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Some Microbiological and Chemical Properties of Poultry Wastes Manure After Lactic Acid Fermentation

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ABSTRACT

Poultry wastes manure was characterized for the presence of enterobacteria, enterococci, *Closrtidium* and *Salmonella*. In parallel, chemical determinations, including, ash, total nitrogen, non-protein nitrogen and total volatile nitrogen, were determined for un-treated and transformed bio-products. Wastes were humidified and mixed with 10% molasses; the mixture then inoculated with a starter cultures of *Lactobacillus plantarum* and *Pediococcus acidilactici* and incubated at 30°C for 7-10 days. While hazardous microorganisms (enterobacteria, enterococci, *Clostridium*, *Salmonella*) are present at high levels in un-treated wastes, a net decrease of all these microbial populations was observed in the bio-manure by the fermentation process. A steady decrease of the pH and low levels of total volatile nitrogen was observed. The application of lactic acid bacteria to poultry wastes manure led to solve the problem the great polluting power of these wastes as well as the bio-manure can be safer and cheaper.

Key Words: Poultry manure; Microbial status; Lactic acid fermentation; Biotechnology; Biopreservation

INTRODUCTION

Poultry wastes (litter & excreta) are generally added to the soil as a fertilizer. The final step in the poultry farming management strategy entails a big risk for the environment due to the nutrients and microorganisms contained in high concentrations in these waste materials (Hall *et al.*, 1994; Kingery *et al.*, 1994; Bagley *et al.*, 1996; Kherrati *et al*., 1998). Huge production of poultry wastes in Morocco (about 8000 Tons) has led to intensive research in the field of waste recycling and bioengineering of this solid waste treatment. In previous studies, manure has been used, directly or after transformation by chemical (acidification) or physical (heating) processes, in feed formulations (Ruffin & McCaskey, 1991; Rankins *et al.*, 1993; Bagley *et al.*, 1996). The treated material was used at low concentrations in feeds (Smith & Wheeler, 1979).

Chemical acidification, carried out by acetic, propionic acids or a mixture of both, was used to eliminate pathogens (Harmon *et al*., 1975). Other authors used formaldehyde for reducing the undesirable microorganisms (Caswell *et al*., 1975). Additionally, several works have described biological processes, such as anaerobic digestion (Nasr *et al.*, 1983; Desai & Madamwar, 1994; Patel & Madamwar, 1996; Salminen & Rintala, 2002), composting (Kashmanian Richard, 1995) and silage of poultry manure (Harmon *et al.*, 1975; Arndt *et al.*, 1979; Caswell *et al.*, 1988) to circumvent contaminating pathogens. Amadi and Ue Bari (1992) reported the ability of poultry manure to improve soil fertility and maize production. However, none of these methods have used a microbial controlled fermentation by acid-producing microorganisms (pure cultures of lactic acid bacteria). Thus, the use of such biotechnological process for recycling and transformation of wastes may be a good way to ensure the safety of the obtained products. Such biotechnological process may be more efficacious for the control of food and feed systems. In this way, not only wastes should be preserved but they should be transformed into a new exploitable ingredient, «bio-products» in particular to supply animal alimentation. The microorganisms involved in the fermentations in silos are isolated from the environment. The anaerobic bacteria, in the absence of oxygen and in the presence of glucosides (molasses) are rapidly multiplied in the whole silo mass, ensuring the silo preservation in very good conditions during the entire ensiling period. The glucosides are the source leading to lactic acid formation. In practice, the silo is supplemented with molasses for a rapid proliferation of lactic bacteria in the whole ensiled mass and for the production of lactic

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acid in large amounts in the shortest time possible (Goodman *et al.*, 1995).

Here we developed a biotechnological process to transform poultry wastes with the hope to face the problem of high contamination of such poultry wastes manure and to a valuable bio product with added values.

MATERIALS AND METHODS

Poultry wastes. Samples of poultry wastes were collected from poultry farms (caged laying hen excreta) every week. One Kg amounts were recovered in plastic bags and transported to the laboratory. The microbiological determinations were performed immediately.

Fermentation process. Poultry wastes were humidified with tap water in the proportions 1:1. The pH was adjusted to 6.5 with 50% sulfuric acid solution. The product was prepared as described in Fig. 1. For Inoculation and microbial strains, 10 kg amounts of the above mixture were placed in disinfected (chlorine) 25 L plastic containers and inoculated with starter cultures of lactic acid bacteria (LAB) as below. The lactic cultures of *Lactobacillus plantarum* and *Pediococcus acidilactici* were previously identifed from different fermenting materials in Marocco (Faid *et al*., 1997). These strains were characterized for their activities (inducing pH decrease & antimicrobial activity) in fermentation wastes. Strains were stored on MRS (Merck, Germany) slants at 4°C. Working cultures were grown on MRS broth at 30°C for 48 h. The cultures were centrifuged at 2800 g to separate the biomass from the medium. The biomass obtained from 1 L of culture was used to inoculate 10 Kg of wastes.

The fermentation process was monitored by determining pH and acidity as well as the microbial counts. Activities of LAB strains were also evaluated to follow up their growth pattern during the fermentation period by pour plating appropriate dilutions (10^{-4} to 10^{-7}) on MRS agar at 30°C for 24 h. All the determinations were daily done.

Inhibition test. The antimicrobial activity of LAB used in this work was analyzed by using the spot test (Harris *et al*., 1989) and the agar diffusion test (Piddock, 1990). The diameter of the clear zone formed was measured after incubation at 37C for 24 h. A diameter of 1.5 mm or greater around the culture was considered as positive inhibition. Test bacteria included *Escherichia coli* (10 strains), *Staphylococcus aureus* (4 strains), *Listeria spp.* (4 strains), *Enterococcus fecalis* (5 strains), *Proteus spp.* (4 strains), *Shigella dysenteria* (4 strains), *Pseudomonas aerugenosa* (5 strains) and *Salmonella enteritica* (4 strains). All of theses strains were obtained from the collection of micro-organisms of the Department of Food Microbiology and Biotechnology, IAV Hassan II (Rabat).

Chemical determinations. The pH was measured by a pH-meter Crison Micro-pH 2000. The dry matter was determined by hot-air oven drying a weighted amount of the product at 105°C to constant weight. Ash was determined by ignition at 550°C. Total nitrogen (TN) was determined by the Kjeldhal method as described by the APHA (1989). Non-protein nitrogen (NPN) was measured, using the same method, in the filtrate after precipitating with a 10% trichloroacetic acid solution. Total volatile nitrogen (TVN) was measured using the method described by Conway (1947). Fat content was determined on the dry matter by the Soxhlet method using hexane as solvent. Reducing sugars were determined by the Bertrand method.

Microbiological analysis. 10 g of each sample were blended in 90 mL of saline water (0.9 % NaCl) with a Warring blender to prepare the initial dilution. Colony forming units (CFU) were determined by standard pour plate methodology. Decimal dilution for total viable counts wre made in (0.85% saline solution) and 1 mL was palced in duplicate on standard plate count agar (Biokar, France).- Enterobacteria were enumerated on MacConkey Agar (Leininger, 1976). The plates were incubated at 37°C for 24 h.

Salmonella species were determined by transferring 25 g portions into 250 mL flasks containing 100 mL of peptone water. The flasks were incubated at 37°C for 18 h and then 1 mL of the culture was transferred to selenite broth (Merck, Germany) and tetrathionate broth (Merk, Germany). The positive tubes were streaked on Hektoen Agar (Merck, Germany). Non-colored colonies with and without a dark center were purified and streaked on trypticase soya agar (Biokar, France) slants and stored at 4°C. The identification was performed according to the method described by Poelma and Silliker (1975).

Clostridium was cultured in reinforced clostridial agar (Biokar, France) in tubes containing 15 mL of the medium. Sodium sulfite (0.1 mL $tube^{-1}$ of a 5% solution) and ammonium ferric citrate (0.1 mL $tube^{-1}$ of a 5% solution) were added after inoculation. The tubes were shaken, allowed to solidify and incubated at 30°C.

For the staphylococci, dilutions up to 10^{-6} were plated on mannitol salt agar (Merck, Germany). Yellow colonies on the medium were counted and checked for Gram and catalase reactions. The isolates were checked for their DNAse and phosphatase reactions. For the phosphatase test, the strains were grown on brain heart infusion (Biokar, France). 0.5 mL of the culture was added to 0.5 mL of the nitrophenylphosphate solution in small tubes and incubated at 37°C for 12 to 24 h. A positive reaction is revealed by a yellow colour of the reagent. For the DNAse test, the deoxyribonucleic acid agar (Merck, Germany) was autoclaved and poured in plates, which were allowed to solidify. The strains were surface spot inoculated (five spots per plate) on the medium and the plates were incubated at 37°C for 24 h. Reactions were revealed by a 2 N chlorhydric acid solution poured on the plate surface. Clear zones around the cultures indicated a positive reaction.

Enterococci were cultured on azide dextrose broth

Fig. 1. Flow chart showing the production of biologic poultry manure silage

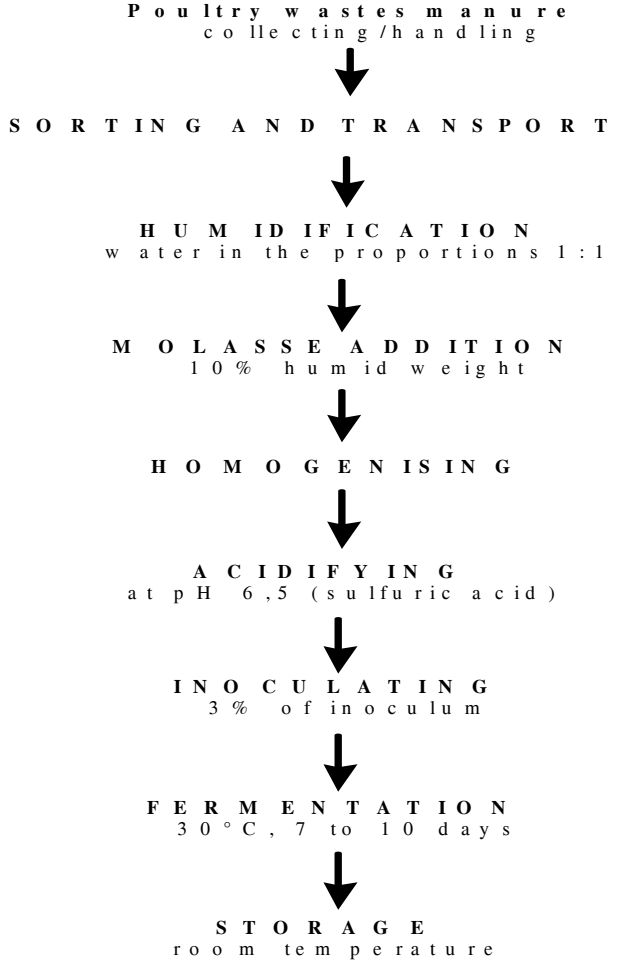
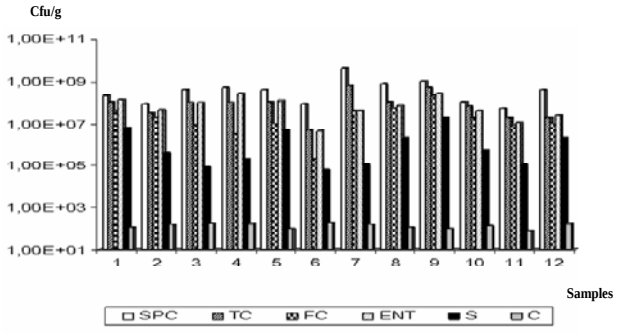


Fig. 2. Microbial profiles of the fresh raw poultry manure samples



SPC: Standard plate count ; C: total coliforms ; FC: Fecal coliforms ; ENT: Enterobacteria ; S : *Staphylococcus* ; C: *Clostridium*

(Biokar, France) using 3 tubes per dilution and incubated at 37°C for 24 h. Tubes showing growth were transferred to azide ethyl dextrose broth (Biokar, France) incubated at 37°C for 24 h. Yeasts were plated on potato dextrose agar (Difco Laboratories, USA) acidified to pH 3.5 with sterile lactic acid. The plates were incubated for 3 days at 30°C.

RESULTS AND DISCUSSION

Poultry Wastes Manure

Microbiological analyses. Hygienic conditions of poultry wastes were evaluated on different samples over three months. The general assessment of the hygienic conditions was achieved by the determination of counts of enterobacteria, enterococci, staphylococci and *Clostridium*.

The evaluation of the standard plate count in the different samples of wastes showed a heavy contamination (10^9 cfu g^{-1}) in the different samples (Fig. 2). The high counts could constitute a problem, which can induce a subsequent degradation of the environment if such wastes were generally discarded in nature without any treatment and/or recycling. These wastes are highly contaminated with ubiquitous microorganisms, this may renders the treatment of such wastes hard in some cases.

The enterobacteria counts, which reach in some samples 1.2 x 10^8 cfu g^{-1} (Fig. 2), is evocative of high polluting capacity of these wastes. Yet, these microorganisms are part of the normal microflora of the animal digestive tract, however among of them hazardous microorganisms may exist and may be dispensed in environment through these wastes. *E. coli* was the most species that were isolated from fresh poultry waste manure with about 55.5% of total isolates (Table I). As shown, other species with sanitary impact were also isolated with low percentage especially *Klebsiella pneumonia* (9.8%), *Pseudomonas aerogenosa* (5.5%), *Schigella dysenterae* (1.3%) *and Campylobacter jejuni* (0.7%). Results suggested also that the indicator organisms could be used fatherly for monitoring the progress and the success of the process used for the treatment of these wastes. *Salmonella* was not isolated in any of the samples and all the suspected isolates were identified as species of *Proteus sp.* However, this does not exclude the presence of *Salmonella* in low numbers, which could not be detected by the method used here.
In addition, coliforms can overgrow *Salmonella* species and render their isolation hard.

The different samples were also highly contaminated with enterococci, reaching 2.4 x 10^8 cfu g^{-1} (Fig. 2). The presence of enterococci in high numbers with the enterobacteria would be expected in the waste samples, since these microorganisms would constitute the normal microflora of the animal digestive tract.

Population of *Clostridium* varied widely in the different samples and counts in some samples reached a maximum of 200 cfu g^{-1} (Fig. 2). However, several samples were found to contain less than 80 cfu mL^{-1}. High counts of *Clostridium* in the waste are also normal

for faecal materials. These microorganisms are not easy to eliminate from contaminated materials and may resist un-favourable conditions by spore formation. Thus, attention will be given to these microorganisms in all treatment procedures to be applied to such materials for a possible recycling.

High counts in staphylococci were also observed in all samples before the fermentation (Fig. 2). These microorganisms are halo-tolerant and can resist to high concentration of sodium chloride and they are usually present at high contents in the solid animal wastes. Isolates of staphylococci were tested for their DNAse and phosphatase reactions. We observed that 56.66% of the isolates were DNAse positive and phosphatase positive. These high proportions in DNAse and phosphatase positive strains would attest for the high contamination of poultry wastes with toxigenic microorganisms.

Chemical analyses. From Table II, the presence of total volatile nitrogen in high concentrations. The initial content in the mixture, which may consist of wastes, molasses and water, ranged between 4.6 and 4.9 mg 100 g^{-1} (Table III). This component would be higher in the crude poultry wastes as it is the source of the nauseating odour of these materials (Pr Faid, personal communication). The high content of volatile nitrogen in the waste would restrict the use of these materials for some purposes such as feeds or other uses. As indicated in Table II, poultry wastes manure show a high level of crude protein and ash, which may constitute an added value for these materials.

Bioproduct Process

Chemical changes. As described in material and methods, the initial pH of the humidified poultry wastes was adjusted to 6.5. During the fermentation process, we observed that pH decreases in the fermenting material (Fig. 3). This may suggest that the strains inoculated to the waste mixtures could grow potently and induces decrease of the pH to 4.0 in a short period (5 days). Thus, the acidity increases during the fermentation period and reached a high level at the end of the process (Fig. 3). The decrease in pH observed here is more important compared to that reported by Harmon *et al*. (1975), who found a pH around 4.68 at the end of the fermentation of poultry manure mixed with corn (silage). The increase in acidity may confirm the pH drop and attest about the growth of the starter culture made of lactic acid bacteria.

Salminen and Rintala (2002) mentioned that in all fermenting processes there are important lactic bacteria producing as much lactic acid as possible, which consume a low amount of glycosides (from molasses) and which survives to high acidity (pH 3-4.5).

These microorganisms are responsible for the acidity produced in the medium, which may play a role of preservation of the waste materials (Raa *et al.*, 1982). The pH decrease in the product gives evidence of a good acidification through lactic acid fermentation by the starter culture. According to Faid *et al*. (1997) and

Fig. 3. pH and acidity pattern during fermentation of poultry wastes manure

—, pH; ---, acidity.

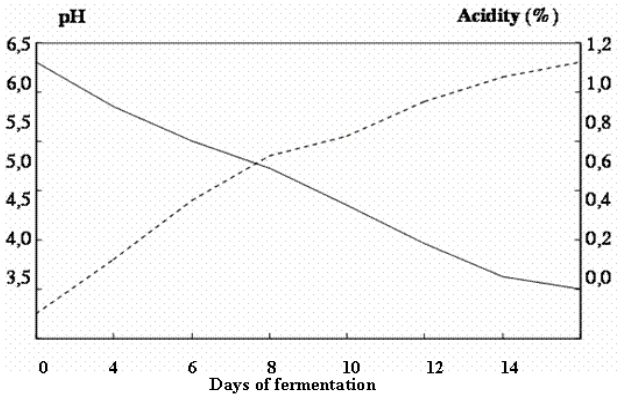


Fig. 4. Behaviour of various microbial groups in poultry manure fermentation

—: standard plate count; .....: enterobacteria; --------: enterococci

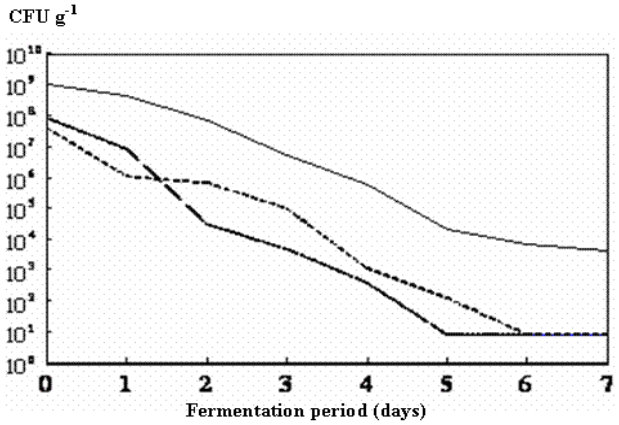
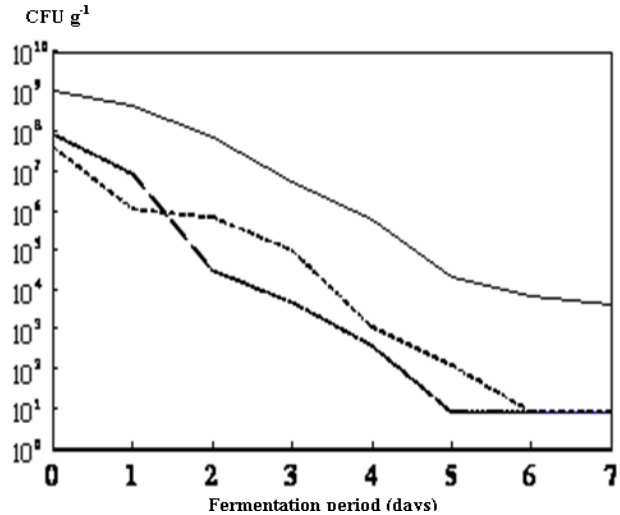


Fig. 5. Microbial profiles pattern of pathogenic and toxigenic bacteria in fermentation period



Haaland and Njaa (1990), the most important factor to control in the biotransformation is the pH decrease, which must be achieved as quickly as possible in order to inhibit the growth of spoilage microorganisms in the product.

The chemical analyses revealed an important level of proteins 22.9%, which was preserved in the transformed product (Table II). The non protein nitrogen level increased from 0.15 to 0.36 of the total nitrogen. Poultry manure may contain alkaline compounds (total volatile nitrogen), especially ammonia, which may avoid a pH drop during the fermentation process. The ammonia completely disappeared in the transformed product (Table II). This result is very interesting since low level of ammonia at the end of the fermentation testifies to a deodorization process (Faid *et al.*, 1997; Kherrati *et al.*, 1998; El Akhdari *et al.*, 2005). The success of the biotechnological method is also beneficial for eliminating the un-desirable volatile compounds. The mechanism by which the total volatile nitrogen is reduced is not yet elucidated and work is now being carried out to study such process and to explain the occurrence of both chemical and microbiological phenomena.

Knowledge of the chemical changes during the fermentation process that result in the disappearance of the manure-like odour could reduce the problem of incorporation of fresh manure in feed formulae by Moroccan farmers. Indeed, huge amounts of ingredients are used for animals feeding in Morocco, due to drought the country has endured during the last two decades; cereal yield production has been dramatically reduced in the range of 25-85% (INRA, 2002). Besides sanitary effects of this incorporation, oganolopetic characters of meat could be affected by bad odor of poultry manure.

Microbial changes. A general hygiene of the product was also evaluated by the total viable bacterial counts during the incubation period. The microbial profiles reported in Fig. 4 showed a good effect of the fermentation process on the initial microbiota. Total viable bacterial counts, reached at the end of the fermentation process, were not high. Microorganisms counted in the last phase may include some yeasts brought out by the molasses used in the mixture as a carbon source to start the fermentation.

A drastic decrease in enterobacteria count was observed in all the trials (Fig. 4). The presence of *Proteus* and other amine-forming species could be prevented by the acidification/fermentation process. The high levels of enterobacteria in the fresh raw materials were greatly decreased to less than 10 cfu g^{-1} in the transformed product and *Salmonella* was not detected. This could ensure a safety of the product by the absence of hazardous microorganisms.

The same drastic reduction pattern as that noted for enterobacteria was also observed for Gram positive bacteria, especially stapphlococci and *Clostridium*, whose counts were less than 10 cfu g^{-1} in the fermented product after 5 days. *Clostridium* species, as well as most spore-forming bacteria, are sensitive to the pH drop (Faid *et al.*, 1997). The suppression of growth of *Clostridium* is very important during fermentation, because these bacteria produce organic acids with offensive odours such as butyric acid (Wang *et al.*, 2001).

Table III showed the inhibitory activity of *Lactobcilus plantarum* and *Pediococcus acidilactici* against gram-posistive abd gram-negative bacteria. A 75% and 25% of strains of *S. aureus* were inhibited by *L. plantarum* and *P. acidilactici* supernatants, respectively while *Listeria spp.* strains were completely inhibited. Although the suppression of growth of putrefactive and food poisoning bacteria was mainly due to the fall in pH during lactic acid fermentation. Callewaert and De Vuyst

Table I. Proportion of bacterial strains isolated from raw poultry waste manure

Strains	Percentage (%)
Escherichia coli	55.5
Klebsiella pneumonia	9.8
Citrobacter freundii	5.5
Pseudomonas aerogenosa	5.5
Enterobacter cloeae	4.2
Enterobacter agglomerans	3.5
Enterobacter sakazaki	2.1
Proteus mirabilis	2.1
Staphylococcus	1.3
Schigella dysenterae	1.3
Clostridium	1.3
Pseudomonas spp.	4.2
Enterobacter fecalis	2.8
Campylobacter jejuni	0.7

Table II. Changes in Chemical indicators between the fresh raw and the fermented poultry waste manure

	RPWM	TPWM
pH	8.7±0.1	4.0±0.5
Dry Matter	79.3±3.5	45.5±6.4
Ash	20.1±1.5	20.2±3.5
Crude proteins	24.6±2.6	22.9±3.4
NPN	0.15±0.1	0.36±0.5
TVN	4.9±0.2	0
Reducing sugar	1.2±0.1	4.5±2.1

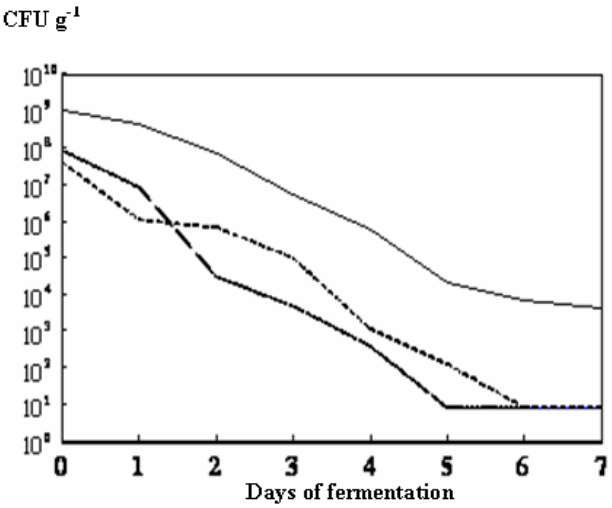
Legends: RPWM; Raw Poultry Wastes Manure: TPWM; Transformed Poultry Wastes Manure: NPN; Non Protein Nitrogen: TVN; Total Volatile Nitrogen: Proteins; calculated as total nitrogen x 6.25, in % of the product.

Table III. Inhibitory effect of *L. plantarum* and *P. acidilactici* supernatants on Gram-positive and Gram-negative bacteria

Strains	Number of strains	Percentage of inhibition *L. plantarum*	*P. acidilactici*
Staphylococcus aureus	4	75	50
Lisrteria sp.	4	100	100
E. fecalis	5	60	40
Escherichia coli	10	80	0
Proteus sp.	4	0	0
Shigella dysenteria	4	50	0
Salmonella enteritica	4	50	0
Pseudomonas aerugenosa	5	60	0

Fig. 6. Population of lactic bacteria and yeasts during fermentation of poultry wastes manure

—, lactic acid bacteria; ---, yeasts



(2000) found that various inhibitory substances (organic acids, diacetyl, bacteriocins, hydrogen peroxide) generated from lactic acid bacteria would also suppress the growth of these putrefactive bacteria.

The lactic bacteria as well as other types of microorganisms are also capable of producing and discharging into the environment a great variety of antimicrobial substances, which inhibit the development of microorganisms not suitable for ensilling. The bacteriocins form a class of antibiotics whose biosynthesis occurs during or at the end of the exponential stage of lactic bacteria strain growth. The use of lactic acid bacteria for the preservation of organic materials is based not only on the production of lactic acid, but also on the production of bacteriocins which show a wide range of antibacterial effects on Gram-positive especially putrefactive bacteria such as *Clostridium* and *Staphylococcus* (Wang *et al*., 2001).

A successful fermentation is caused by the LAB growth and activity in the mixture. The growth curve plotted in Fig. 6 shows the characteristics of the strains used in the fermentation. A maximum of 10^9 cfu g^{-1} was reached in the product after 7 days. Yeasts reached to 10^7 at the end of the fermentation (Fig. 6). The high counts of yeasts would result in alcohol and some metabolites production, which could hide the effluent odor in the final product. Fermentation by yeast may require high amounts of sugar relative to growth. Faid *et al.* (1995) demonstrated that fish waste silage in a controlled fermentation by LAB can be improved by yeasts addition for a combined alcohol/lactic fermentation.

It is assumed that fermentation of food and feed materials with LAB would have some properties related to the nutrition and safety (probiotic) of the obtained product. This product, which is obtained by some complicated biological and biochemical reactions, is not easily compared to products processed with physical or chemical methods, such as drying, heating, acidifying etc.

CONCLUSION

Biotransformation of wastes by fermentation may be a very convenient process for treatment of the huge amount of wastes produced in the different food treating units (poultry industry, poultry processing, slaughterhouses etc.). The process may yield highly valuable bio-products. Preserving properties of LAB are widely used in the field of food bio-preservation. These properties should first be studied in the laboratory to select the most suitable strains according to their growth and antimicrobial activities. An appropriate procedure for transformation and soil safety recycling of poultry wastes should be suitable for both the protection of environment and for the economical income. It would be interesting to learn more about the mechanism by which poultry wastes manure can not only be preserved but also by which the manure odour is removed. The application of LAB should be also extended to the feed products preservation and improvement. Simple and cheaper processes can be found to solve the problem of the high polluting power of these wastes. The application of poultry manure would encourage the research in progress.

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