RESULTS

Fall in pH during fermentation of poultry intestine

From fig. 1 it is evident that desired pH (4.2) has been achieved in poultry intestine by fermentation process in 6h at 37 °C and in l8h at 27 ± 2 °C when molasses and propionic acid were used as additives. At 37 °C which is the suitable temperature for Lactic acid bacterial growth, pH has decreased more rapidly than at ambient temperature (26 ± 2 C).

Microbial Succeesion **of poultry intestine during fermentation at ambient** temperature (26 ±2 C).

Trends in inhibition of various pathogenic and spoilage microorganisms at ambient temperature are present in Fig. 2a, and 2b and Table 1. At ambient temperature pathogenic organisms like E.coli, Coliform, Staphylococcus and Enterococci are inhibited at 48h, while counts of Yeast decreased from log 8.57 to log 4.95 at the end of fermentation (l44h), while at 37°C E.coli, Coliform, Staphylococcus and Entereococci are inhibited between 12 to 24h. Table 1 clearly ihows that incubation at 37°C, the counts differ significantly over ambient among all microorganisms. Results of microbial succession reveal the changes in microflora from an aerobic population to a microaerophilic one. Since poultry intestine consists of a mixed culture, it intially consists of mainly aerobic pathogens and spoilage organisms. As fermentation proceeds, the oxygen content in the silage is reduced

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favouring a microphilic condition. It is this condition which favours the proliferation of Lactic acid bacteria and hence an increase in growth pattern as seen in Fig. 3.

pH decreased due to acid production of Lactic acid bacteria. This has further produced favourable condition for Lactic acid bacteria to dominate. While anaerobic microorganisms cannot tolerate this decrease in pH and acid condition and were inhibited before the next life cycle could begin. It is also noted that pH remains constant at 4.2 and slightly decreases to 3.9 to 4.0, thus preventing even the small population (undesirable) which might be present to grow within the silage, thus paving the way to Lactic acid bacteria to dominate. This type of microbial change facilitating the Lactic acid bacteria to succeed over other undesirable organism leads to lactic fermentation.

Microorganisms associated with microbial succession during poultry intestine fermentation are presented in Table 2. The results revealed that from the 130 isolates identified as Lactic acid bacteria from poultry intestine, the Lactobacilli were predominant. The other Lactic acid bacteria were Enterococcus, Pediococcus and Leuconostoc. The Lactobacilli identified are listed in table 2. Apart from Lactic acid bacteria enterobacteria, Staphylococcus, fungus comprising of Yeast and Molds were found. Coliforms were also identified where in E.coli was predominant. As fcrmentation proceeded, the aerobic microflora switched over to the aerobic microflora consisting of mainly Lactic acid bacteria and Yeast. By 24h all other microorganisms were eliminated and Lactobacilli remained predominant over [Streptococcus lactis and Pediococcus. From 50 isolates of Lactic acid bacteria, 45 species were of the Lactobacilli group, 3 species of S.lactis. and 2 Pediococcus could

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be obtained, showing Lactobacilli were succeeding over the other Lactic acid bacteria. But Yeast did remain in the silage. Towards the end of l44h, Yeast were present while Lactobacilli dominated among other Lactic acid bacteria. From 45 Lactobacilli isolates identified, 25 were L.plantarum. 15 were L.acidophilus and 5 were L,fermenti. Thus studies on microbial sucession can reveal the trend in changing microflora from one species to another and even among the particular species itself. This is justified by the results represented in table 2 were Lactic acid bacteria dominated other microflora, while within the Lactic acid bacteria, Lactobacilli predominated and within the group of Lactobacilli L.plantarum was having highest identified counts when compared to L.acidophilis and L.fermenti.

Growth **pattern of Lactic acid bacteria during fermentation of poultry intestine**

From the counts obtained from the microbial sucession during fermentation of poultry intestine, the growth pattern of the Lactic acid bacteria can be eaisly derived. It has been found in the study that Lactic acid bacteria were the major component (10 to 10^{10} / g) of the microflora of poultry intestine. It can be seen in Fig 3, that the intital count of the Lactic acid bacteria was log 9.3 increasing steadily upto 12h and maintaining an almost constant count between log 9.8 and log 10 till 96h, thereon it decreased to log 8.4 at l44h. This pattern of Lactic acid bacteria growth signifies its ability to reduce pH to 4.2. By viewing the graph it is evident that counts have increased by 12h, thus pH decrease as indicated in Fig 1 is rightly between l8h to 24h. This increase in count of Lactic acid bacteria was directly related to decrease in pH in poultry intestine silage. Subsequently pH remained constant. So also the Lactic

bacteria counts. Thus growth of Lactic acid bacteria led to acid production and simultaneously a fall in pH. Thus, it could be stated that Lactic acid bacteria are mainly responsible for the fermentation process.

Survival of Salmonella during poultry intestine fermentation

From the study it is well recognized that Salmonella is harboured in the intestine of birds. Isolates of Salmonella have been represented in chapter 1. From Table 3 it is evident that the Salmonella inoculated into the homogenized poultry intestine cannot tolerate the low pH and acid produced by native Lactic acid bacteria in poultry intestine. This condition is not conducive for the growth and multiplication of Salmonella. Thus death of Salmonella is evident and Salmonella could not be found in the silage. It is interesting to note that at 37 °C, acid production was faster with the fall in pH to the desired 4.2 level at 6h and the elimination of Salmonella in 12h. While at ambient, the pH decrease was within 18 to 24h, thus Salmonella was inhibited at 48h. Thus it is possible that fermentation technique that can conserve proteinaceous material in poultry intestine free from Salmonella.

DISCUSSION

Much work on the ensiling of grass legumes, fish waste, whole fish and other vegetable silage with their microbiological aspects have been published. But there are rare or no reports on the microbiology of poultry silage. A similar trend on the microbial succession in poultry intestine silage was noticed as in grass silage. Where there is a major change in microbial profile from an aerobic one to a facultative,

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obligate anaerobic or microaerophillic silage, provided such conditions are established. A gram negative microflora will be replaced by a gram positive microflora. In the present study bacterial microflora shows an overtaking of one genera from the mixed culture already present in it during fermentation process. The dominating genera in silages are the Lactic acid bacteria. The inhibition of the other undesirable genera is due to the production of acid with the ultimate fall in pH. All these factors are very important in contributing to a desirable stable good quality silage.

In response to the changed environment imposed by ensilage, the early dominance of the microflora by Lactic acid bacteria could be due to their elaboration of substances (bacteriocins). These bacteriocins are inhibitory to undesirable organisms in the raw material. (Narashima Rao, 1995). This happens because the proportion of acid producing microorganisms antagonistic to other undesirable microorganisms increases as silage matures. The microbial succession seen in poultry intestine is of a heterofermentative type. Its activity is of paramount significance as it ensures the on set of a conventional fermentation.

After a few days it is difficult to distinguish between multiplication and survival within the silage microflora. Counts of bacteria reveal little information on the state of preservation, and in conventionally fermented silage, pH is a more reliable indicator of whether or not microbial development will occur.

Among the animal product silages, microbiological investigations have centered on silages made from fish. Reports on microorganisms associations with

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poultry intestine have been reported. Shapiro and Sarles, (1949); Shapiro *et al,* (1949); Fuller (1973); Peter and Sneath, (1986).

During succession in poultry intestine silage, pathogenic Salmonella, E.coli. Coliform, Staphylococcus, Enterobactetriaceae and spoilage organism fungi were predominant during the early hours of ensilage. These organisms could not succeed further when once PH reached 4.2 during poultry intestine ensilge process. These results are in accordance with the findings reported by Russell *et al,* (1993). Once again it is stressed that temperature has a pronounced effect as inhibition is rapid at 37 °C than when compared to ambient (26 ± 2 °C). Thus any benefit to be gained from storing silage at a temperature above ambient will have an influence by the temperature optima for the microorganisms within each group represented in the silage.

Reports to confirm the present results are available to support the effectiveness in the use of a lactic acid fermentation and its succession during fermentation against other undesirable microorganisms. Work of Hercules *et al,*(1985) in naturally fermented fish silage reports production of lactic and results in a pH reduction to below pH 4.0. This pH reduction inhibits the growth of bacteria such as Staphylacoccus (Bartholomeo and Blumer, 1980), E. coli (Tramer, 1966), Serratia, enterobacteria, Psuedomonas, Citrobacter and Achromobacter (Dubois *et al,* (1949). Enterobacteriaceae occuring during the first fermentation cycle are common on plant material and were found also in natural fermentation of eg. Soya beans (Mulyowidarso *et al,* 1989) and early stages of the Sour dough fermentation (Lonner *et al,* 1986). Their disappearance in the following cycles with pH 4.5 is in

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accordance with the death Kinetics of Enterobacteriaceae in similarly fermented material (Nout *et al,* 1989). Similarly reported in milk. (Juffs and Babel, 1975; Rutzinski and Marth 1980).

Not withstanding the `Non-sterile' character of the fermentation technique, naturally occuring filamentous fungi appear to be unable to compete in the combination of acidity and poor access of atmospheric oxygen prevailing in the fermenting poultry intestine. This implies that there is no risk that mycotoxins will be formed during the fermentation process. This confirms with earlier results on lactic fermentation of Sorghum-based infant foods. (Nout 1991).

In poultry intestine silage, growth of lactobacilli along with pathogenic microorganisms were till about 24h after which lactobacilli dominated as seen in the present study. A similar trend is reproted by Suhaime *et al,* (1987), where the growth of the Lactobacilli and Enterococcus in ensiled ammonia treated straw was rapid luring the first two days. After the fourth day, they stablized and declined. At the end ofthe ensiling period, the Lactobacilli were present in all silages at about log 8 cfu/g wet silage Enteriococci could not be recovered. Hurbant (1975) has studied the microbial population changes during fermentation of feedlot waste with corn. In this type of fermentation the coliform count, which remained constant for the first l2h of the fermentation, then dropped to one hundredth of the original number by 24h. This pin was similar to the results in the present study. The role of LAB in inhibiting S.aureus has also been reported by many an investigators (Branen *et al,* 1975; Daly *et al,* 1972; Haines and Harmon, 1973; Gilliland and Speck, 1974, 1977; Lindgren and Clevstrom, l978a,b).

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The pH range for the production of enterotoxin is very much the same as that in which Staphylococci will grow, namely pH 5.2 - 9.0. Staphylococci do not grow as well as at the lower end of the pH range. Hence, not as much enterotoxin is produced under those conditions. The optimum range for production of enteriotoxin appears to be pH 6.5 - 7.5. Although the Staphylococci will grow at the higher pH range, laboratory experiments indicated that enterotoxin is not likely to be produced much above pH 8.0. (Merlin, 1990). Generally Staphylococci do not grow well in the absence of other organisms unless the Staphylococci count is very high than that of other organisms. Therefore during ensiling of poultry intestine the predominance of LAB, the reduction of pH, the production of acid, alcohol and antimicrobial substances (bacteriocins) are all necessary for effective production of a good silage..

The work of Hrubant (1975) reports the 24h to 72h period of the fermentation lias one of transition for both lactics and yeast populations. By 48h, the pH reached a minimum of 4.21, Coliforms were no longer isolated, and the number of Lactics was stabilized. Where total numbers of Lactobacilli remained constant, heterolactics declines to 20% of the population. The persistent group of streptobacteria became the most numerous lactic, representing one third of the total. In the present study it was observed that l44h the pH had decreased again to 4.2. Both lactic and yeast populations were stable changes in microflora of poultry intestine silage experienced a similar pattern where yeast and Lactic acid bacteria were predominantly associated. In many cases, L.plantarum was present in the fermenting product and dominated at the final stage of fermentation, due to its high acid tolerance (Akinrele, 1970; Noat, 1980; Mbugua, 1984; Kotzekidou and Roukas, 1986; Oyewole and Odumfan, 1990). A

mixed culture heterolactic fermentation prevails in poultry intestine silage, as seen in the present study.

The associations between yeasts and lactic acid bacteria are very common in a wide variety of traditional food and beverage fermentations. It has also been reported in Animal feed at USA (novel process) B.J. Wood (1981). A further remarkable and technologically important characteristic of these associations, is their ability to achieve dominance over the organisms naturally present in the raw materials used in the fermentations.

According to Wood (1981) the modern application of the yeast / Lactic acid bacteria association is perhaps the most remarkable example of its capacity to overwhelm other organisms to the benefit of the people operating the technology.

Lactobacilli especially L.plantarum predominated in the micorbial succession during fermentation of poultry intestine. Coliforms, E.coli. Staphylocuccus, Enteriococcus and Salmonella have been elimanated from the silage. The studies lead to the development of a stable and safe poultry silage. Fermentation techiniques developed in the present study is simple and inexpensive for conserving poultry intestine in tropical countries.

The technological importance of this lactic fermentations resides in the ability to economize on nitrogen, converting the nitrogenous compounds normally to a form acceptable to the animals as part of their diet while at the same time getting rid of a potentially highly objectionable product of intensive animal rearing, converting a foetid material into something with an odour likened to that of silage.

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In some lactic fermentation this is going to make important contributions both on a practical level and to our knowledge of the biochemical capabilities of the microbes participating in it.

Stable mixed cultures of yeasts and LAB are wide spread in a variety of seemingly unrelated environments. They are apparently able to outgrow, dominate and even destroy microbes which were originally present in much greater numbers than were yeasts and LAB. In doing so they cause changes which can be used for the benefit of humanity and be consequence they have been so employed in diverse ways for many centuries.

Since some bacterial pathogens are ` obligate' parasites and do not survive for any length of time outside the host, whereas others, although they are principally associated with animals, can survive in the environment for long periods and, in some instances, multiply therein. The survival of any bacterium in the environment is dependent on many factors including availability of nutrients, Eh, moisture, pH and temperature. The presence of competing microorganisms is also important, particularly since some produce antibacterial substances and some bacterial species are capable of forming either spores or dornant cells as part of their survival strategy (Hinton and Bale, 1991). From the present study it is seen survival of Salmonella the most potent pathogen in the intestinal tract. These organisms survive in the intestinal tract, and become established carriers (Williams,' 1981). These microorganisms are then transmitted to feed if proper treatment is not given during the processing of the animal by-products into animal feed. This ultimately is eaten by the domestic birds (Morris *et al,.* 1969). Thus the poultry industry continues to be plagued by

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Salmonella contamination in the poultry feed and ingredients. A lot of work has been done regarding Salmonella in poultry feeds. (Edwards *et al,* 1958, Erwin 1955, Burr and Helmboldt, 1962; Taylor *et al,* 1965). The U.S. Advisory committee on Salmonella (Anon, 1978b) stated that feed is perhaps the single most important source of Salmonella contamination of live stock and poultry.

The primary cause for the contamination of poultry feed is the improper handling of the animal by-product or ingredients from which feed has been prepared. Several investigators have reported that animal feed and particularly animal feed constituents of animal origin frequently are contaminated with solmonella. (Pomeroy and Grady, 1960). From the present study, it is well recognised that Salmonella is harboured not only on the skin or feathers of birds, but also in the intestine of the feds. Hinton *et al.,* (1991) has tried the reduction of S. typhimurium colonization in chicks with or without dietory lactose. Therefore occurrence of Salmonella is a well known factor. To exclude Salmonella from the intestine by competitive establishment of non pathogenic microflora had been investigated. (Nurmi and Rantala, 1973; Rantala and Nurmi, 1973; Snoeyenbos *et al.,* 1979). These workers have added cecal content of older birds into the diets of chicks to establish the microflora of the two day old chicks so as to prevent Salmonella colonization. Samanta and Biswas (1994) worked on the addition of probiotics consisting of viable lactobacillus organisms. These organisms colonize in the intestinal tract and produce different acids mainly the lactic and as a metobolite. This lowers the pH and produce beneficial effects. Thus any methods have been investigated in order to free feed and birds from contamination of pathogenic microorganisms.

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The Salmonella are relatively resistant organisms and can survive for months outside the gastro intestinal tract in harsh environments. (Thomason *et al.,* 1978) as well as in dried form and animal feed. Salmonellas may grow in solid composted manure if the temperature is in the range of 20-40 °C, the moisture content exceeds 120% and the carbon to nirogen ratio is in excess of 15 on the other hand, although Salmonella numbers decline in stored slurry. With the rate being dependent on temperature and the concentration of solid matter (Jones 1980).

The present study has achieved inhibition of Salmonella mainly due to the Lactic fermentation, this is evidenced by the presence of large numbers of LAB. Their ability to produce acid and decrease the pH to the desired level of 4.2-within 6 h for 37 °C and the elimination of Salmonella by 12 h as compared at 26 ± 2 °C (ambient). Thus temperature is an important parameter for the accelaration of the growth of LAB followed by production of acid, in order to control fermentation, similarly Hargrove *et al,* (1969) has reported the variables significantly affecting survival of Salmonella are pH and the type and amount of the starter. Further more work on Manchegicheese by Medina *et al,* (1982) reports reduction in number of Salmonella during manufacturing could be attributed to the combined effect of pH and lactic acid concentration. In the associative growth experiments (Mara *et al,* 1991) however, inhibition of Salmonella growth was recorded only when the pH of milk dropped from 6.07 to 4.77.

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Conclusion

It is found that microbial succession resulted in the predominance of L<u>actobaciilus species</u> during fermentation of poultry intestine. Microbial fermentation resulted in a silage free from undesirable microorganisms including <u>Salmonella</u>.

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Table 1.1

Microbial sucession of **poultry intestine during fermentation at Ambient and** 37ºC

TPC n = 6

Log cfu / g

Hours / Temp.	0	6	12	24	48	72	96	120	144	**
Ambient	8.58	8.31	8.59	8.65	7.45	7.27	7.16	6.68	6.24	7.65^{y}
37°C	8.28	8.96	7.82	7.63	7.43	6.61	5.18	5.22	5.29	6.93^{x}
*	8.43^{f}	8.63^{f}	8.20^{f}	8.14^{f}	7.44^{e}	6.94^{d}	6.17^{c}	5.95^{b}	5.76^{a}	

SE = 0.0159 df=85 SEm=0.05147

Table 1.2

Microbial sucession of poultry intestine during fermentation at Ambient and 37ºC

Coliform n = 6

Log cfu / g

Hours / Temp.	0	6	12	24	48	72	96	120	144	**
Ambient	7.59	6.51	6.40	5.50	0	0	0	0	0	2.88y
37°C	7.78	6.87	0	0	0	0	0	0	0	1.60x
*	7.58^{d}	6.69^{c}	3.2^{b}	2.75^{a}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	

SE = 0.0101 df=85 Sem=0.04102

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Table 1.3

Microbial sucession of poultry intestine during fermentation at Ambient and 37ºC

E.coli n = 6

Log cfu / g

Hours / Temp.	0	6	12	24	48	72	96	120	144	**
Ambient	7.56	7.33	6.37	5.49	0	0	0	0	0	2.97^{y}
37°C	7.33	6.91	0	0	0	0	0	0	0	1.58^{x}
*	7.44^{d}	7.12^{c}	3.18^{b}	2.74^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	

SE = 0.0220 df=85 Sem=0.06055

Table **1.4**

Microbial sucession of poultry intestine during fermentation at Ambient and 37ºC

Staphylococc us n = 6

Log cfu / g

Hours / Temp.	0	6	12	24	48	72	96	120	144	**
Ambient	5.79	5.72	5.24	4.75	0	0	0	0	0	2.38^{y}
37°C	5.58	4.88	2.22	0	0	0	0	0	0	1.40^{x}
*	5.68^{d}	5.3^{d}	3.73^{c}	2.37^{b}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	

SE = 0.3411 df=85 SEm=0.023843

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Table 1.5

Microbial sucession **of poultry intestine during fermentation at Ambient and** 37°C

Enterococci **n** = 6

Log cfu / g

Hours / Temp.	0	6	12	24	48	72	96	120	144	**
Ambient	8.57	7.95	7.7	5.0	0	0	0	0	0	3.24^{y}
37°C	5.3	7.33	5.02	0	0	0	0	0	0	1.96^{x}
*	6.93^{d}	7.64^{e}	6.36^{c}	2.5^{b}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	

SE = 0.0355 df=85 SEm=0.0769

able **1.6**

Microbial sucession **of poultry intestine during fermentation at Ambient and** 37°C

Yeast **and** Mold **n** = 6

Log cfu / g

Hours / Temp.	0	6	12	24	48	72	96	120	144	**
Ambient	8.60	7.43	7.78	7.4	7.38	6.88	6.1	4.96	4.95	6.83^{y}
37°C	8.04	7.58	6.87	5.86	6.61	5.43	4.42	4.7	4.1	5.95^{x}
*	8.32^{g}	7.50^{f}	5.32^{ef}	6.63^{d}	6.99^{de}	6.15^{c}	5.26^{b}	4.83^{a}	4.52^{a}	

SE = 0.1172 df=85 Sem=0.13976

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Table 1.7

Microbial sucession of poultry intestine during fermentation at Ambient and 37°C

Lactic acid bacteria

n = 6

Log cfu / g

Hours / Temp.	0	6	12	24	48	72	96	120	144	**
Ambient	9.24	9.46	9.9	9.85	9.81	9.8	9.98	8.97	8.41	9.49^{x}
37°C	8.92	10.51	10.2	10.5	9.3	9.26	9.0	8.51	8.29	9.38^{x}
*	9.08^{c}	9.98^{e}	10.05^{e}	10.17^{e}	9.55^{d}	9.53^{d}	9.49^{d}	8.74^{b}	8.35^{a}	

SE = 0.0414 df=85 Sem=0.08306

Legends:

*	**Mean Rows**
**	**Mean Column**
SEm	**Standard Error of mean**
df	**Degrees of freedom**

Row Means is bearing different superscript a,b,c,d,e,f,g differ significantly

Column Means is bearing different superscript x,y,z differ significantly

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Table 2

Succession **of microorganisms isolated during fermentation of poultry intestine**

0 h	24 h	144 h
E.coli	Lactobacilli sps	L.plantarum
Coliform	S.lactics	L.acidophilus
Staphylococcus	Pediococcus	L.fermenti
Enterobacteria	Yeast	yeast
Fungi		
Yeast		
Lactic acid bacteria		
L.salivarus		
L.brevis		
L.acidophiles		
L.plantarum		
L.fermenti		
L.jensenni		
Enterococcus sps.		
Pediococcus		
Leuconostoc		

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Table 3

Changes **in Salmonella, Lactic acid bacteria and pH during the silage fermentation of poultry intestine (without addition of Salmonella culture)**

Period of Fermentation	37 ± 1 °C			26 ± 2 °C		
	pH	*	**	pH	*	**
0 hour	5.2	2.0	7.2	5.1	3.1	6.8
6 hours	4.2	0	8.7	5.0	2.8	7.3
12 hours	4.0	0	8.8	4.9	2.2	8.0
18 hours	3.9	0	9.4	4.7	1.3	8.7
24 hours	3.9	0	9.0	4.4	1.0	9.2
48 hours	3.8	0	9.6	4.2	0	9.8
72 hours	3.7	0	8.6	4.0	0	8.4

'Salmonella MPN /g '*

Lactic acid bacteria / g

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Table4

Changes in Salmonella, Lactic acid bacteria and pH during

Period of Fermentation	37 ± 1 ^{0}C			26 ± 2 ^{0}C		
	pH	*	**	pH	*	**
0 hour	5.2	5.2	7.7	5.1	5.1	7.3
6 hours	4.2	3.3	8.9	5.0	4.9	8.6
12 hours	4.0	0	9.4	4.9	4.0	9.0
18 hours	3.9	0	9.6	4.7	3.4	9.4
24 hours	3.9	0	9.2	4.4	2.2	8.0
48 hours	3.8	0	8.8	4.2	0	9.2
72 hours	3.7	0	8.4	4.0	0	8.7

the silage fermentation of poultry intestine inoculated with S.typhimurium.

'Salmonella MPN / g

"Lactic acid bacteria / g

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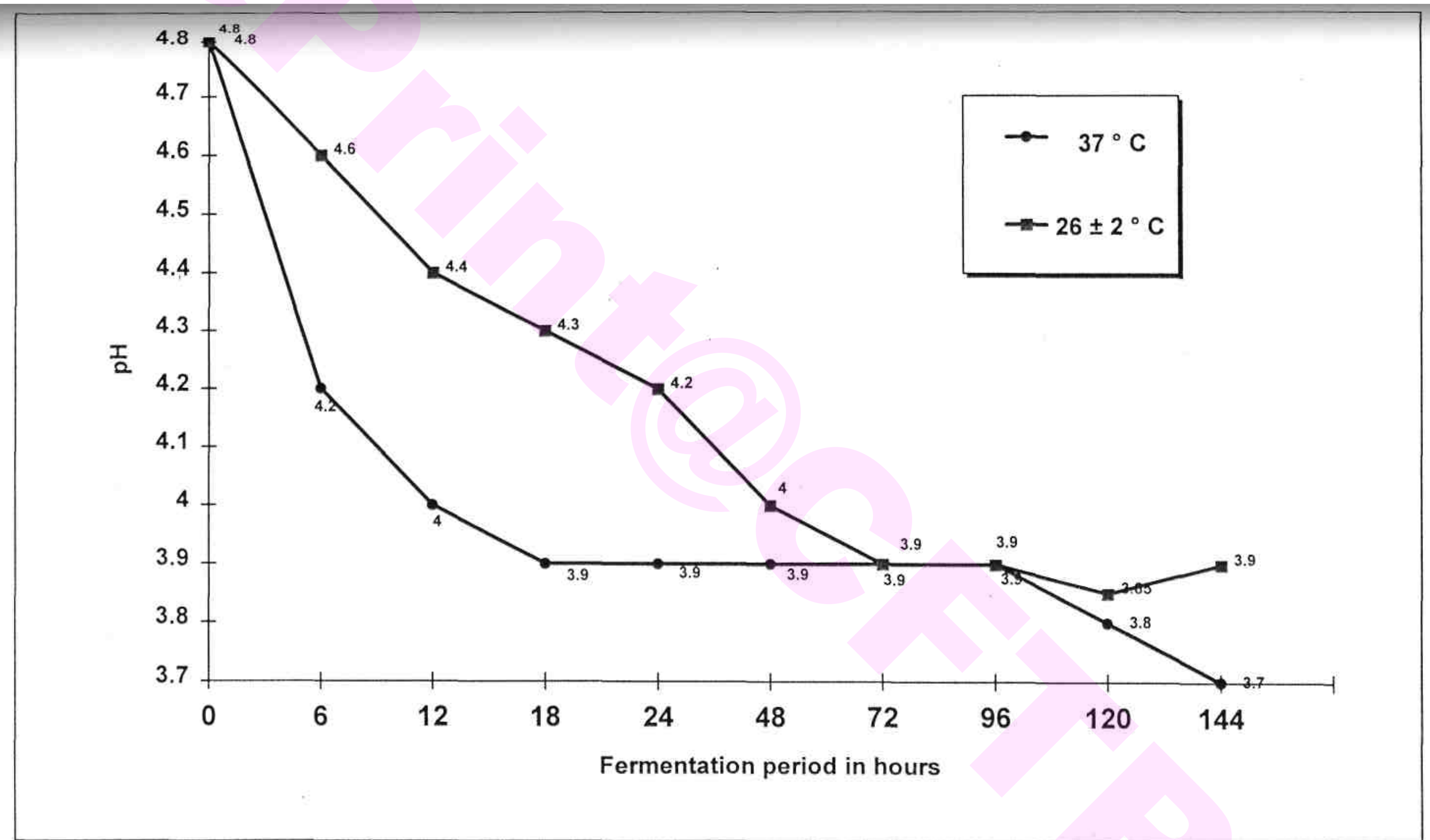

Fig 1: FALL IN pH IN POULTRY INTESTINE DURING FERMENTATION

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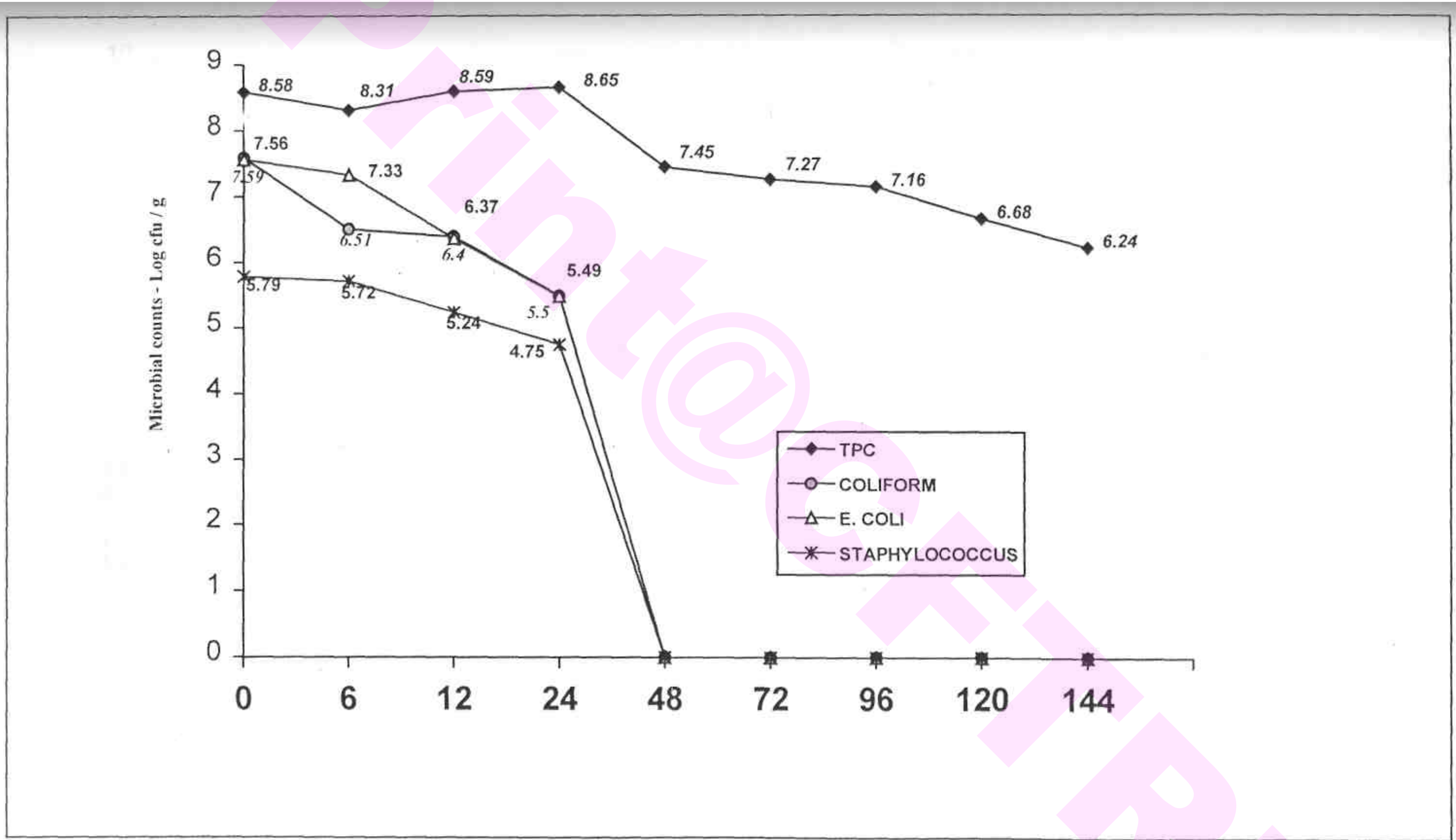

Fig 2a: Microbial succession of poultry intestine during fermentation with 10% of Molasses and 0.5% propionic acid at

26 ± 2 ° C (Ambient)

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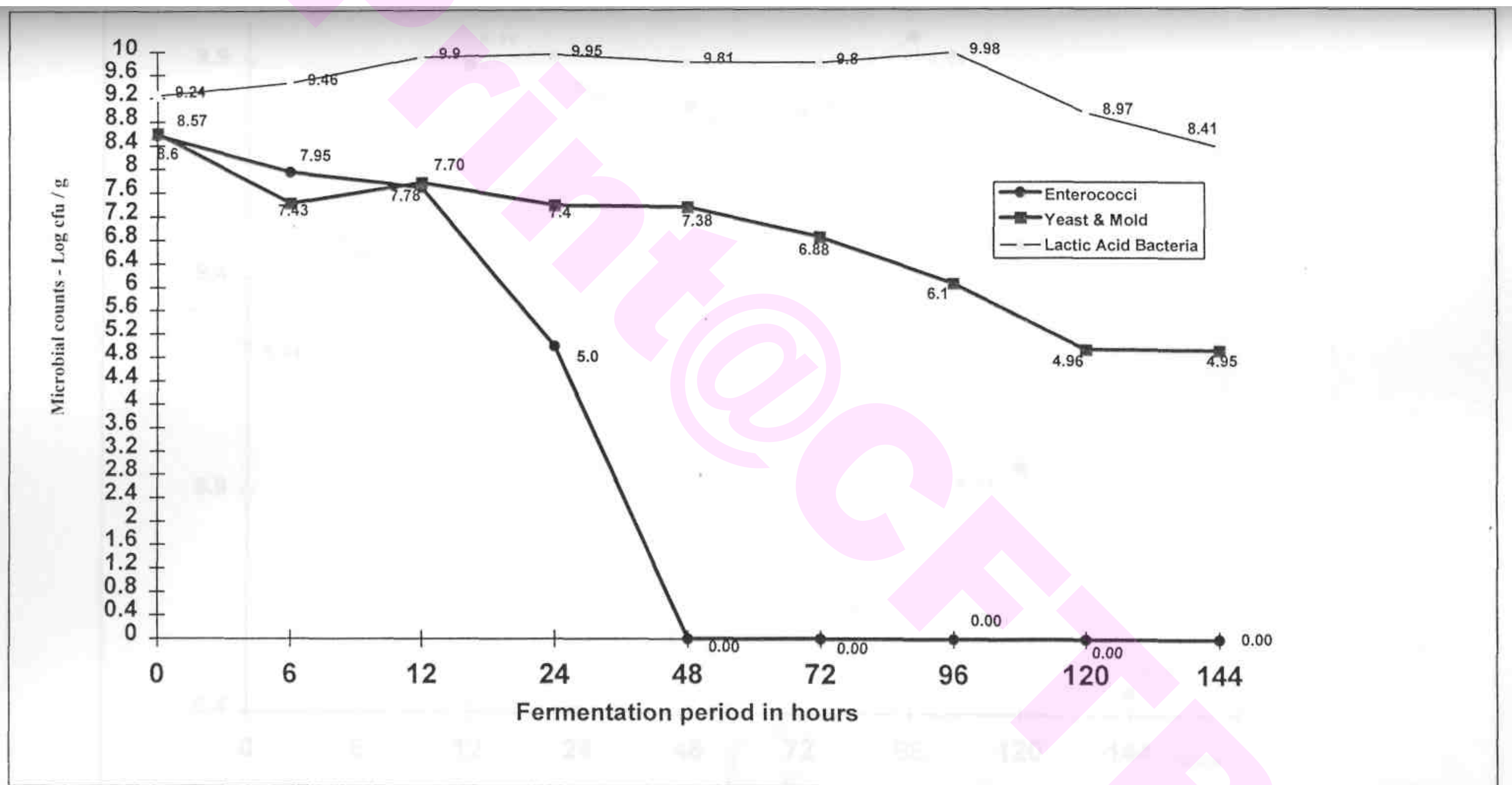

Fig 2b: Microbial Succession of poultry intestine during fermentation with 10% Molasses and 0.5% Propionic acid at $26 \pm 2\ ^{\circ}C$ (Ambient)

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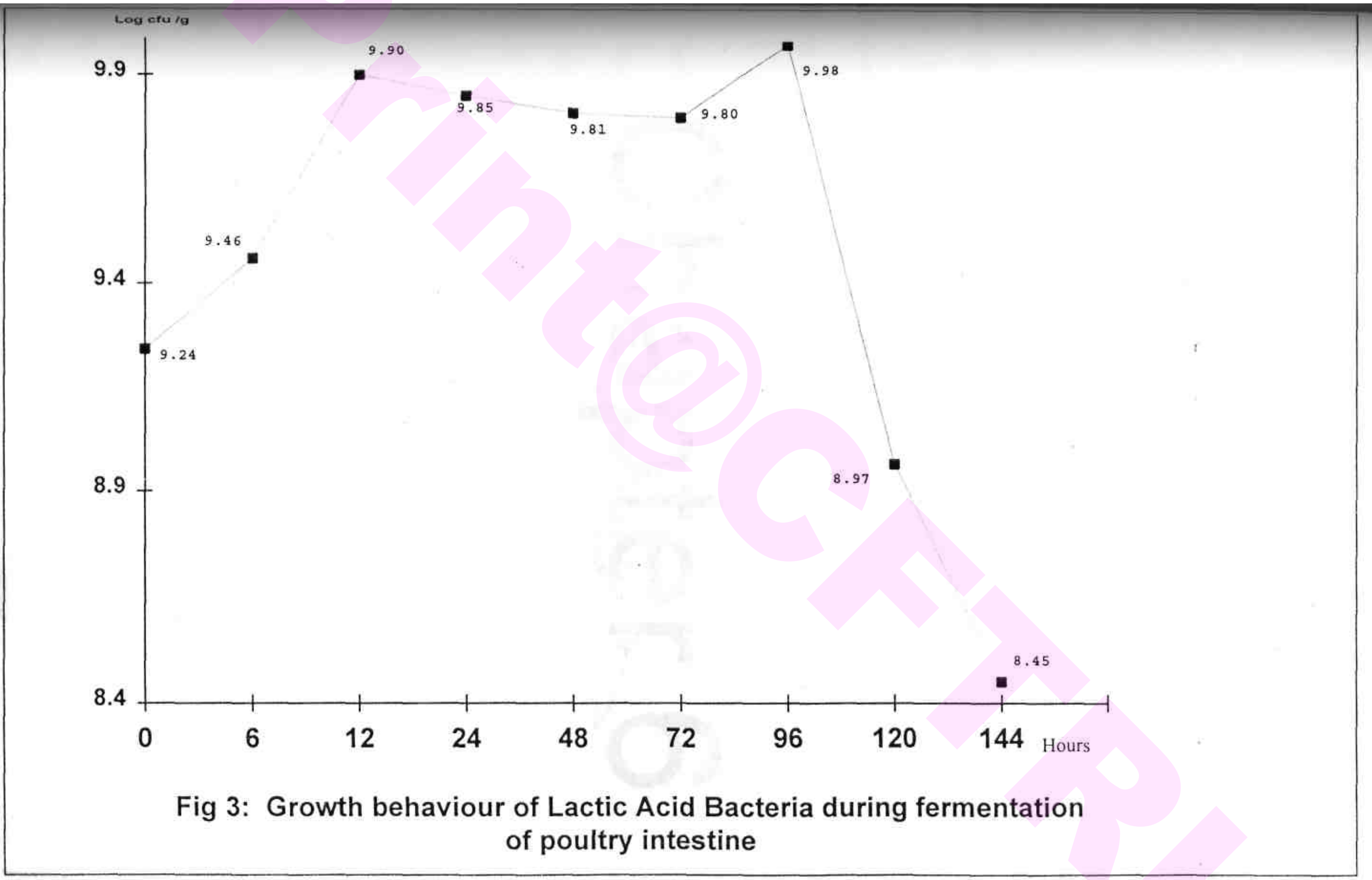

Fig 3: Growth behaviour of Lactic Acid Bacteria during fermentation of poultry intestine

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Chapter 6

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ACCLERATION OF FERMENTATION ENSILING OF POULTRY WASTE

INTRODUCTION

Fermentation has emerged as a simple technique for preserving of biological wastes. Ensiling is directed towards evaluating the potential process for the preservation of slaughterhouse offals that are the rich sources of proteins in animal feeds. It is an alternative to the energy dependent techniques. Hence the simple techniques which are economic, easy and one which does not require skilled know-how are ideal for conserving poultry intestine in tropical countries.

Conservation of materials like poultry intestine by ensiling is based on natural fermentation in which the natural flora of Lactic acid bacteria (LAB) converts sugar ¡under anaerobic conditions into Lactic acid, ethyl alcohol and carbon dioxide, since it is not purely a homofermentation but of the heterofermented type. As a result the pH decreases with the production of acids and antimicrobial substances produced by Lactic acid bacteria during ensiling and the silage is preserved.

In order to improve fermentation during ensiling it is suggested that suitable Lactic acid bacteria inoculents are added before initiating fermentation, so that with the addition of the starter cultures, it will aid in increasing the rate of fermentation and these favourable environmental conditions may prevent the growth of pathogenic and undesirable microorganisms. Unlike other silages like that of grass, wheat, alfalfa or other herbages, including animal waste like fish, poultry intestine has an added advantage due to the presence of abundant naturally inhabited Lactic acid bacteria.

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therefore addition of pure cultures or specific starter cultures of Lactic acid bacteria are not required, but to increase the rate of fermentation, backslop material (BSM already fermented material) has been added. BSM containing high levels of Lactic acid bacteria would bring rapid and efficient utilization of the added carbohydrates, followed by rapid decrease in pH. Thus, poultry intestine can rapidly be stabilised. kelerated natural lactic fermentation can be achieved by repetitive use of 5 to 10% (w/w)of a previously fermented batch as a starter. It as shown earlier (Nout *et al,* (1989 a) that mixtures of cereals and pulses fermented in this way have a strong antimicrobial effect towards a range of pathogenic bacteria.

As important strategy in production of high quality animal feed is to control and minimize growth of various microorganisms at the earliest. The important word here is 'control' since microorganisms are ubiquitous contaminants of raw materials and finished feeds. The growth of these microorganisms have serious consequences on the animal feed like - loss in nutritive value, reduction in pallatability, change in physical properties, disemination of pathogenic organisms and production of mycotoxins.

The objective in this chapter was to evaluate factors that lead to a rapid lactic fermentation, ie.a rapid decrease in pH (<4.2); a rapid proliferation of Lactic acid bacteria with contaminant decrease in spoilage microorganisms count. The present study proposes to unravel a strategy and technology to rapidly eliminate the harmful effects of microorganisms, by way of determining the effect of temperature and addition of backslop material as a starter culture on the fermentation ensiling of

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poultry intestine. This could result in the development of rapid fermentation for Ensiling of poultry intestine.

MATERIALS AND METHODS

Collection and sampling of poultry intestine

Intestines were collected from Cobb broiler birds (6 to 8 weeks of age) slaughtered in the local market and brought within lh of slaughter to the laboratory. About three kg intestine were homogenized with 10% (w/w) molasses and 0.5% (v/w) Propionic acid. Molasses was added as a source of energy for microorganisms while Propionic acid served as an antimycotic agent. This was distributed into 2 latches of 1 kg each in plastic buckets, the mixture layered with polypropylene sheets, bucket covered tightly with the lid to create microaerophillic condition and incubated at ambient (26±2°C separately for fermentation.

Microbiological analysis

Fifty gram of the sample (of intestine homogenate) and 450 ml sterile physiological saline were blended for 2 minutes. This formed the first decimal dilution. Further , serial dilution's were made upto 10 using 90ml sterile physiological saline. Escherichia. coli: Coliforms; Staphylococcus, Enterococci Streptococcus, Yeast and Molds, Lactic acid bacteria (LAB) and total plate count were estimated as per standard procedures (APHA, 1984).

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Backslop material (BSM)

When the desired pH of 4.2 was reached the material was considered stable, which contained large number of LAB (10^9 to 10^{10}/g) and free from pathogenic and spoilage microorganisms. This fermented material (backslop material) was used to inoculate the fresh batch of poultry intestine containing 10% molasses (w/w) and 0.5% Propionic acid (v/w).

Fermentation of poultry intestine with addition of backslop material

Backslop material (stabilized poultry intestine at 37°C) was inoculated into two sets of fermentation mixture. Backslop material was inoculated at different percentages (5-25%) into the mixture One set was held at 26 ± 2°C and the other at 37°C.

The fermented material was mixed thoroughly for 2 minutes. Fifty gram of the material was removed at regular intervals (0, 3, 6, 12, 24, 48, 72, 96, 120, l44h) into 450 ml sterile physiological saline and serially diluted till 10 for the enumeration of the microorganisms.

Estimation **of reducing sugar**

A calorimetric method of Michael Dubois *et al,* (1956) was followed for the determination of reducing sugar.

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pH determination

The pH of the fermenting material was recorded using pH meter, Radiometer openhagen, Denmark).

Statistical Analysis

Experimental data (count of microorganisms) obtained from washed and unwashed poultry intestine, were subjected to ANOVA. Significant difference between means within the sample were tested by Duncans multiple range test Puncan, 1955) and between washed and unwashed samples by t-ratio test.

RESULTS

Temperature effect on pH of poultry intestine silage on addition of backslop material as starter culture

The results presented in Table 1 and Fig.la and b indicate that when BSM was Bed at 5% to 20% levels, the material did not attain safe pH of 4.2 till l0h, during the (fermentation at 26 ± 2 °C whereas at 37°C, desirable pH reached in the material with 5% BSM level at 4h itself. This indicates temperature has a vital role to play with the pattern of growth in microorganisms specially LAB. From chapter 5 it was evident that 37ºC pH decreased drastically within 6h as compared to ambient which took 18 to 24h. Thus an elevated temperature i.e. 37°C might be favouring rapid pltiplication of Lactic and bacteria for poultry intestine silage. Moreover, BSM as a starter culture (additive) or fermentation stimulant enabled a more rapid decrease in pH. Even at ambient pH decrease has hastened by 14h which is remarkable. More so

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at 37 ºC with only 5% BSM, pH drastically reduced within or even less than 2h. This proves that temperature rise improves the rapidity of fermentation. From these studies it is evident that 37°C can be considered ideal for the growth of LAB. Temperature together with addition of BSM go hand in hand to produce effective and safe stable silage. This decrease in pH is attributed to production of acids with concomitant reduction of unwanted microflora for production of good silage. Microbial studies were conducted with 5%; 25% and 35% levels of BSM.

Microbial succession in poultry intestine with and without addition of BSM

Microbial succession in any given ecosystem or fermentation leads to competition with the dominant species succeeding. In poultry intestine silage an aerobic microflora is switched over to a microaerophillic microflora. Results of microbial succession of poultry intestine during fermentation at 37°C is given in Fig.2a and b, where Fig 2a shows fermentation of poultry intestine without BSM. It is ascertained that coliform and E.coli are eliminated within l2h while counts of Staphylococcus and Enterococci were reduced to zero level by 24h. The growth pattern of TPC shows between 6 and l2h there is an increase of total counts with slight decrease till 96h from log 10^7 to log 10^5 , remained constant subsequently. While Yeast and Molds decreased from 6h onwards. It was observed that there was an increase in Lactic acid bacteria upto 24h and subsequently, the count reduced very marginally. This decrease may be due to depletion of nutritients and substrate within the silage.

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The results on the addition of BSM at 5%, 25% and 35% levels revealed that pirable pH was attained with 5% at 4h while with 25% and 35% within 2h. Microbial profile as revealed in Table 3 indicated that 5% BSM adequate to inhibit E.COLI, coliform, Staphylococcus and Enterococci within l2h. TPC was reduced to Log 5.6 in all three percentages of BSM. Silage attained stability in 24h with 5% BSM. Yeast and Molds were lowered to log 4 at l44h with 5% BSM, while in 25% and 35% Yeasts and Molds were lowered to 6.1 only at l44h, indicating that 5% BSM was adequate to inhibit pathogenic and spoilage microorganisms and stabilize the silage. The statistical analysis in Table 3 also shows the signficance of BSM in reduction of pathogenic E.coli. Coliform, Staphylococcus, Enterococci. A slight decrease in Lactic acid bacteria counts has been noticed towards the end of fermentation.

The trends in inhibitaion of various pathogenic and spoilage microorganisms at 37 °C temperature are presented in fig 2a and 2b. Lactic acid bacteria increased rapidly both at ambient and 37 °C during fermentation ensiling of poultry intestine, production of acid is very evident by high count of Lactic acid bacteria at 6 to 24h. This means increase in Lactic acid bacteria mass and acid production resulted in decreased pH.

The rapid decrease in pH and elimination of undesirable microorganisms in poultry intestine silage can be attributed to firstly the temperature and secondly BSM. From Table 2, it is evident that microbial profile of BSM is safe and stable.

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Effect of reducing **sugar during fermentation of poultry intestine**

Results from fig 4 clearly indicates, microorganisms require sources of substrate which are readily availble in order to grow and multiply. BSM was added to fresh poultry intestine containing 10% molasses and 0.5% propionic acid because inoculum added from previously fermented material does not contain enough of the sugar source. From figure 4 it is seen from the intial log 12 count within 6h the counts lave reduced to log 8.9, and then within on span of l2h there is a 9 log decrease, dearly showing that microorganisms have been effectively utilizing the sugar in molasses. Thus BSM will have little sugar. Therefore if BSM is directly put in poultry intestine it would result with no growth of Lactic acid bacteria and spoilage of the material takes place. Intial studies to reduce molasses content with addition of BSM, showed 10% molasses is the optimum requirement for rapid proliferation of lactic acid bacteria, rapid reduction of pH followed by increase in acid. But with the addition of the sugar source it enabled the Lactic acid bacteria to proliferate and help lostabilize the fresh poultry intestine material. Therefore it is a fact that the value of moculants for silage is the availability of the substrate and product. At 37 °C depletion of sugar content is higher as recorded by Lactic acid bacteria counts. Reducing sugars in poultry intestine silage were reduced to 4 g from 12 g (intial) at 72h in ambient, while the same amount at the 37 °C was reduced in 18 - 28 h.

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DISCUSSION

Fermentation aims in conservation of material whether it be food stuffs prepared of animal or plant, or feed stuffs from grain, grass and herbage or waste products of both animal or plant. As the word fermentation implies, production of acids in homofermentation or acid and alcohol as in heterofermentation by specific microorganisms capable of fermenting into the desired product. Thus research had advanced in using desired microorganisms to develop a particular product of once own choice. As stated by Henderson and Mc Donald, (1984), the success of inoculant as a silage additive depends on many factors, such as the type and properties of the plants to be ensiled, climatic conditions, epiphytic microflora, ensiling techniques and the properties of the inoculant. Much work on silages have been initially carried out on crop and grain since silage preparation started. This ensiling mechanism was then adopted for waste fish and now made use for poultry wastes. Even in animal waste the material for ensiling makes a difference whether it fish or poultry, the microflora in poultry is different from that of fish, where Lactic acid bacteria are low in number in fish when compared to poultry (Wirahadikusunaah, 1968; Smith and Adamson, 1976). It has been observed in the present study that, Lactic acid bacteria are more predominant from the beginning of fermentation since, the poultry intestine contain high count of Lactic acid bacteria. It has demonstrated that crop and intestine of chicken contained a large number of lactobacilli (Shapiro and Sarles, 1949; Barnes *et al,* 1971; Fuller and Turney, 1971; Fuller, 1973). As fermentation proceeds Lactic acid bacteria increased by 1 log and maintained their population till the depletion of nutrients and substrates. This is indicated in the fall in

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pH during poultry intestine fermentation, where the rate of microbial metabolism was increased by the rise in temperature. Any benefit to be gained from storing silage at a temperature above ambient will have an influence by the temperature optima for the microorganisms within each group represented in the silage. It has been revealed from the present study temperature also plays a vital role. At ambient (26 ± 2 °C) fermentation was complete in 24h with desirable pH (4.2) while at 37 °C fermentation was achieved in 6h without BSM. Fermentation was complete in 3h with BSM. This indicates inoculum thrives well and promoted the growth of Lactic acid bacteria and inhibits both spoilage and pathogenic microorganisms at the lunperatures studied. Work done by Ohyama *et al,* (1973) have shown a more rapid dcvelopment of lactobacilli and decline in streptococci at 30 º C than at 15 º C, and on storage the count was greater at the lower temperature than at the higher temperature. Rydin *et al,* (1956) found that silage with sugar addition stored at 37 º C and had a high content of Lactic acid and lower pH than at 16 º C. Beck and Wieringa, (1964) and Laniga, (1963, 1965) have worked on temperature / growth relationship and have shown optimum growth temperature of 30 to 35 º C for L.plantaram and L. Curvatus.

Before a culture can be considered as having potential as an inoculum lament for silage, it should satisfy the criteria laid down by Whittenbury, (1961) these are: It must have a high growth rate and be able to compete with, and dominate leer organisms likely to occur in silage. It must be homofementative. It must be acid Blerant and produce a final pH of 4.0 quickly. It must be able to ferment glucose, fructose, sucrose. It should have no action on organic acids. It should have a good

growth - temperature range, preferable upto 50 °C inorder to survive any risk in temperature during the early stage of ensilage.

Basically similar criteria where employed by Wieringa and Beck, (1964) in the selection of microorganisms suitable for inoculation trials. Though BSM contained heterofermentative culture, it was able to fulfill all requirements as a potential inoculun according to criteria of Whittenbury, (1961). While 0.4% of Lactic acid bacteria in poultry intestine as reported in chapter three was capable of fermenting glucose, fructose and sucrose.

The rate of application of culture is also very important. The recommended rate for several commercial applications is so low in terms of viable microorganisms applied that additional stress is placed on the requirement for an microorganism to grow rapidly and compete successfully with the indigenous microflora. In the present the addition of 5% BSM into the fermentation mixture (poultry intestine) at 37 °C shows rapid decrease in pH. With this change, many other changes - chemical and physical composition of poultry intestine would automatically follow, faster than when taking place without BSM. Here an important point is to be stressed that for a conventional silage a rapid decline in pH is more important. The provision of efficient acid producing cultures (Lactic acid bacteria) could be considered as one means. But one attraction of the use of silage as culture would be relatively the low cost of production compared to current additives.

The qualitative changes in the microflora particularly in Lactic acid bacteria are attributed to the powers of survival and not to one group growing more rapidly than another (Gibbson and Stirling, 1959). This power of survival referred to acid

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tolerance and acidifying potential of the genera concerned. As Rogers and Whittier, (1928) demonstrated that the accumulation of acid is responsible for cessation of growth. The finding in the present study are in accordance with the above work.

The fermentation efficiency of the indigenous Lactic acid bacteria is important |in terms of their abilities to initiate a rapid fermentation and sustain a rapid fall in pH. Wieringa and Beck, (1967) pointed out that only a small proportion of indigenous population possess these abilities and considered that there could be advantages borne by addition of selected strains of bacteria to silage. But the present study reveals previously fermented material containing heterofermentative culture of indigenous Lactic acid bacteria is able to bring rapid fermentation with concomitant rapid fall in pH in poultry intestine silage. Thus addition of BSM had improved fermentation in poultry intestine. These findings agree with those of Weinberg *et al.,* (1988) where inoculation with H / M + inoculant improved the alfalfa silage as judged by chemical composition; helped to reduce dry matter losses and repressed undesirable microorganisms such as Enterobacteria and clostridia. Also the work of Carpintero *et al,* (1979), who used an inoculant (L.plantaram. L.mesenteroides and S.faecalisì in laboratory rye grass - clover silages. Inoculant eliminated clostridia. This has resulted in lower pH due to Lactic acid bacteria during fermentation. Much work on improved ensiling has been done in plants alone Weinberg *et al,* (1987).

The subject of the value of inoculant for silage is highly controversial (Woolford, 1984). Often work has been undertaken on laboratory scale using combined inoculum / fermentation substrate treatments and it is therefore difficult to decipher the effect of each or the interaction between the two components of such treatments. An overriding factor to consider in any assessment of the value of inoculant for silage is the ability of substrate, an inoculum will have little chance to

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exert its influence on silage if there is insufficient sugar for fermentation. Stirling, 1954) stated that failures with inoculated silage generally for this reason. Weringa, (196 1, 1962) showed that low dry matter grass inoculated with L·.plantaram would produce stable silage provided it contains the sugar content at the outset is atleast 80 g **of** sugar per kg of the dry matter. Some benefits were brought to silage made from orchid grass having only 42 g/k g-' sugars in the dry matter (Mc Donald *et al.,* (1960). This work strongly suggests that an inoculum will only assist the ensilage of crops low in sugar. An efficient utilization of substrate is essential to secure preservation. The results of the present study are in aggrement with Neelakantan and Singh, 1976); Singh and Neelakantan, (1976) found that the contents of Lactic acid and **Lactic** acid bacteria in silages made from corn, sorghum or berseem were higher when molasses was used as a source of sugar supplement. Studies conducted by other workers have shown the merits of the use of mixtures of cultures and sugar in silage (Orla-Jensen *et al,* 1947; Weringa, 1960, 1961; Gross, 1969). Addition of molasses into fresh homogenate poultry intestine is essential in order to obtain desirable fermentation. Fish treated with an inoculum of Lactic acid bacteria and with molasses at about 90 g/kg^{-1} of fresh weight underwent a more rapid acidification than similar material treated with molasses alone (Kreuizer and Boyes, 1953; Kreuizer 554). A similar trend has been observed in rye grass treated with an inoculums Lactobacilli and molasses at 20 g / kg^{-1} fresh weight when compared to the same crop treated with either with the inoculum or with molasses alone (Mc Donald., 1964). During the building-up of sour dough starters (Nout and Creemers-Molenarr, 1986) and accelerated natural lactic fermentation of soya beans for tempe manufacture (Nout *et al,* 1987 b), it was observed that the process of acidification stabilized after **3-5** consecutive fermentation cycles. However it took much longer (30, resp. 60 cycles) for natural fermentation to result in a rather stable microbial composition. Since BSM is free from all pathogenic and spoilage microorganisms, it is a stable material having a pH of 3.8 - 4.0 and contains active Lactic acid bacteria. Russell *et al.,* (1993) reported Lactic acid bacteria would bring out the rapid fermentation of silage, which is an effective means of inhibiting pathogens and indicator microorganisms in broiler chicken offal. All lactic cultures can to some latent repress the growth of other bacteria. This property can be a result of acid

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production and pH reduction (Rolf Anderson, 1986). By this it may be possible to reduce the number harmful microorganisms prior to processing of offal, thereby reducing the possibility of cross contamination, retaining the nutritive value in poultry intestine.

For the present work, the Lactic acid bacteria are the only group of microorganisms likely to be useful in the production of silage. Inspite of the lack of inequivocal evidence on the merits of cultures as additives for silage, many preparations containing Lactic acid bacteria as the active principle are being vigorously promoted by several commercial concerns. Their sole selling point is that they are safer to handle and environmentally more desirable than additives in current use. Most of these preparations are based on a single culture of Lactic acid bacterium. In view of differences in the tolerance to acid and rates of growth among the various genera, silage Lactic acid bacteria in combinations have generally given better results than single cultures.

Conclusion

Rapid fermentation can be brought about during ensiling of poultry intestine the addtion of BSM along with molasses. Higher incubation temperatures would further enhance fermentation.

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Table l Microbial profile of backstop material

Microorganisms	Count (Log cfu/g)
E.coli	0
Coliform	0
Staphylococcus	0
Enterobacteria	0
Fungi	0
Yeast	5
Lactic acid bacteria	10

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Table 2.1

Microbial sucession of poultry intestine during fermentation using BSM at 37°C

n = 6, Log cfu / g

Hours / % BSM	0	6	12	24	48	72	96	120	144	**
5	10.45	8.94	9.52	7.26	6.31	5.5	6.54	5.68	5.6	7.31^{y}
25	8.73	7.8	7.69	6.02	.5.21	5.48	5.67	5.95	5.24	6.46^{x}
35	8.12	7.2	7.33	6.38	5.53	5.45	5.64	5.60	5.64	6.32^{x}
*	9.1^{g}	7.98^{e}	8.18^{f}	6.55^{d}	5.68^{b}	5.47^{a}	5.95^{c}	5.74^{b}	5.62^{d}	

SE = 0.0124 df=130 SEm=0.04546

Table 2.2

Microbial sucession of poultry intestine during fermentation using BSM at 37°C

Coliform **n = 6, Log cfu / g**

Hours / % BSM	0	6	12	24	48	72	96	120	144	**
5	8.82	7.22	0	0	0	0	0	0	0	1.78^{y}
25	8.45	5.27	0	0	0	0	0	0	0	1.52^{x}
35	7.41	5.58	0	0	0	0	0	0	0	1.44^{x}
*	8.22^{c}	6.02^{b}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	

SE = 0.0060 df=130 SEm=0.03162

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Table 2.3

Microbial sucession of poultry intestine during fermentation using BSM at 37°C

E.coli n = 6, **Log cfu / g**

% BSM \ Hours	0	6	12	24	48	72	96	120	144	**
5	8.40	7.40	0	0	0	0	0	0	0	1.75[x]
25	8.60	5.9	0	0	0	0	0	0	0	1.61[x]
35	7.76	5.83	0	0	0	0	0	0	0	1.51[x]
*	8.25[c]	6.37[b]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	

SE = 0.0005 df=130 SEm=0.009128

Table 2.4

Microbial sucession of poultry intestine during fermentation using BSM at 37°C

Staphylococcus n = 6, **Log cfu / g**

% BSM \ Hours	0	6	12	24	48	72	96	120	144	**
5	5.75	5.09	0	0	0	0	0	0	0	1.20[x]
25	5.88	5.87	0	0	0	0	0	0	0	1.30[x]
35	5.83	4.74	0	0	0	0	0	0	0	1.17[x]
*	5.82[c]	5.23[b]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	

SE = 0.0002 df=130 SEm=0.00577

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Table **3.5**

Microbial sucession of poultry intestine during fermentation using BSM at 37°C

Enterococci **n = 6, Log cfu / g**

Hours / % BSM	0	6	12	24	48	72	96	120	144	**
5	8.4	5.11	0	0	0	0	0	0	0	5.50^{y}
25	8.41	6.05	0	0	0	0	0	0	0	1.60^{z}
35	8.03	4.23	0	0	0	0	0	0	0	1.36^{x}
*	8.28^{c}	5.13^{b}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	

SE = 0.0008 df=130 SEm=0.01154

Table **2.6**

Microbial sucession of poultry intestine during fermentation using BSM at 37°C

Yeast **and Mold** **n = 6, Log cfu / g**

Hours / % BSM	0	6	12	24	48	72	96	120	144	**
5	8.47	7.333	6.0	5.77	5.23	5.18	4.76	4.36	4.0	5.66^{x}
25	7.77	7.34	7.6	5.59	6.13	6.76	7.2	7.6	6.2	6.91^{y}
35	8.02	8.26	7.71	6.41	5.38	5.5	7.62	7.63	6.10	6.95^{y}
*	8.08^{g}	7.64^{f}	7.10^{e}	5.92^{c}	5.58^{b}	5.78^{b}	6.52^{d}	6.53^{d}	5.43^{a}	

SE = 0.0831 df=130 SEm=0.01961

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Table 2.7

Microbial sucession of poultry intestine during fermentation using BSM at 37°C

Lactic acid bacteria n = 6, **Log** cfu / **g**

Hours / % BSM	0	6	12	24	48	72	96	120	144	**
5	9.26	9.77	9.15	9.02	9.05	8.76	8.13	7.87	6.54	8.61^{y}
25	9.21	9.74	9.9	10.01	8.18	7.8	6.72	6.33	6.56	8.27^{x}
35	9.72	7.88	10.15	9.16	8.39	7.64	6.56	6.19	6.47	8.24^{x}
*	9.38^{d}	9.79^{c}	9.73^{c}	9.39^{d}	8.54^{c}	8.06^{b}	7.13^{a}	6.79^{a}	6.52^{a}	

SE = 0.0031 df=130 SEm=0.02273

Legends:

*	**Mean Rows**
**	**Mean Column**
SEm	**Standard Error of mean**
df	**Degrees of freedom**

Row Means is bearing different superscript a,b,c,d,ef,g differ significantly

Column Means is bearing different superscript x,y,z differ significantly

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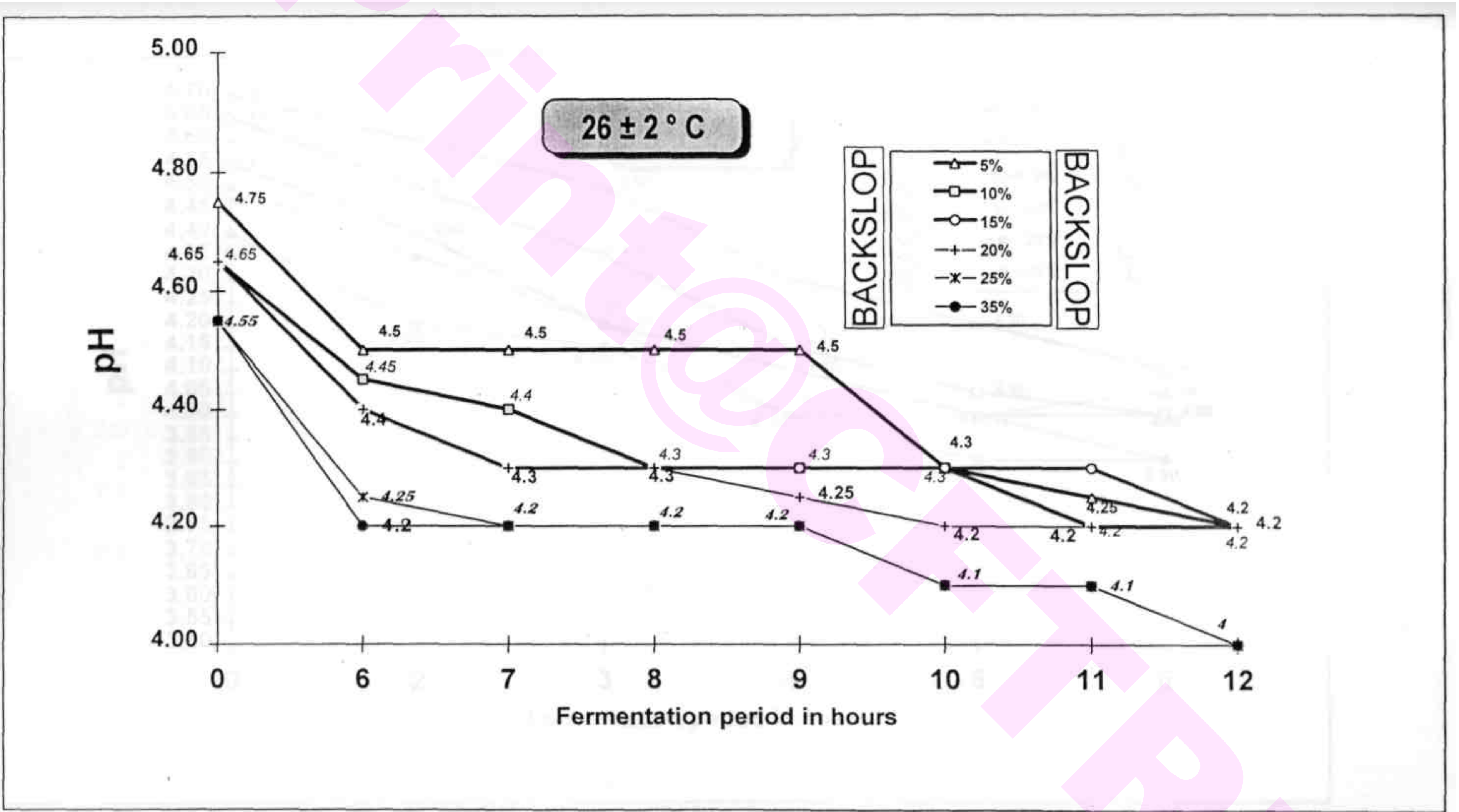

Fig 1a: Effect of temperature on pH with addition of different percentages of BACKSLOP Material

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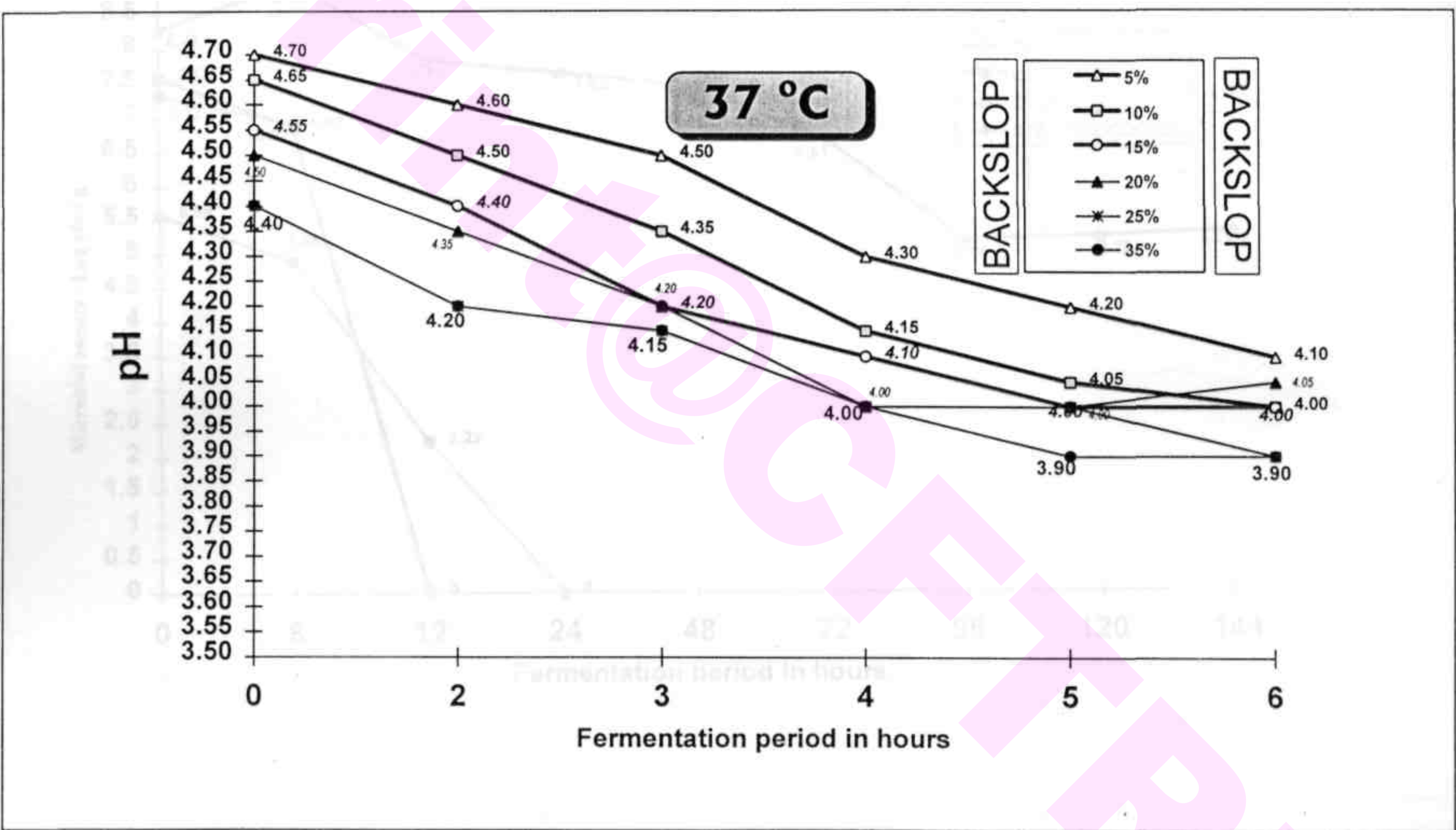

Fig 1b: Effect of temperature on pH with addition of different percentages of BACKSLOP Material

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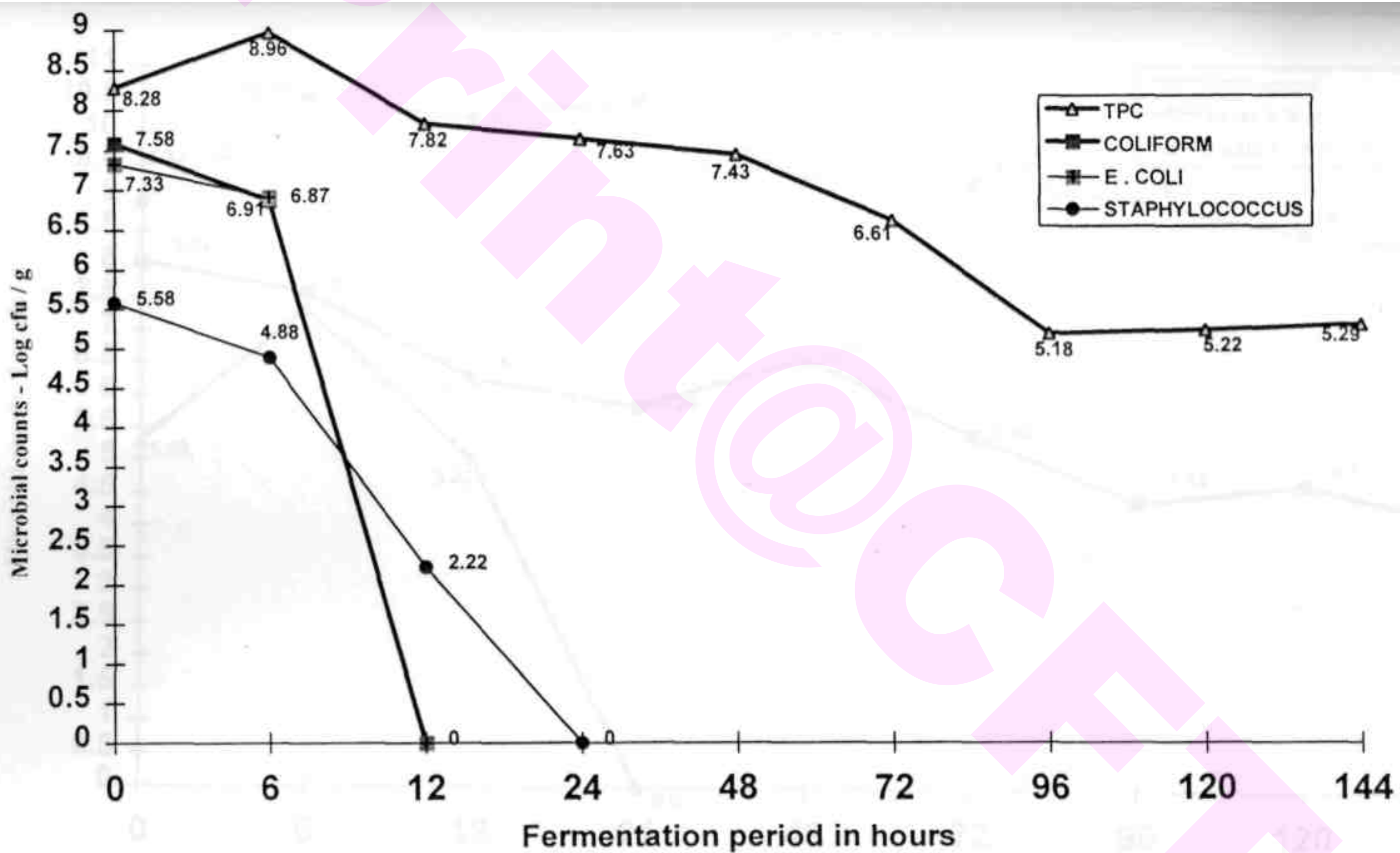

Fig 2a: Microbial Succession of poultry intestine during fermentation with 10% Molasses and 0.5% Propionic acid at 37 ^{0}C

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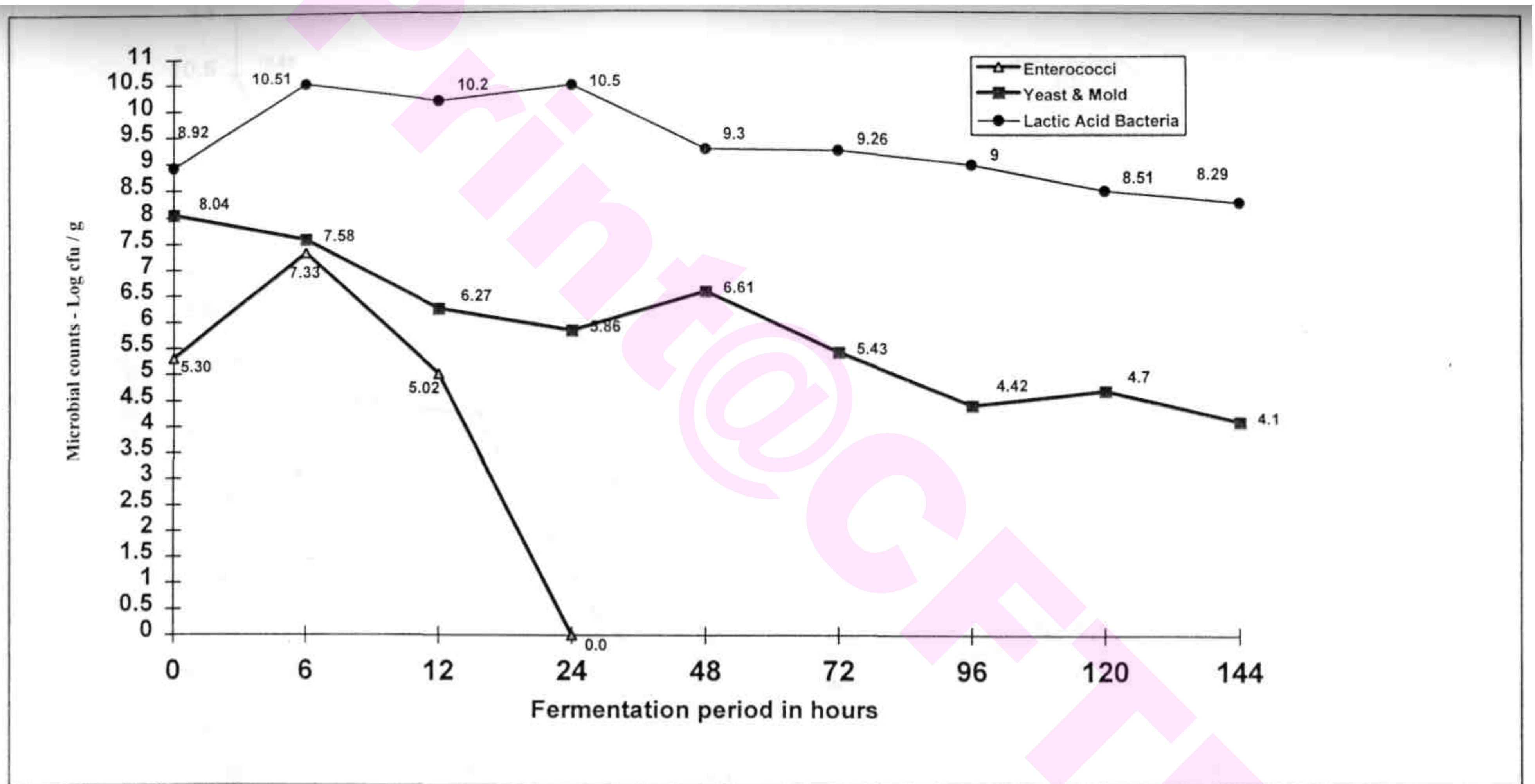

Fig 2b: Microbial Succession of poultry intestine during fermentation with 10% Molasses and 0.5% Propionic acid at 37 ^{0}C

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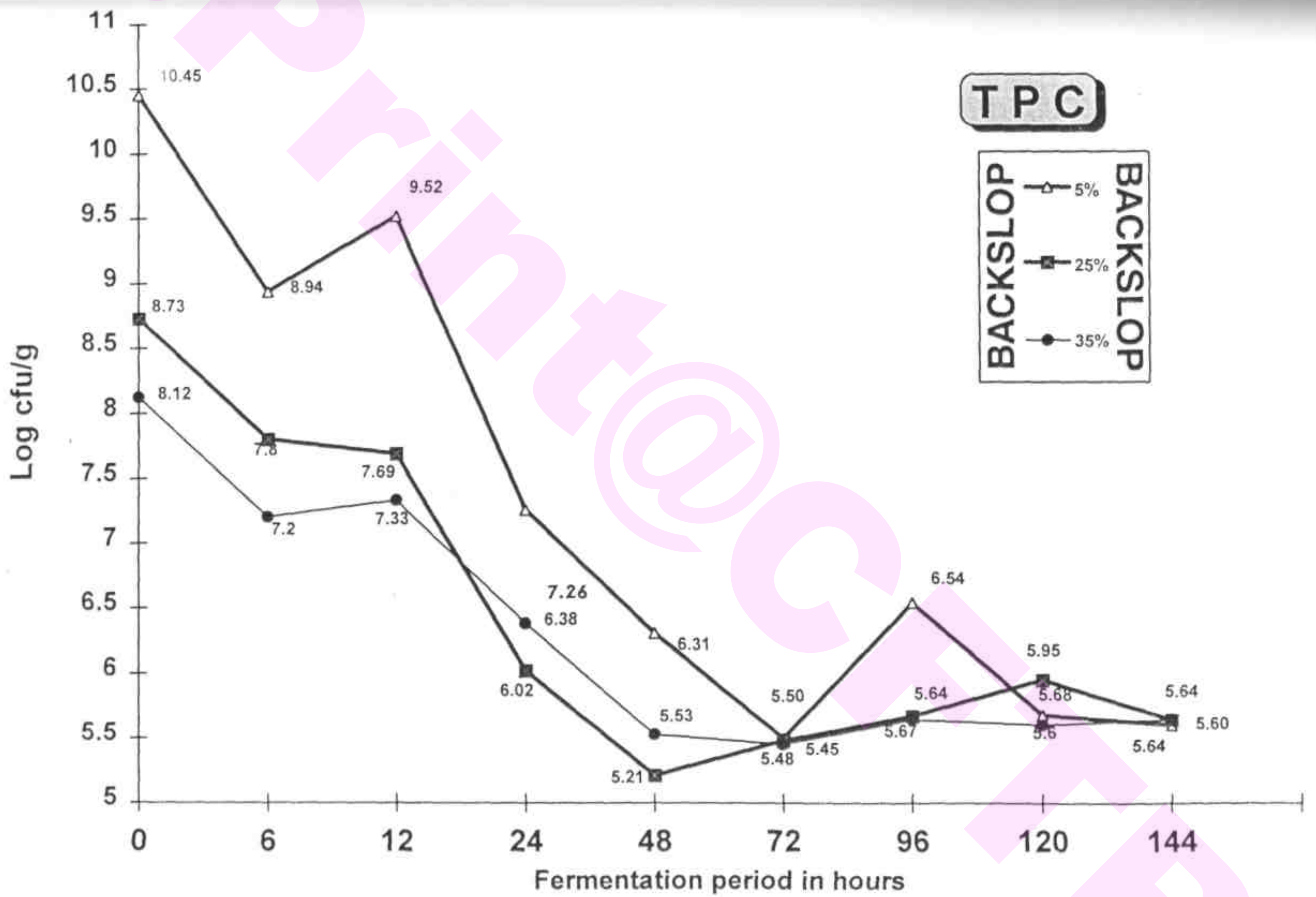

Fig 3a: Effect of different percentages of BACKSLOP on fermentation of poultry intestine

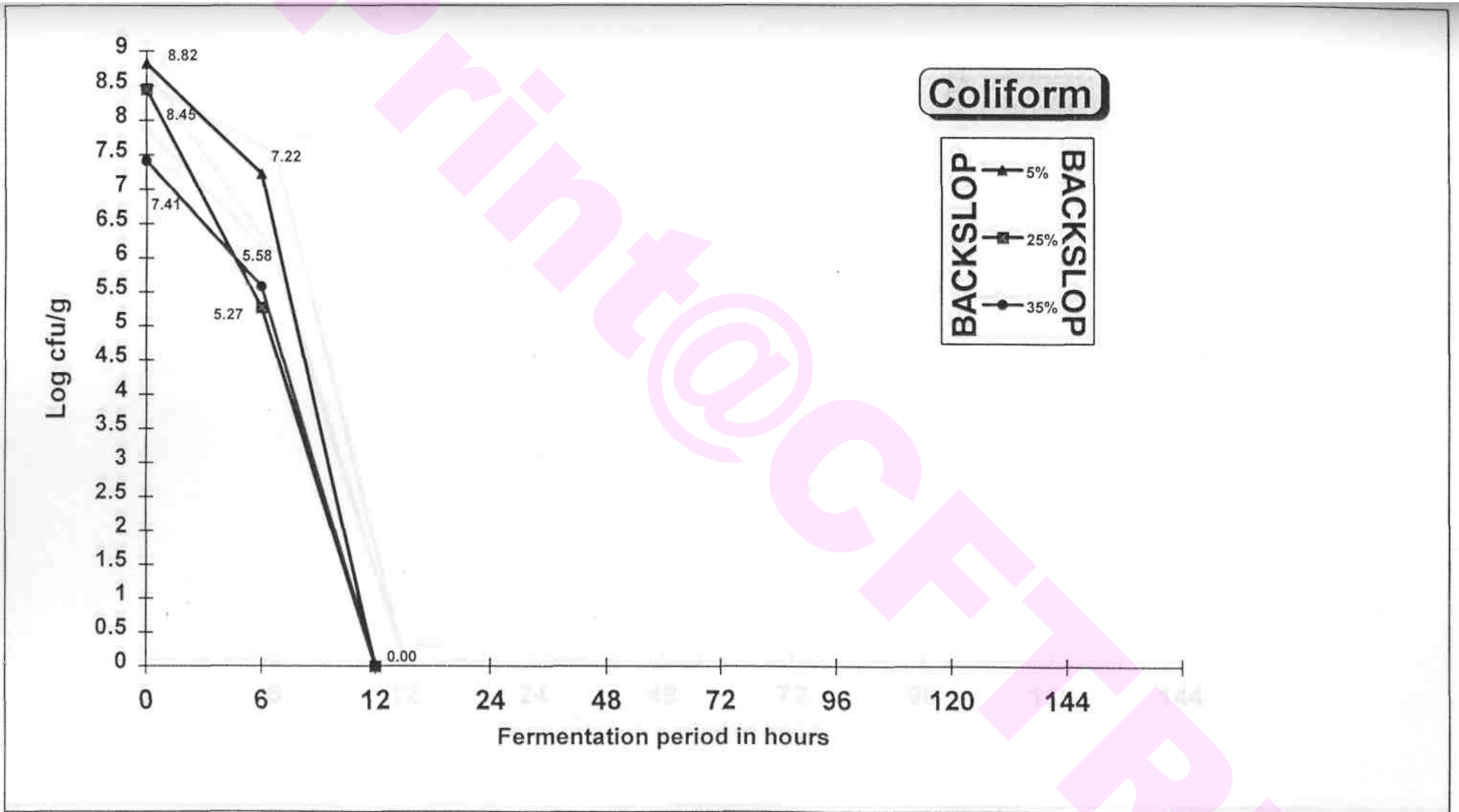

Fig 3b: Effect of different percentages of BACKSLOP on fermentation of poultry intestine

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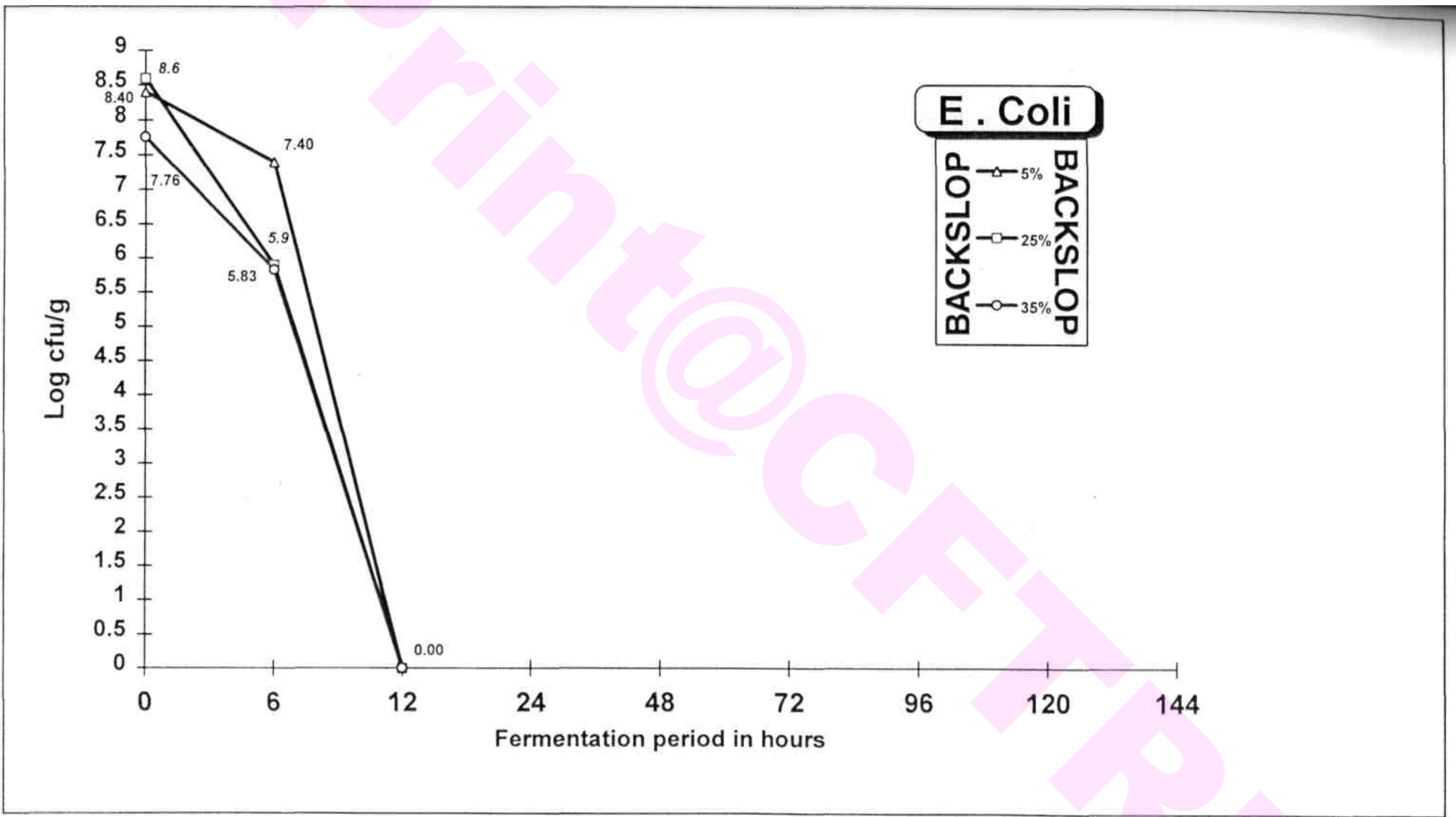

Fig 3c: Effect of different percentages of BACKSLOP on fermentation of poultry intestine

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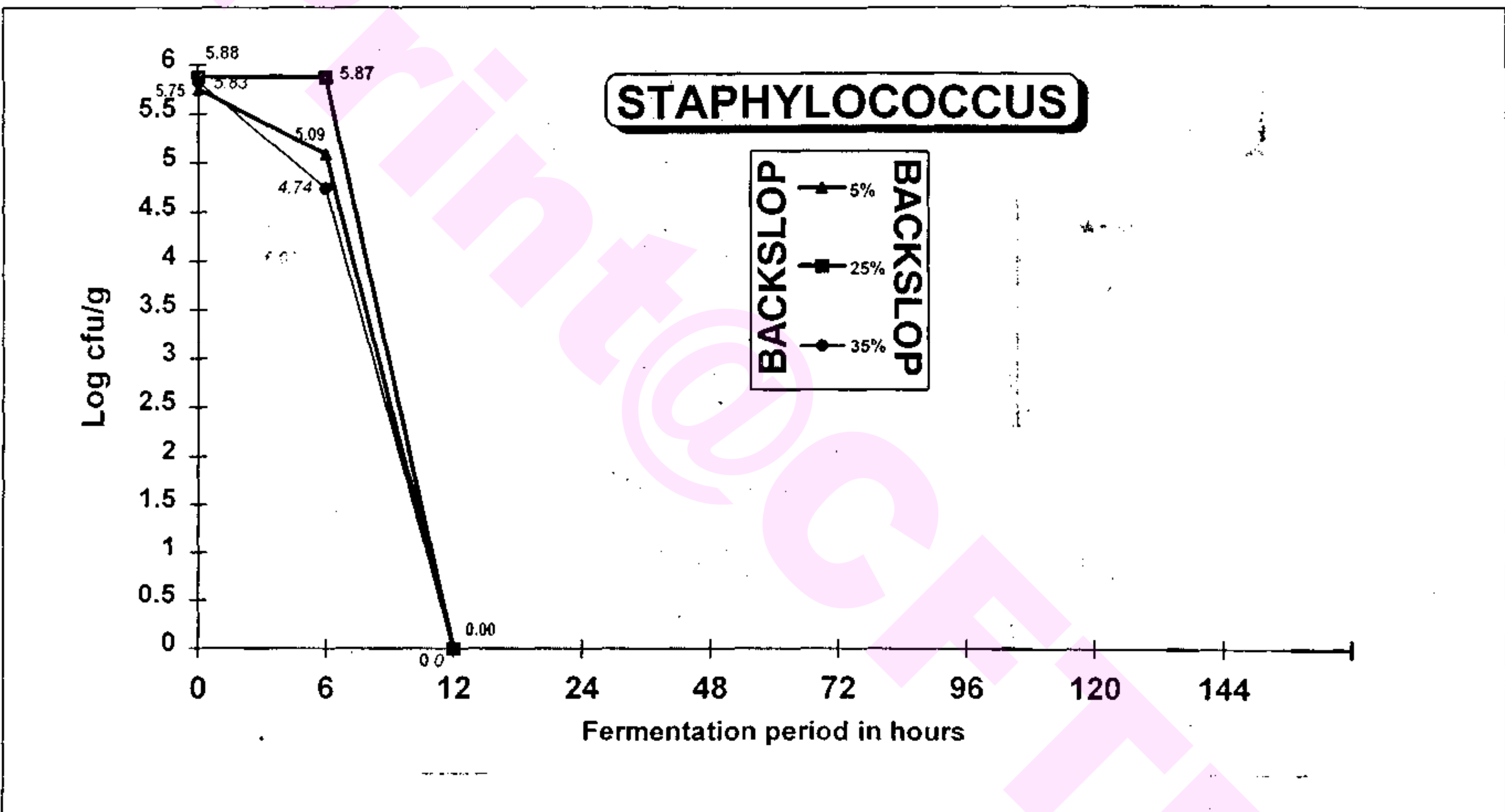

Fig 3d: Effect of different percentages of BACKSLOP on fermentation of poultry intestine

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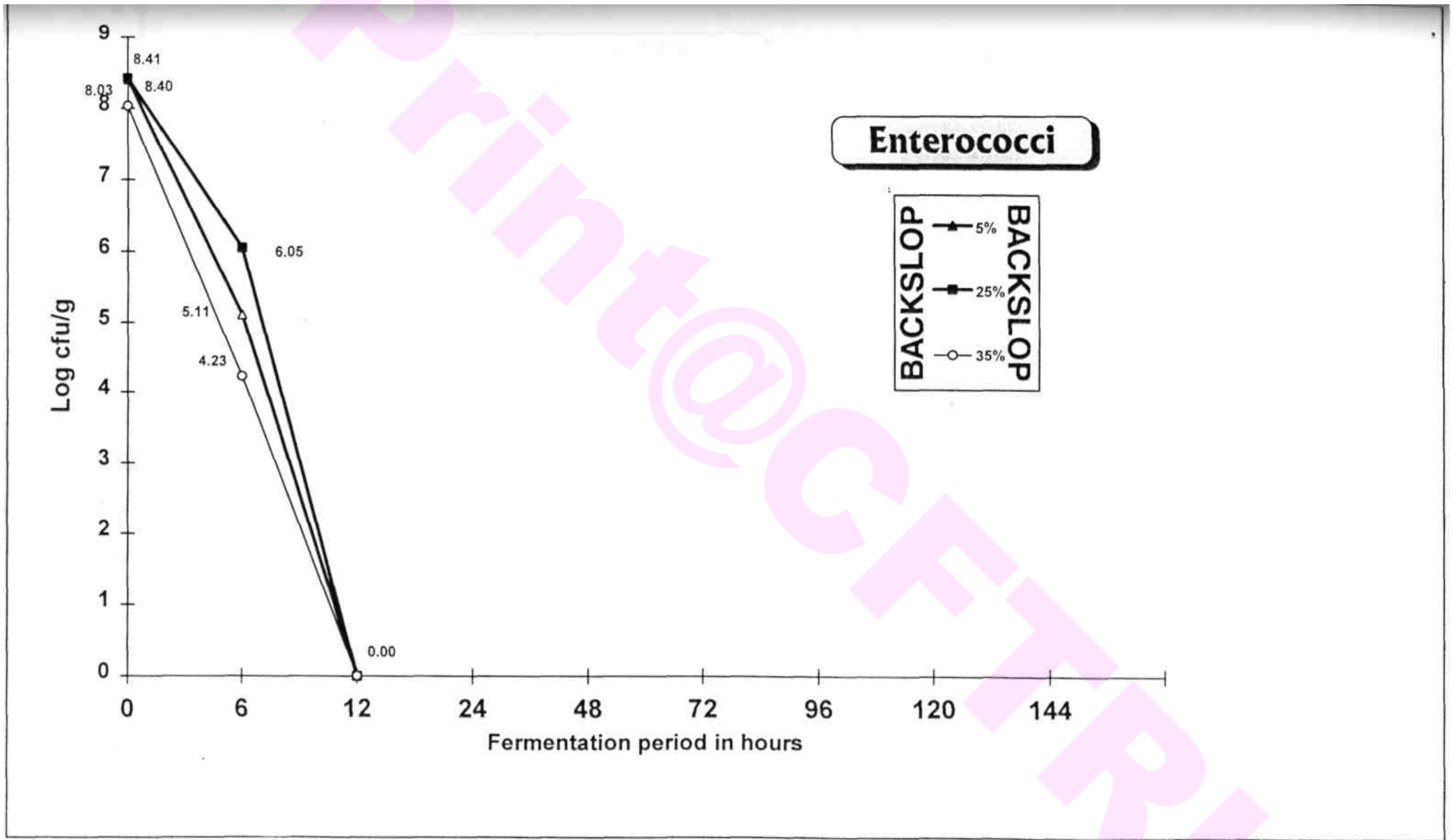

Fig 3e: Effect of different percentages of BACKSLOP on fermentation of poultry intestine

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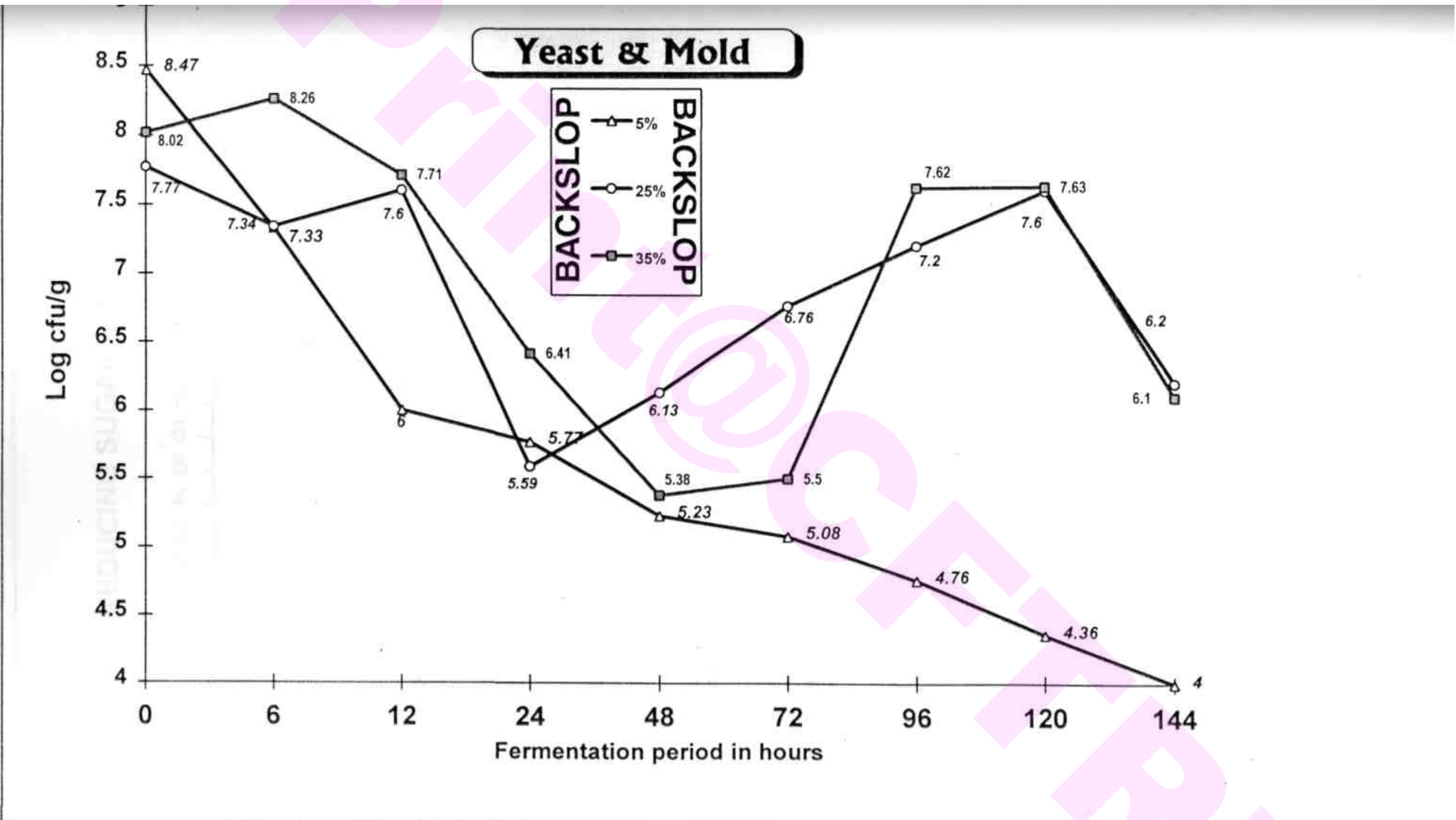

Fig 3f: Effect of different percentages of BACKSLOP on fermentation of poultry intestine

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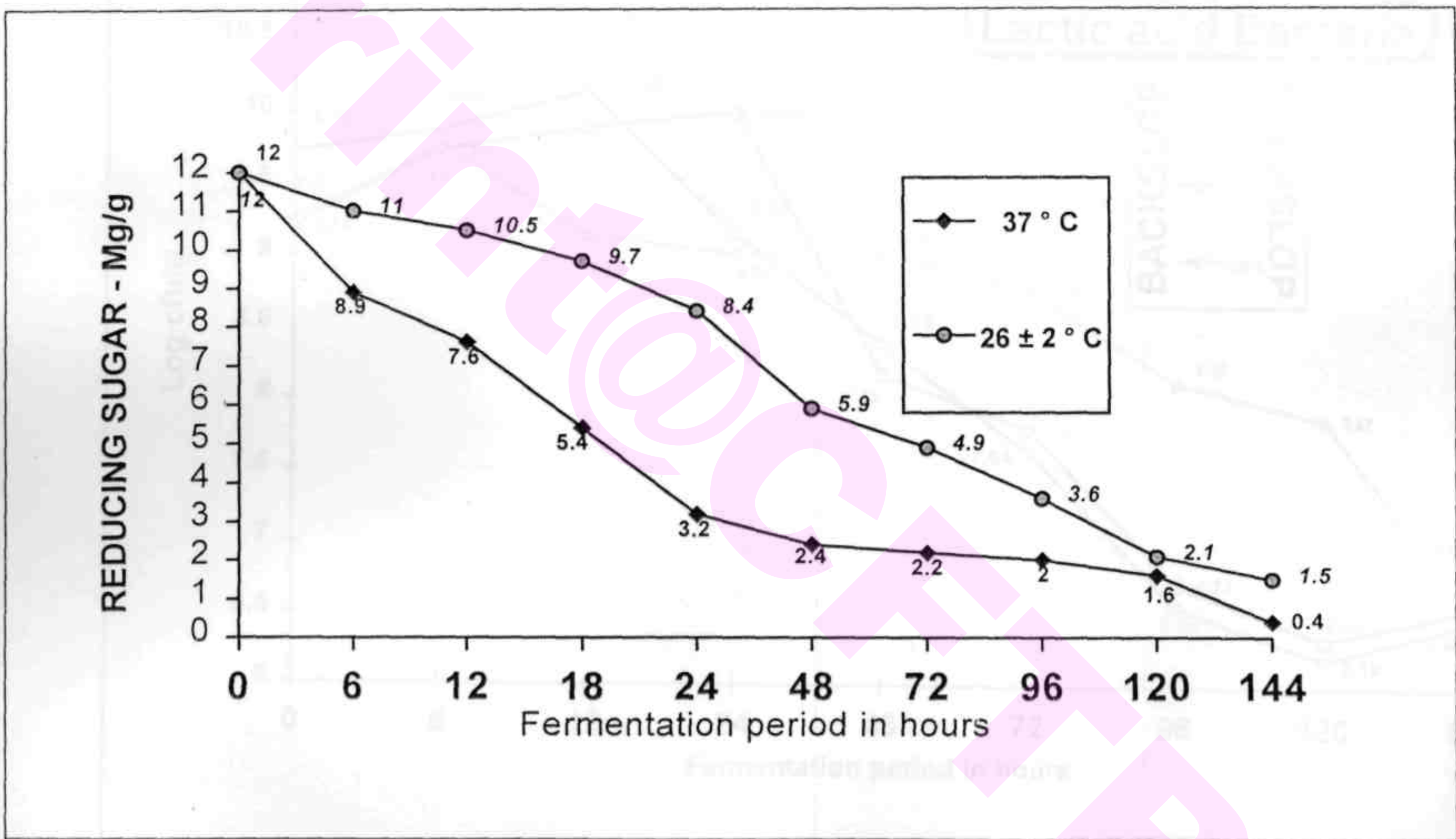

Fig 4: Fall in Reducing Sugar content in poultry intestine during fermentation

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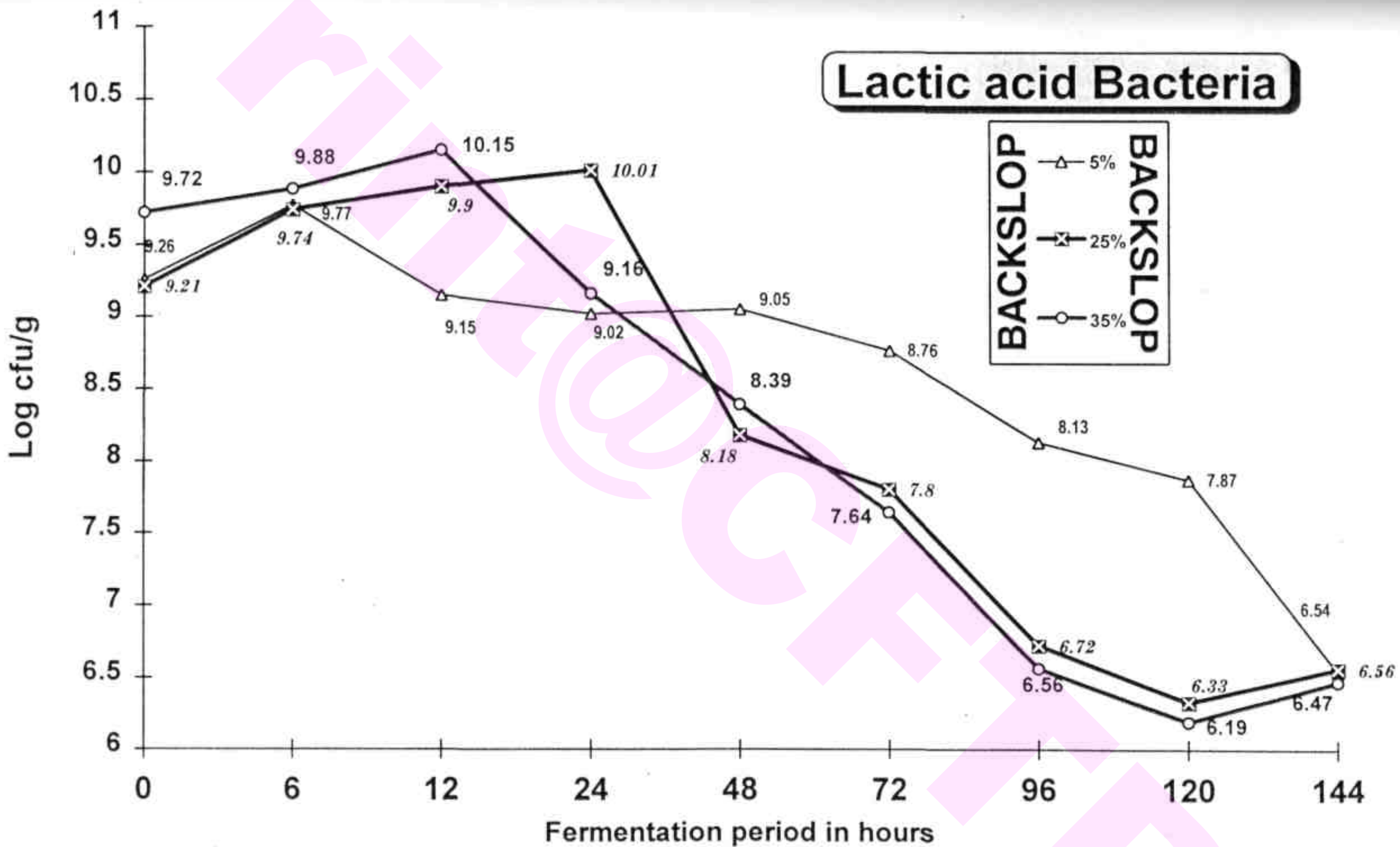

Fig 3g: Effect of different percentages of BACKSLOP on fermentation of poultry intestine

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Chapter 7

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USE OF THE FERMENTED SILAGE FOR THE FORMULATION OF FEED. ITS EFFECT AS DIET IN BROILER CHICKENS.

INTRODUCTION

Silage is not a new product. The process of ensilage has become a common practice for the production of animal and poultry feed from animal wastes. Ensilage process as dealt earlier involves the use of chemicals or microorganisms to hasten the process and bring about the conversion of animal waste into a value added product which can be given to poultry as feed.

A day to day surplus of poultry waste can occur in many poultry industries. Its presence causes environmental pollution and more so its removal from the poultry industrial premises invovles labour, time and money. Therefore the precise and simple technology for utilization of this proteinaceous non- marketable material would be its conservation by ensilage as studied previously in the present investigation. The outcome of this fermentation process would be a useful ingredient in poultry feed which can replace fish meal.

Poultry silage has a number of advantages over fish meal.

0 Relatively small quantities can be handled from day to day.

0 Little capital equipment is needed.

0 Only low level of skill is required.

0 The process could be repeated daily and product / feed formation could be attained fast to meet the daily requirements of the poultry industry.

A large number of feeds have been formulated from various animal wastes. Shrimp waste has been found useful in preparation of protein powders.

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(Lekshmy et al, 1989). Fermented fish waste as poultry feed. (Emmanuel et al, 1988; Tatterson, 1974, Arul James, 1966) A combination of maize-soyabean meal forms an excellent ration for chicks and broilers along with animal protein like blood meal or meat meal or defatted silk worm pupae meal (Gopalakrishnan *et al,* 1982). Erdos, (1984) reported that poultry by-product meal was suitable in broiler rations. Shrivastava and singh, (1985) indicated that poultry offal meat can satisfactorily be used in chicken diets. Kim and Rhee (1977) fed diets to broiler chicks containing fermented dehydrated poultry waste. Work of Hazarika *et al,* (1993) reveals that poultry by-product meal (PBM) blood meal (BM), hatchery byproduct meal (HBM) were prepared to feed to poultry. Studies on poultry offal meals have also been conducted by Potter and Fuller (1967); Jackson and Fulton, (1971). Work on Poultry by-product and hydrolysed feather meal have been conducted by Bhargava and O.Neil (1975). Poultry waste as feed has been investigated by Pieczynski *et al,* (1985); EL Boushy *et al,* (1985).

Considerable data have been accumulated concerning the value of fish meal as an ingredient for broiler diets. The literature concerning the value of fish meal has been extensively reviewed by Menge *et al,* (1952). Branion and Hill, (1953); Rusmussen *et al,* (1957) and Harns *et al,* (1961). In commercial broiler diets, the amount of fish meal is usually limited due to the possibility of producing off-flavours in the meat (Fry *et al,* 1965)

Fish meal is a common source of animal protein in poultry rations. Fish meal is in short supply and a good quality fish meal is very expensive, therefore alternative feed ingredients to replace fish meal are to be found.

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Utilization of animal waste and slaughter house by-products is extremely vital conomise the cost of feeding. At present, in India most of the animal by-ucts are getting wasted or under utilized. India has to use crores of rupees ally due to non-utilization of animal by-products (Hazarika et al, 1993). acement of costly protein feed supplement by utilization of animal by-products not yet received due attention. Therefore, scientific utilization of these by-jcts as a potential source of animal protein should be initiated to recover that colossal loss.

The objective of this chapter was to test the stability of the poultry intestine (in animal rations by partially replacing common animal protein "fish meal" their diet and then to study the growth and carcass characteristics in poultry.

MATERIALS AND METHODS

Preparation of Silage

Poultry intestine procured from the local market was homogenised with (w/w) molasses as a source of fermentable sugar as ã substrate; 0.5% propionic as an antimycotic agent and 0.02% ethoxyquin as an antioxidant. The genate was held at ambient temperature (25 ± 2°C) under microaerophillic tion and allowed to ferment within 24 hours. The pH of silage was below 4.2. was allowed to ferment another 24 h so that all pathogenic and spoilage organisms would be inhibited. This ensiled product was added with other feed lient as a replacement of fish meal.

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Diet formulation

Isonitrogeneous (21% protein) and isocaloric (ME = 2900 K cal /kg) diets were formulated. Fish. meal at 8% level was used in a control diet and two experimental diets replacing 25 and 50% fish meal were formulated using poultry silage. The silage was mixed with all ingredients and dried in an air flow drier at 55°C for 4 to 5 h to reduce the moisture content to below 10%. Dried feed in mash form was used in the feeding experiments which were conducted involving cobb broiler chicks obtained locally.

Experimental design

One hundred and twenty, one day old broiler chicks were distributed statistically in 12 groups of 10 birds each so as to get the same average live weight. Three diets (A,B and C) were assigned to four groups each (quadruplicate). `A' group diet consisted of 25% and `B' group diet consisted of 50% replacement offish meal by poultry intestine silage. Birds were raised on deep litter. Birds were fed with previously weighed feed, which were daily recorded before putting in the feeders and the body weight and feed intake were monitored at fortnightly intervals for eight weeks in order to obtain the growth performance of chicken. Water feeders were cleaned morning and evening before filling with water. Water was provided at libertum. Medicines were administered only on the advise of the veterinary doctor.

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Slaughter **and** dressing

At the end of 8 wks feeding period, all the birds were starved for 18 to 20 h prior to slaughter. Water was provided abundantly. Slaughter and dressing were carried out by cutting Jugular Veins, bleeding for 2 minutes, scalding at 58 - 60 °C minutes, singeing and evisceration. The eviscerated birds were washed mghly, drained and placed individually in LDPE bags, washed and cleaned meats were packed separately and stored under frozen condition for future **quality studies** Frozen carcasses (4 birds from each diet group) were thawed by holding at ambient temperature (25 ± 2 °C). Deboned meat from breast and leg portions were minced separately in Hobart Mincer. A portion of minced meat was used for proximate composition (AOAC, 1990) and other portion cooked and organoleptic quality attributes were attributed by ranking method (Mahendrakar *et al,* 1991).

Histological Studies

Liver, kidney and intestines of birds of each diet group were fixed in formaldehyde solution, the tissues embedded in paraffin, cut into 5 strips using microtome, stained by haematoxylin and eosin techniques (Girdley, 1960). Microscope photographs were taken of these meat organs in order to evaluate the experimental (A & B) along with control fed birds.

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Microbiological Analysis of Formulated Feed

Microbial profile of the feed were analysed of all three control 25% and 50% replaced diets. The profile _ analysed consisted of only pathogenic and spoilage microorganisms so as to evaluate the microbial safety of the feed. Microbial analysis pas conducted on the serial dilution technique and organisms were enumerated typour plating differential media and counting colonies after incubating at 37 °C for 24-48h. Aflatoxin analysis of inidividual ingredients for addition into feed, mainly corn and groundnut extract was conducted at AQCL, CFTRI, Mysore as they both are oil containing material easily attacked by aflaoxin producing organisms.

RESULTS

Feeding trials of poultry intestine silage to broiler chicks and their growth **and carcass traits:**

The effect of substituting fish meal with poultry intestine silage at 25% level and 50% levels on the growth and carcass traits are presented in Table 2, Fig. 1 and Table 3. Significant difference was observed between A diet containing 25% poultry intestine silage and that of control in gain in body weight (live weight) and carcass weight indicating superiority of A diet to B diet and even control rations. A similar trend was also observed with respect to percentage carcass yield and organ meats at the end of 8 weeks were feed intake was higher by 4.7% and simultaneously better growth was observed by 5.34%

On comparing B diet 50% replacement with poultry intestine silage with that of control diet as shown in Fig 1, that birds fed on B diet initially had a lower weight

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gain than control by 10%, but finally equalled that of control diet fed birds. Similarly carcass weight and live weight were lower than control diet birds. But percentage carcass yield and organ meats showed better yield than control birds, almost equal to the control diet fed birds. Meat: Bone ratio was same in all A,B and C diet birds.

It was observed from the results that better performance was obtained from experimental diet A birds and B diet birds equalled that of control diet fed birds. This clearly indicates that poultry intestine silage when substituted with fish meal had no adverse effect on the growth performance of birds or on carcass weight and percentage carcass yield. Cumulative FCR was found to be in the range of 2.1 to 2.3 in all the diets as shown in Table 2 and was not influenced by diet.

The mortality of birds was in the range of (2.5 to 10.0%) of poultry intestine silage diets (Table 2). Postmortem examination of visceral organs soon after death revealed that the death was due to Infectious Bursal Disease (Gumbaro disease) even though birds were vaccinated against IBD on the 18th day. The cause was due to outside contamination from other birds, obtained from the local market which was incidentally kept overnight adjacent to the rearing pens. This was the only main reason for the mortality. If it were due to poultry intestine silage in feed, the recurring mortality would be observed only in experimentally fed birds, but it is common among control birds also.

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Composition and Nutritive value of poultry intestine silage feed and feed fed birds:

From Table 2 it is observed that poultry intestine silage feed contained 20 to 23% protein (dry basis), 3 to 5% fat, 7.5% ash and a moisture content below 10% land it contains 2924 - 2966 k.cal; ME per kilogram.

The chemical composition of breast and legs have been analysed and it was found, that moisture content was high in leg than in breast muscle (5%), both in experimental diets as well as in control. Fat content in A diet breast was 9.94% while leg 7.0% in B diet, breast contained 7.71% and leg 11.9% fat, while control birds breast had a fat content of 6.5% and leg 9.37%. Protein content of A diet and B diet birds, was not found to be different from that of control, but was higher in leg muscle than breast muscle. Protein content ranged between 19 to 21% in breast and 21 to 23% in leg. Ash in A diet was 0.7%, B diet was 0.8% and control contained 0.8% in breast and leg respectively. The results are shown in Table 4. From these results it is evident that no adverse effect or difference in chemical composition is noticed between either control or experimental birds. Indicating 25% and 50% poultry intestine silage diets are in par and better than control diet and can be conveniently replaced by fish meal. Therefore meat quality was not influenced by the inclusion of silage in the diets for broiler chickens.

Mircobial Analysis of Formulated Feed

Microbial profiles of the feed were analysed and results obtained are presented in Table 5. Results revealed that feed is microbiologically safe. From the

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microorganisms isolated and enumerated only E.coli was present at log 2 in the experimental diets, while all other pathogens were totally eliminated at the feed level, thus preventing the birds as carriers for the transmission of pathogenic strains into the body of the consumers, which would otherwise cause detrimental effects. Thus silage from poultry intestine can be prepared for the production of a value added product like animal and poultry feed as all pathogenic and spoilage organisms have been inhibited by the process of fermentation.

Histology of organ meats

The plates 1 to 4 reveal no structural variations, in the visceral organs of experimental as well as control birds. Malformation or sclerosis of cells, shrinkage or swelling due to toxic substances of epithelical cells of intestine were not noticed. Cell size and shape showed no defect of any kind. Thus histologically also experimental diets had no effect on broiler chicks. Once again it can be stressed that poultry intestine silage can be effectively substituted for fish meal upto 50% level in broiler chicken diet.

DISCUSSION

In a tropical country like India, a poultry intestine silage product could offer tonsiderable benefits as a means of converting waste poultry intestine into animal feed, particularly where expensive fish meal must be imported. However, it must be established that the product is not a potential hazard to animals and that it is nutritionally adequate as a feed supplement. This was the main objective of the

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feeding trials and the results obtained are appealing in line with the meagre piown literature on the use of poultry by-products as an animal feed.

Formulation and preparation of feed has to be done with extensive care in order to balance all ingredients. Protein and energy are the most important in order to gain efficient poultry production. Care has to be taken during the fermentation and post fermentation processess.

A number of publications deal with the use of fish silage as an animal feed but few give details of the feeding trials. Lisac (1961); Arnesen and Emersson (1967); Sikorski *et al,* (1969); Luscombe (1973); Jensen (1973) and Smith and Adamson (1976) have all reported on the successful feeding of acid silage to pigs and for poultry. Silages prepared by lactic acid fermentation of fish have been tested by Nilsson and Rydin (1963) and Krishnaswamy (1974 - personal communication), on the other hand, found weight gains with poultry to be lower than with control diets aid silage products were more suitable as feed supplements than as complete diets. Products made by enzymatic hydrolysis have been reported to give feed efficiencies lower than these with control diets (March and Biely, 1961, Sripathy *et al,* 1963; Higashie et al., 1965)

In the present study it has also been observed that lactobacilli are the predominance species which has been found in poultry intestine fermentation. Though it is not of the homofermentative type of fermentation, lieterofermentation is likely, and hence production of lactic acid to some amount. It can be ensured due to the presence of <u>Lactobacilli</u>. It may be the presence of lactic acid in the fermented silage. When fed to the chickens helps lower the intestinal

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pH, since reports of Biswas and Samanta (1993) reveals lactic acid in feed lowers intestinal pH, it creates an unfavourable environment for the pathogenic organisms ike E.coli. Staphylococcus, Pseudomonas, Salmonella, Shigella etc and supports lie proliferation of normal beneficial gut microflora which leads to establish a proper microbial balance. This microbial balance keeps the intestine in a healthy condition and favour's for normal functioning of the intestinal tract which ultimately insure proper digestion and absorption of the nutrients. This presence of lactic acid nd its effect on intestinal pH could explain to some extent the results in the present tudy which led the birds to show efficient growth rate and an increase in carcass ield (25% - A feed and 50% - B feed showed no adverse effect when compared rath control. Therefore it can be considered that, upto 50% of poultry intestine silage could be conveniently utilised as an ingredient in poultry feed, by replacing fish meal.

Another important observation found in fermented fish scraps fermented herring and in the herring fish meal is the difference in amino acid composition which probably resulted from the inevitable supplementation of fermented products with the amino acids of the yeast or the bacterial culture used in fermentation. (Emmanuel and Jeong, 1988). Hassan and Heath, (1987) suggested that fermenting fish or fish waste increases the soluble nitrogen content because the complex protein structure is degraded and also increases the level of free amino acids and short chain peptides. Thus, these changes are expected to improve the digestibility of fish silage or fermented fish products. This may be the trend, likely to take place in all fermented animal by products.

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Though the most important animal protein supplement incorporated in the poultry ration is fish meal. High quality fish meal is among the most valuable ingredient. It is the costliest item of feed constituent in the ration. Considerable data have been accumulated concerning the value of fish meal as an ingredient for broiler diets. The literature concerning the use of fish meal has been extensively reviewed by Menge *et al,* (1961) But firstly in commercial diets for broilers the amount offish meal is usually limited due to the possibility of producing off-flavours in the meat (Fry *et al,* 1965) and secondly the cost of good quality fish meal ranges from Rs. 3000 - 3500/tonne. One of the disappointing poor quality fish meal which is an important protein supplement in poultry feeds (Gopalakrishnan and Lai, 1982). Thus from the results obtained by using poultry intestine silage as feed ingredient in broiler diet does not bring about off-flavour though used at 50%. Poultry intestine is cheap as it is a waste product at the same time high in protein. The importance of protein in poultry ration cannot be set aside, they are required in large amounts next to energy. The very young chicks requires more than 20% in the ration. (Gopalakrishnan and Lai, 1982). Since, protein sources are mostly costlier than energy sources, a judicious selection of feed stuff becomes imperative. A wide range of protein supplements of animal origin is available. But are these wastes microbiologically safe and its chemical composition suitable for feed? This question has been answered in the present work of poultry intestine having undergone the process of ensilage and effectively utilized as a protein supplement in comaprison to fish meal as ingredient in broiler diets, resulting in good growth performance of chickens.

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Several experiments were conducted at the university of Georgia to determine the effect of an antioxidant on the nutritional value of poultry by product meal and meat meal. In the experiments reported by Kirkland and Fuller, (1969 and 1970), ethoxyquin treatment was shown to reduce the rate of oxidation of the residual fats in these materials indicated by iodine number (I_2NO). Samples of poultry offal fat were found to be remarkably stable and no changes in iodine number, Iperoxide value or fatty acid composition occured whether or not ethoxyquin was added at any level. Ethoxyquin offered excellent protection from changes in fatty acid j composition as shown by GLC analysis. The use of ethoxyquin as an antioxidant offered greater protection against oxidation, than other antioxidants (Brown *et al,* 11957; and Lea *et al,* 1960) therefore used in the present study.

A review on the proximate composition of poultry by Keshri et al (1989) reveals meat has a number of desirable nutritional properties. It contains several classes of nutrients. Poultry meat contains high proportions of protein than other meats but the fat content varies according to age, sex and species in carcasses. Carcasses of younger birds contain more moisture than older birds.

Marini and Gyles (1973) reported the moisture contents in 8 week old chicken broiler's breast as 73.37% for males and 72.40% for females. Robertson *et al,* (1966) observed 23.29 to 24.10% protein in white meat of broilers, where as the protein content of red meat was only 19.70 to 20.80%. The results of moisture and protein was similar to the present findings.

Singh and Essary (1974), however, stated that age and sex had no significant influence on percentage moisture in raw or cooked breast and thigh muscles.

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However muscles from 4 to 6 week old broilers contained higher moisture content than did muscles from 8 to 10 week old birds. The percentage fat in the breasts and thighs was not affected significantly with age but between sexes. Chambers *et al.,* (1981) found in modern chicken broilers the eviscerated carcass fat, expressed on wet basis, ranged from 14.26 to 19.04%

Ghosh *et al.,* (1982) concluded that the proximate composition of muscles of broiler chicken at 6 and 8 weeks of age were not affected although there was significant difference between thigh and breast.

Marini and Goodwin (1973) reported that the moisture content in 8 week old broiler chicken representing two commercial lines and four mating types derived from them, the breast moisture lower than thigh moisture in raw state (72.6 Vs73.7)

Danky and Hill (1952) observed that the growing chickens fed a high energy ration depostied more of fat on the carcass than did the birds on a ration with moderate amount of energy.

Donaldson *et al.,* (1956) investigated that the dietary energy levels in chicks influenced the body fat. As the ratio of energy to protein in the ration was increased the energy intake and carcass fat deposition were increased and the water content of the carcass decreased.

Summers *et al,* (1965) observed that the carcass protein of poultry was increased and the fat was decreased in a linear manner with increasing levels of

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dietary,protein. Conversely, increasing levels of dietary resulted in decreased larcass protein and increased carcass fat.

Kubana *et al,* (1972) found that the dietary energy level did influence the lody fat in chicks. Protein and ether extract content of carcasses increased with age of the birds and the moisture content decreased.

The composition and nutritive value of poultry by-product meals have been reported by Potter and Fuller (1967) that poultry offal meal contains 63.3% total protein, 21.3% fat, 7.2% ash and 92.3% dry matter. Poultry offal meal contains 3340 K, cal, ME per kilogram, 61.5% protein, 21.5% fat and 93% dry matter (Jackson aid Fulton, 1971). Jackson (1971) also found that crude protein in offal meal was 55.9% while in peruvian fish meal it was 63.4% As per the reports of Pieczynski *et al,* (1985) the true digestibility and protein efficiency ratio values for poultry waste were 85.0 to 89.6% and 1.32 to 1.64 respectively.

From the above references cited above and the present study it can be stated that poultry waste is a useful by-product of that throw away material, since it has the potency and the naturally available resources of protein and useful lactic microorganisms, which can make it easily convertable into a value added product, such as a feed ingredient which could substitute upto 50% of fish meal. Thus the results obtained from the feeding trials of broiler chickens fed with fermented poultry intestine silage revealed that waste can be effectively recycled and protein material valuably utilised

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Conclusion

The inclusion of 50 % ferementled poultry intestine silage in broiler chicken ts as a replacement of fish meal had no pronounce effect on the growth / performance, carcass characteristics, meat quality, sensory attributes of meat and histological structure of the visceral organs.

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Table 1
Feeding Trials on **chicken** Using **Poultry Intestine Silage in Poultry Feed as a Replacement of Fish**

FEED FORMULATIONS

(Weights in Kg)

Ingredients	A (25%)	B (50%)	C Control
Fish meal	6.0	4.0	8.0
Poultry intestine silage	2.6 * (10.0)	5.2 * (20.0)	-
Maize	49.7	49.1	51.1
GNE	20.0	20.0	20.0
RRB	10.0	10.0	10.0
DORB	6.0	6.0	5.0
SFE	3.2	3.2	3.0
Mineral Mix	2.0	2.0	2.0
Salt	0.5	0.5	0.5
S.F. Oil	-	-	0.4
Total	100.00	100.00	100.00

Chemical **Composition**
%) estimated)

Ingredients	A (25%)	B (50%)	C -Control
Moisture	7.22	8.13	10.15
Ether Extract	3.4	3.0	5.52
Protein	22.50	20.79	22.35
Ash	8.57	7.71	9.57
ME, K cal/Kg (Calculated)	2924	2966	2914

* 10.0 or 20.0 kg wet silage correspond respectively to 2.6 or 5.2 kg dry silage containing approximately 10% moisture.

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Table 2

Feed consumed, Weight Gain, FCR and Mortality (during 8 weeks)

FEED	WEEK	FEED CONSUMED PER BIRD (g)	WEIGHT GAIN PER (g) BIRD	FCR	* MORTALITY
	II	432.9	192.4	2.2	5
	IV	1487.6	749.7	2.0	2.5
A	VI	3191.1	1452.2	2.1	NIL
	VIII	4484.8	2105.6	2.1	NIL
	II	382.4	178.5	2.12	10
	IV	1364.2	605.4	2.2	2.5
B	VI	2681.7	1258.7	2.07	NIL
	VIII	3921.7	1759.6	2.15	NIL
	II	382.5	210.4	1.83	5
	IV	1580.2	731.9	2.1	2.5
C	VI	3195.4	1470.3	2.15	NIL
	VIII	4010.4	1858.3	2.2	NIL

* Mortality was found to be due to Infectious Bursal Disease (IBD)

(n=4 batches)

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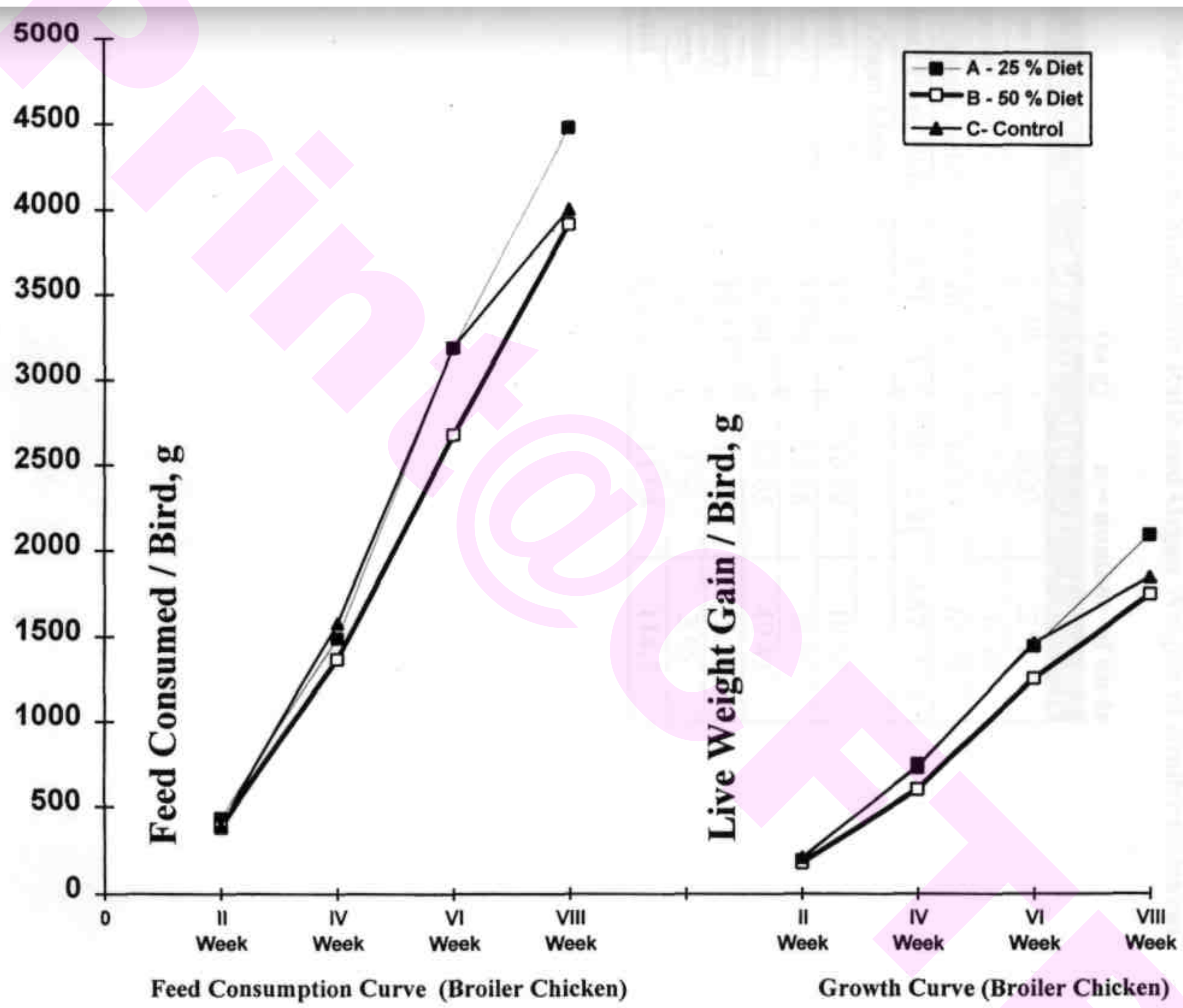

Fig. 1: Feed consumption and growth curve of Broiler chickens fed on poultry intestine silage

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Table 3

carcass Characteristics, Meat-Bone Ratio and Organ Weights of Broiler Chickens

(in g) **n = number of birds**

Feed	A (n = 37)	B (n = 33)	C (n = 34)
Live weight	2240	2020	2251.3
Carcass weight	1585	1385	1439
% Carcass yield	70.78	68.43	65.25
Meat : Bone Ratio	4.71 : 3.3	5.07 : 2.91	4.67 : 3.18
Organ Meats			
Heart	11.55	10.72	10.66
Liver	37.59	35.18	36.39
Gizzard	53.04	52.05	49.43
Kidney	16.47	16.87	15.58
Spleen	2.7	2.56	2.65
Total	**121.4**	**117.4**	**114.7**

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Table 4

Microbial **profile of feed using poultry intestine silage**

Micro organisms	Log cfu /g n = 6	Log cfu /g n = 6	Log cfu /g n = 6
TPC	4.91	4.91	5.3
E.coli	3.40	3.64	0
Coliform	0	0	0
Enterobacteria	0	0	0
Staphylococcus	0	0	0
Salmonella	0	0	0

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Table **5**

sensory characteristics of chicken muscles
No of panelists
8. Rank sums

Feed	A		B		C	
Muscle	Breast	Leg	Breast	Leg	Breast	Leg
Colour/ appearance	16.5	15.13	16.6	15.4	14.88	17.63
Odour	17.25	15.5	15.5	15.88	14.75	16.37
Aroma	17.5	16.75	15.13	15.13	16.5	
Overall Quality	17.0	16.38	14.88	15.25	15.5	

Mean of 4 replicates (n = 4)
Rank Sum : 13-22 (P< 0.05)

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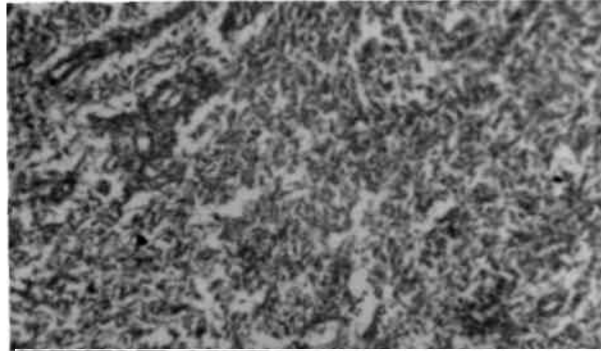
A B C

HISTOLOGY OF EXPERIMENTAL DIETS (25 % AND 50 %) AND CONTROL

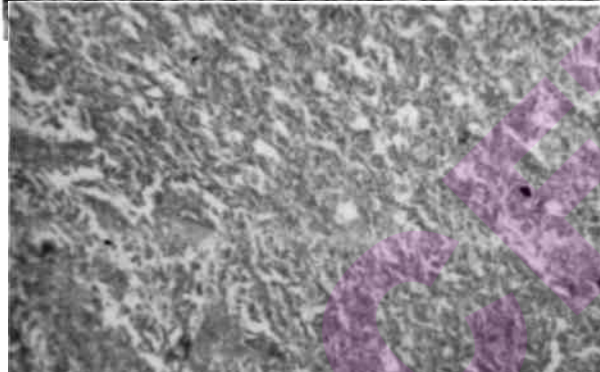
LIVER

—

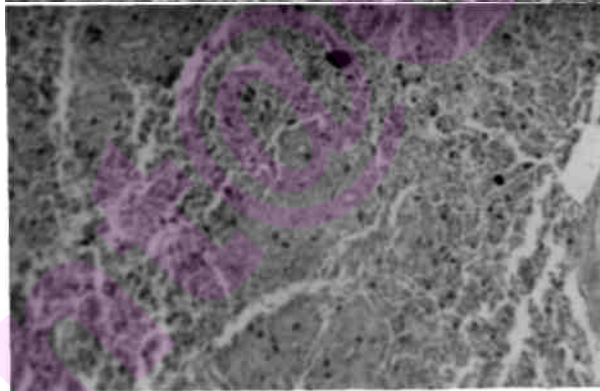
A



B



c



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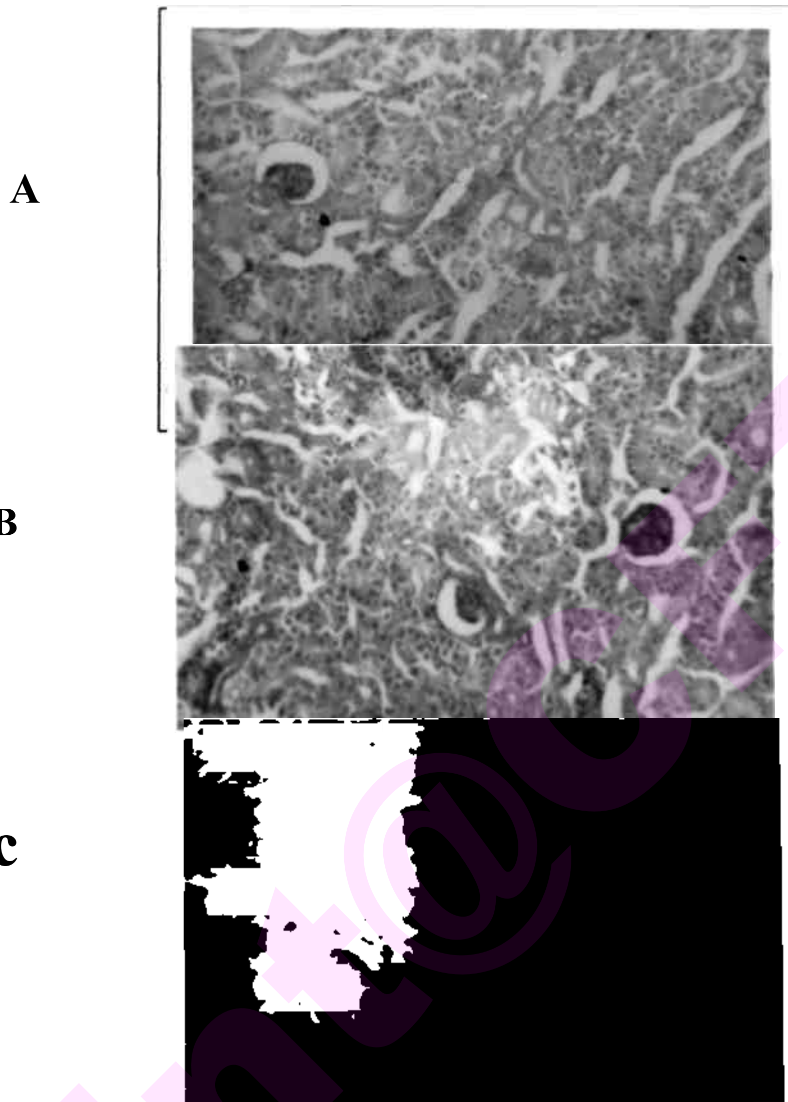
A B C

HISTOLOGY OF EXPERIMENTAL DIETS (25 % AND 50 %) AND CONTROL

KIDNEY



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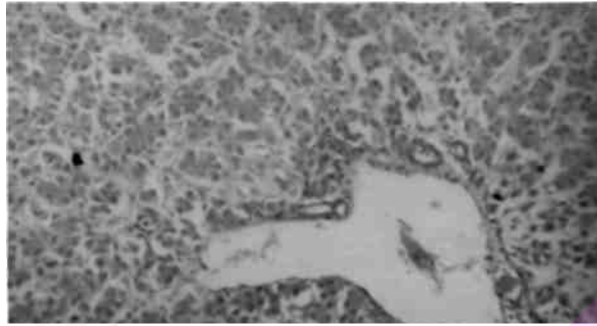
A B C

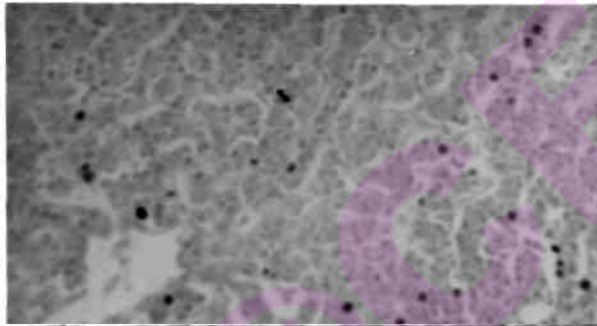
HISTOLOGY OF EXPERIMENTAL DIETS (25 % AND 50 %) AND CONTROL

SPLEEN

A



B



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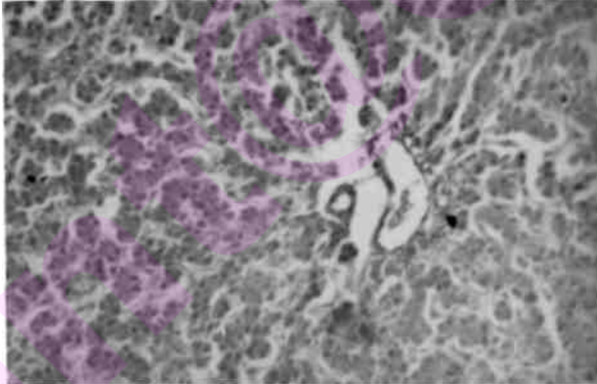

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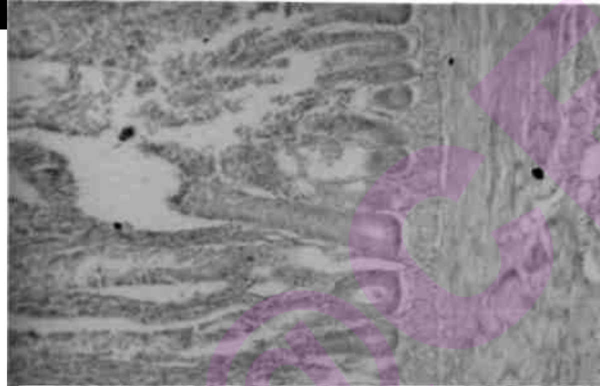
A B C

HISTOLOGY OF EXPERIMENTAL DIETS (25 % AND 50 %) AND CONTROL

INTESTINE

A

B



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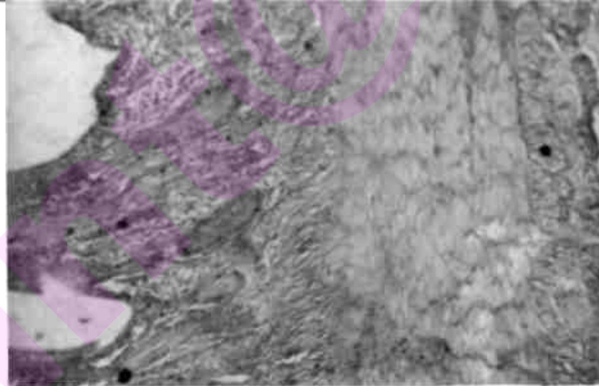

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Plate 5

1 day old broiler chickens

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Plate 6

4 weeks old Broiler Chickens

Chickens fed on

A. 25 %
Feed

B. 50 %
Feed

Control

B

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A

B

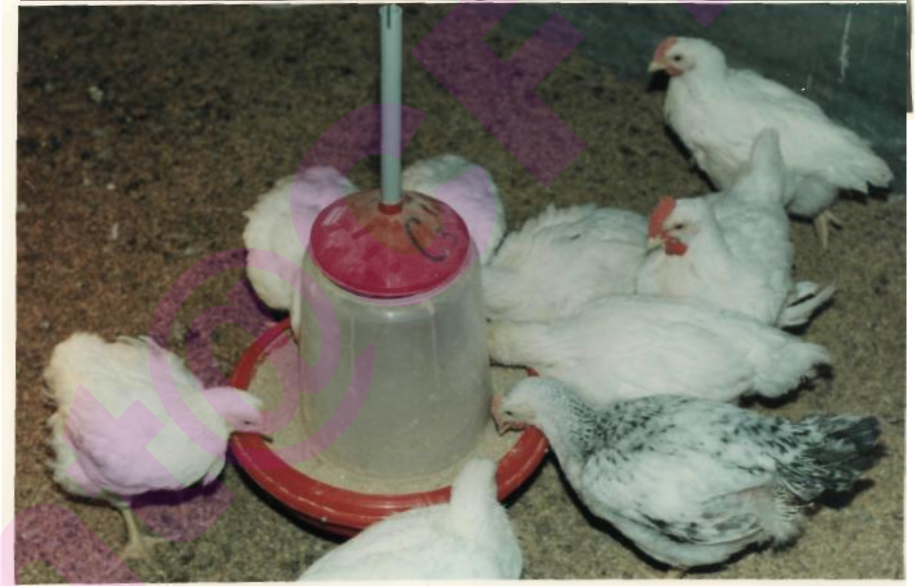

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Plate 7

8 weeks old Broiler Chickens

A. Fed on 25 % Poultry intestine silage

B. Fed on 50 % Poultry intestine silage

C. Fed on Control diet

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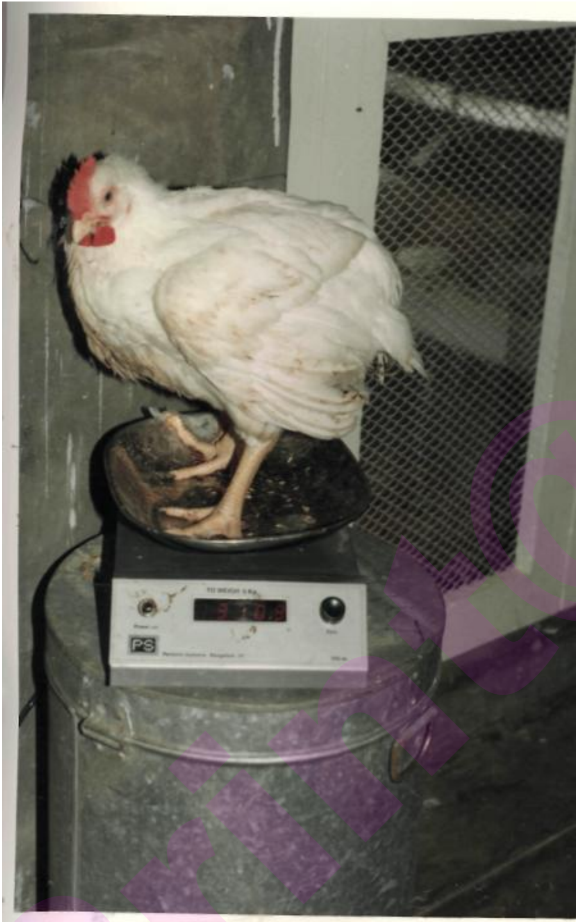

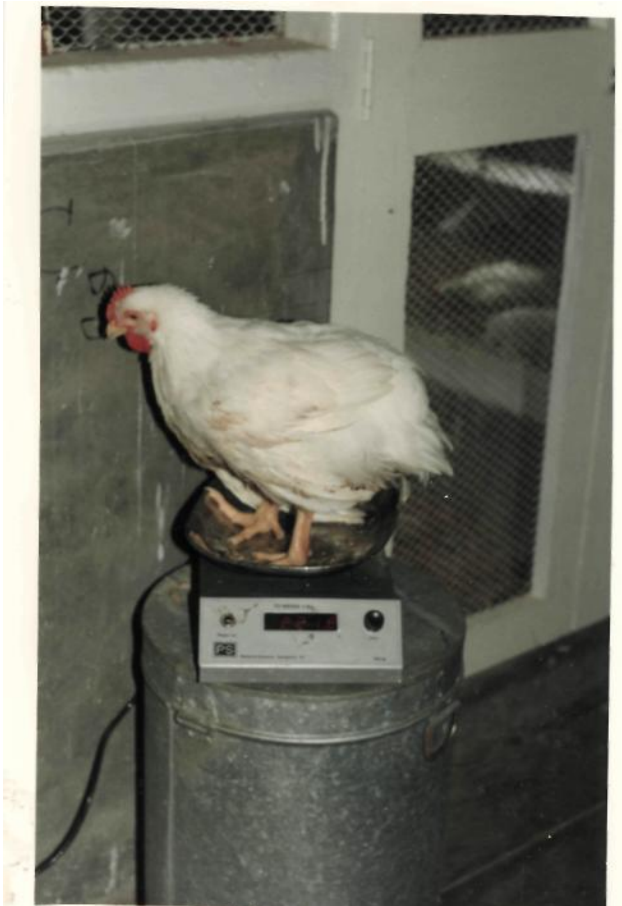

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Plate 8

Comparision of visceral organs between

A: 25% * P.I. Silage Diet	B: 50% * P.I. Silage Diet	C: Control Diet

Poultry Intestine

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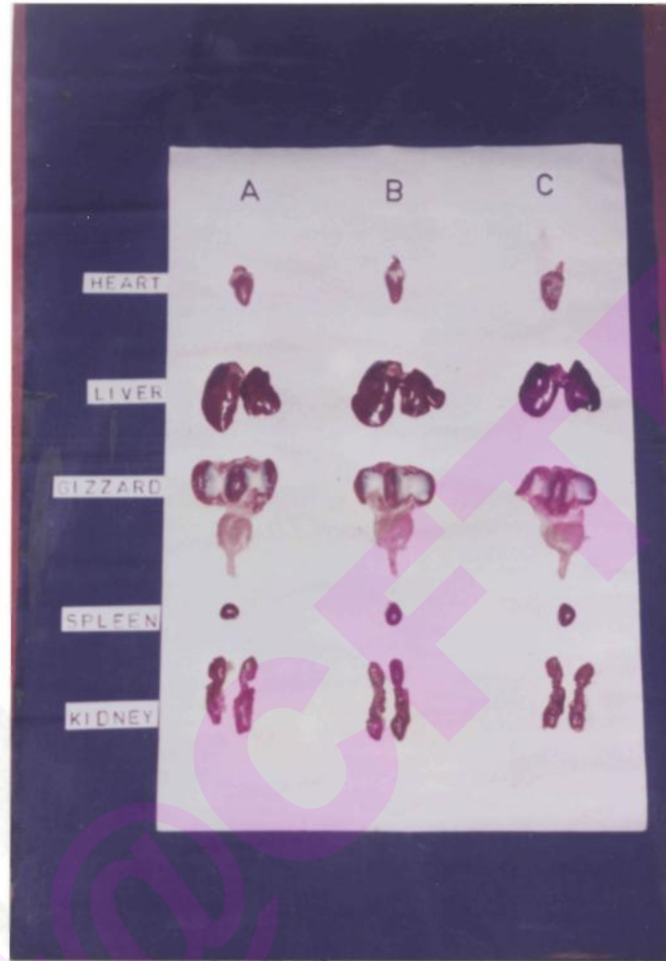

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Part - III

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CONCLUSION

The main objective, theme and aim of the present study was to utilise non-marketable proteinaceous poultry intestine and conserve it by a simple, economic yet dynamic technique like that of fermentation/ensilage to convert it into a useful product, for replacement offish meal, so that it could be incorporated into poultry feed.

The highlights during the study were as follows:

Poultry intestine though a fastideous, unpleasant odoured waste, has been found to be generated in tonnes every year. This usually found no value in day-to-day usuage as it is a waste, therefore it was thrown away, buried, mostly dumped and sometimes burned. All this leading to environmental pollution.

It has been found that poultry intestine is a rich source of protein, but lacks carbohydrates. It is its proteinaceous character that has drawn attention for its usuage, as a substitute for fish meal, which is at present, the only animal protein available for feed. Feeds prepared of high quality fish meal is very expensive. As most times, fish meal is not of good quality and therefore a source of pathogens hampering the feed industry.

Studies in the microbial profile of poultry intestine reveals an interesting note - the presence of large numbers of lactic acid bacteria (LAB). LAB are the primary sources used as starter cultures in various fermentation processes. Therefore poultry intestine has

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greater advantage over other animal offals, for natural fermentation process due to the presence of natural inhabitants of LAB.

From the study it was also, found •that washing of poultry intestine did not have any adequate advantage over the unwashed samples, but rather an uneconomic, time wasting technique.

Isolation and identification of various pathogenic and spoilage microorganisms has helped us understand why putrification in poultry intestine is very fast. It is found in the present study, Pseudomonas species were present which helped bring about its rapid deterioration and bad odour. The species of Pseudomonas that could be identified were: Ps. maltophila. Ps. cepacia. Ps. pseudomälleli. Ps. stutzeri. Ps. diminuta. Ps. mallei.

Potential pathogens such as E. coli. Salmonella and Staphylococcus were isolated from poultry intestine. E. coli serotypes are 0166, 0144, Rough, 064, 057, 0106, 039, 084, UT, 042, 054, 016, 020, 0116, 0103, 0100, 0101, 0157, 081, 0154, 062, 027, 03, 0146, 0147, 0132, 0159, 0134, 0169, 0116, 09, 0129, 021, 011, 0130, 045, 0100. Salmonella serotypes are S. typhimurium. S. cerro. S. gallinarium. S. enteritidis. S. virchow.

LAB has brought about the inhibition of pathogens and spoilage organisms which are undesirable in conserving poultry intestine. This was due to the unique characteristics of LAB species 1. to reduce PH 2. to produce acid 3. to produce antimicrobial substance.

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It has also been recorded, that Lactobacillus organisms are the dominant species in a poultry intestine fermentation; along with the association of yeasts. The major species of Lactobacillus identified are L.plantarum. L.acidophilus. L fermenti. Thus fermentation of poultry intestine is of the heterolactic type.

Suitable substrate and its optimum percentage - Molasses at 10% and antimycotic agent - propionic acid at 5% and an antioxidant common used ethoxyquine - 0.02% have been identified as appropriate for fermentation of poultry intestine, which did not effect the growth of LAB, acid production, decrease in pH during fermentation, but rather boosted the processes towards conservation of poultry intestine material.

An interesting fact, rapid fermentation noted by reduction of pH to the desired level of 4.2 regarded as safe level for all fermentation processes has been achieved in just 24h at ambient (26 ± 2 °C) and in 6h at 37 °C temperature with a large leap to 3-4h with the addition of 5% backslop material. This assures rapid elimination of unwanted microorganisms both pathogenic and spoilage. And also helped to retain the nutritive value of poultry intestine.

The present study has been able to achieve the target of effectively using poultry intestine silage upto 50% replacing that of fish meal in broiler chicken diet, without any adverse effects on the growth performance of chickens, FCR value and meat quality.

In conclusion the present study study resulted in the development of an appropriate, economic yet simple technique - fermentation ensilage for conserving the

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protein rich poultry intestine. The study further highlighted that poultry intestine ensilage can partly replaced fish meal in the diet of broiler chickens. Effective utilization of poultry intestine could prevent environmental pollution and boot up the country's economy.

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1. Doris. M. Shaw, Narashima Rao, D., Mahendrakar, N.S. and Dani, N.P. (1993). Studies in conservation of nutrients in poultry waste by fermentation process. Presented in II International Food Convention (IFCON -93), Food Technology for prosperity and Health, 7-12 Sept. 1993., AFST(I), CFTRI Campus, Mysore, Mysore, India.

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