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RESEARCH ARTICLE

ANTAGONISTIC ACTIVITY OF ACETIC ACID BACTERIA AGAINST POTENTIAL PATHOGENIC GERMS ISOLATED FROM POULTRY LITTER IN CÔTE D'IVOIRE

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Abstract

Poultry litter is an important element in poultry production, influencing animal welfare, environmental impact and production efficiency. However, bedding is a potential source of contamination by potentially pathogenic microorganisms causing diseases that are responsible of huge economic losses. The aim of this study is to use acetic bacteria of various origins to control pathogens isolated from farm litter in the District of Abidjan. A total of 16 strains of acetic acid bacteria were used. Acidification capacity and antimicrobial activity of these strains were determined by different methods. Results showed that all 16 strains (T9I10, T9N3, T10I4, T11G3, T9N5, T11G6, T9G6, T7N8, T11G6, T0N5, T3G3, T3N7, T6D121, T2N5, T3G10 and T4G7) were capable of producing acetic acid with titratable acidity percentages ranging from 0.200±0.00 to 1.010±0.07%. These strains had strong antimicrobial activity, with inhibition diameters ranging from 15.800±1.21 to 23.533±1.15 mm for Escherichia coli, 13.667±3.05 to 28.667±1.15 mm for Salmonella spp, and 77.137 ± 1.82 to 91.037 ± 4.24 mm for Aspergillus spp. Therefore, these strains can be used for litter treatment in poultry farming and could be applied in other industrial fields and furthermore evaluated as potential probiotics.

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Introduction:-

Poultry farming plays an important socio-economic role and occupies a place of choice in Côte d'Ivoire for food self-sufficiency in terms of animal protein **(Ehouman, 2022)**. Indeed, modern poultry production in this country is the only sector to cover local needs among animal production, with a production of 97,000 tonnes of poultry meat in 2022 and 1.680 billion units of table eggs. On the socio-economic front, poultry industry also generated sales of 380 billion CFA and 280 000 direct and indirect jobs **(N'guessan, 2024).**

However, this upward trend in poultry production is threatened by the presence of pathogenic germs, which affect the health of chickens during breeding **(Skóra et al., 2016; Abreu et al., 2023; Joseph et al., 2023)**. These pathogenic microorganisms have several sources of origin including food for chickens, farm staff, drinking water for animals and especially the quality of litter during breeding **(Mustedanagic et al., 2023; Wang et al., 2023).**

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Litter is a necessary and important element in breeding chickens and its quality can influence animal health, welfare and production efficiency **(Dunlop et al., 2016; Jim, 2021; Sáenz, 2021**). However, the quality of the litter changes during breeding and becomes a potential source of contamination by pathogenic microorganisms such as Escherichia coli, Salmonella and Aspergillus (**Ostovic et al., 2021; Pal et al., 2021; Rogovski et al., 2021; Dunn et al., 2022; Lawrence et al., 2022**). These pathogens are responsible of diseases such as collibacillosis, salmonellosis and aspergillosis which cause huge economic losses for farmers (**Zhao et al., 2005; Koffi, 2015; Brou et al., 2018; Djoman et al., 2020; Abrol et al., 2022).**

In Côte d'Ivoire, no study has been carried out on the control of potentially pathogenic germs isolated from litter by other bacteria to improve sanitary conditions of poultry farming, in order to promote biological control against the misuse of antibiotics implicated in antibioresistance of microorganism in this area. Therefore, the aim of this study is to use acetic acid bacteria of various origins for the control of pathogenic microorganisms isolated from poultry litter in the District of Abidjan.

Material and Methods:-

Acetic Acid Bacteria culture

A total of 16 acetic acid bacteria strains from cocoa beans and palm wine during fermenting process were used in this study. Acetic acid bacteria strains stored at -80°C were cultured in Luria Bertani (LB) broth consisting of (glucose 1%; soybean tryptone 1%; yeast extract 0.5%, meat extract 0.5%; sodium chloride 0.05%) and then on Duthathai agar of the following composition: glucose 0.5%, casein peptone 1%, yeast extract 1%, glycerol 2%, calcium bicarbonate 1%, agar 1.5%, bromocresol green 0.0016% and ethanol 4% (**Duthathai and Wasu, 2007)** to check their purity. After verifying purity, a pre-culture is carried out by aseptically introducing a colony of acetic acid bacteria taken from Duthathai agar into 3 mL of sterile LB broth. The whole set is incubated at 30°C for 48 hours. Acetic acid bacteria are cultured by aseptically removing 100 µL of the preculture and inoculating it into 5 mL of sterile LB broth. Incubation took place at 30°C for 24 hours. From this culture, samples are taken for titratable acidity and antimicrobial activity tests.

Revivification of stored microbial strains isolated from poultry litter

For the revivification of Escherichia coli and Salmonella sp strains stored at -20°C, successive plating was carried out in hemolysis tubes containing 3 mL nutrient broth, and incubated at 37°C during 24 h. These broths were then plated onto TBX agar (Escherichia coli strains) and Hektoen agar (Salmonella sp). A succession of streak plating operations was carried out on agar plates poured into sterile Petri dishes. After incubation at 37°C during 24 hours, the colonies obtained were used to test antibacterial activity.

For Aspergillus spp stored in test tubes at 4°C, inoculations were carried out on OGA (Oxytetracycline-Glucose-Yeast Extract Agar) poured into Petri dishes. After incubation at 30°C for 48 to 72 hours, the obtained strains were used to test antifungal activity.

Evaluation of Acidification Capacity of Acetic Acid Bacteria

Acidification capacity of the strains was assessed by monitoring pH of the different acetic acid bacteria cultures after 24 h and 48 h using a pH meter (Hanna Hi 2223, France) previously calibrated. Acidity of the different culture media was determined using sodium hydroxide (NaOH) in presence of two (2) drops of phenolphthalein (color indicator). For this purpose, NaOH solution (0.1 N) was added dropwise to culture media supplemented with phenolphthalein. Once a change in the coloration of the culture medium had occurred, the total volume of NaOH added was used to calculate the percentage of titratable acidity according to the established relationship **AOAC (1990)**:

$$\textbf{\% titratable acidity} = \frac{\mathbf{V(NaOH) \times N \times 0.06}}{\mathbf{Vt}} \times \mathbf{100}$$

V (NaOH): Volume of NaOH solution used for titration
Vt: Test volume
N: Normality of NaOH solution
0.06: Molar mass of acetic acid

Antibacterial Activity of Acetic Acid Bacteria

Antibacterial activity was demonstrated by inhibiting the growth of potentially pathogenic enterobacteria. This test was carried out using the agar diffusion method described by **Tadesse et al. (2004)**. In fact, 200 µL of Escherichia coli and Salmonella sp suspensions produced over 24 hours at 37°C were inoculated into 15 mL of nutrient agar, then homogenized. After solidification, 6 mm diameter wells were aseptically drilled into the agar plates using sterile tip of a Pasteur pipette. Finally, 20 µL of each pre-culture of acetic acid bacteria was deposited in each well. The whole set was refrigerated at 4°C for 2 hours, then incubated at 30°C for 24 to 72 hours. Growth inhibition of Escherichia coli and Salmonella sp strains was determined by measuring diameters of the inhibition zones around the wells; and results obtained were interpreted according to the method of **Bahri (2014)**: (-) no inhibition; (+) weak inhibition for a diameter between 0 and 3 mm; (+ +) good inhibition for a diameter between 3 and 6 mm and (+ + +) strong inhibition for a diameter greater than 6 mm.

Anti-fungal activity of acetic acid bacteria

Antagonism or confrontation test is carried out to verify the existence of any inhibitory activity of acetic acid bacteria towards strains of Aspergillus sp. Using a sterile platinum loop, two lines of acetic acid bacteria are seeded onto Duthathai agar in the form of a straight streak, then the Petri dishes are incubated at 30°C during 48 hours. Afterwards, Aspergillus strains isolated from poultry litter are deposited onto the same Petri dishes by spots on either side of the streak, 1 cm from the edge of the Petri dishes, then incubated at 30°C during 72 hours. Thus, on the same dish, the spot between the straight lines is the test and the other spot represents the control **(Bezert et al., 1996)**. Percentage of mold growth was determined by using **Korsten and Jager (1995)** method, and inhibition rate was deduced according to the following formula:

$$\textbf{Inhibition rate } (\%) = \frac{(\mathbf{C} - \mathbf{T})}{\mathbf{C}} \times \mathbf{100}$$

C= radial growth of Aspergillus without antagonism confrontation
T= radial growth of Aspergillus with antagonism

Statistical analysis of data processing

Results for inhibition diameters and titratable acidity percentages of the acetic acid bacteria were expressed as mean plus or minus with ecartypes. Analysis of variance (ANOVA) was used to process the results. Statistical analyses of the data obtained were carried out using XLSTAT 2016 software. Duncan test at the 5% threshold was used to determine significant differences between means.

Results:-

Acid Production Capacity of Acetic Acid Bacteria

Acid production capacity of acetic acid bacteria is the step highlighted to determine which strains are capable to produce acid. It was evaluated in liquid medium. Results show that all 16 strains studied were able to produce large quantities of acid, with titratable acidity values ranging from 0.200±0.00 to 1.010±0.07% (**Table 1**).

Antimicrobial Activity of Acetic Acid Bacteria

Results of the antimicrobial activity of acetic acid bacteria strains against selected Escherichia coli, Salmonella sp, Aspergillus sp showed that inhibition diameters ranged from 15.800±1.21 to 23.533±1.15 mm for Escherichia coli; 13.667±3.05 to 28.667±1.15 mm for Salmonella sp and inhibition diameters against Aspergillus sp, expressed as inhibition rates, ranged from 77.137±1.82 to 91.037±4.24 % (**Table 1**). The inhibition diameters are illustrated by **Figures 1, 2 and 3**.

Table 1:- Inhibition diameter and Titratable acidity of selected strains.

Strain codes	Inhibition zone diameters against E. coli (mm)	Inhibition zone diameters against Salmonella (mm)	Inhibition rate (%) against Aspergillus	Titratable acidity (%)
T9I10	$18.867^{bcd} \pm 1.09$	$27.333^{ab} \pm 3.05$	$91.037^{a} \pm 4.24$	$0.370^{e} \pm 0.06$
T9N3	$19.867^{bc} \pm 0.57$	$25.333^{ab} \pm 1.52$	$83.743^{bc} \pm 1.79$	$0.200^{f} \pm 0.00$
T10I4	$20.567^{bc} \pm 1.09$	$27.333^{ab} \pm 1.52$	$88.907^{ab} \pm 3.07$	$0.370^{e} \pm 0.06$

T11G3	15.867^{e} ± 1.27	13.667^{c}±3.05	86.933^{abc}±1.90	0.970^{a}±0.06
T9N5	18.067^{cde} ± 1.61	26.000^{ab}±2.64	90.500^{a}±0.85	0.570^{cd}±0.06
T11G6	17.800^{cde} ± 1.38	21.666^{b}±3.05	88.653^{ab}±2.66	0.500^{d}±0.00
T9G6	16.867^{de} ± 1.44	26.000^{ab}±3.00	88.237^{ab}±2.36	0.530^{cd}±0.06
T7N8	15.833^{e} ± 1.15	23.000^{ab}±6.00	87.740^{abc}±1.22	0.330^{e}±0.06
T11I5	19.667^{bc} ± 0.92	25.667^{ab}±3.05	88.963^{ab}±4.72	0.770^{b}±0.06
T0N5	23.533^{a} ± 1.15	26.667^{ab}±1.52	86.817^{abc}±3.31	0.330^{e}±0.06
T3G3	16.933^{de} ± 1.67	23.667^{ab}±3.05	77.137^{d}±1.82	0.400^{e}± 0.00
T3N7	20.200^{bc} ± 1.73	28.333^{a}±3.05	90.773^{a}±3.73	0.300^{e}± 0.00
T6D121	18.867^{bcd} ± 0.57	28.667^{a}±1.15	85.503^{abc}±3.46	0.800^{b}± 0.10
T2N5	16.767^{de} ± 1.09	25.667^{ab}±2.08	88.963^{ab}±4.72	0.500^{d}± 0.00
T3G10	21.200^{b} ±1.73	26.667^{ab}±4.04	86.573^{abc}±3.48	0.600^{c}± 0.00
T4G7	15.800^{e} ± 1.21	15.333^{c}±1.52	82.170^{cd}±2.91	1.010^{a}±0.07

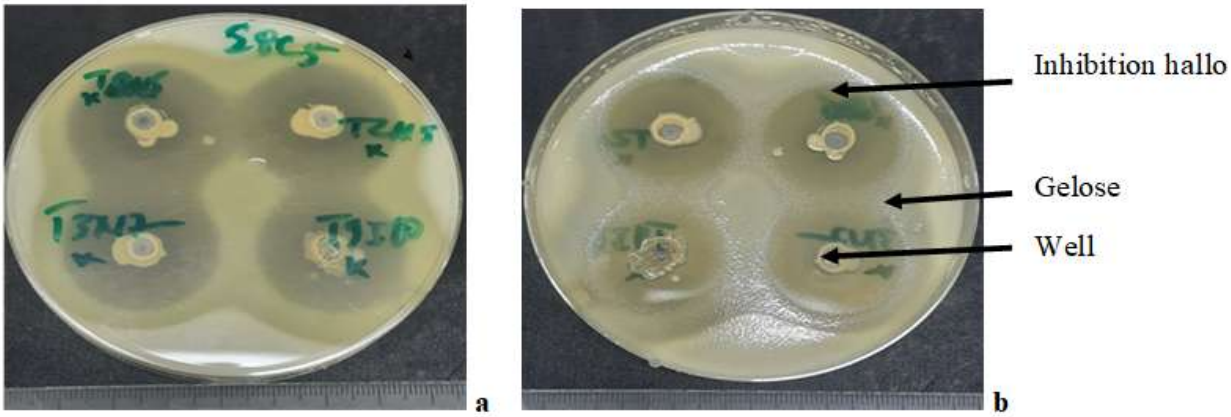

Figure 1:- Growth inhibition of E. coli strains by acetic acid bacteria.

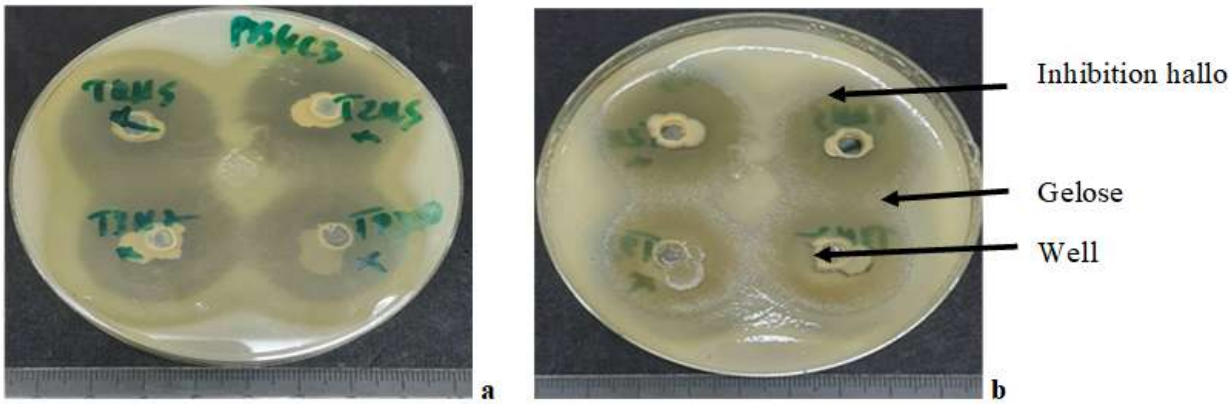

Figure 2:- Growth inhibition of Salmonella spp strains by acetic acid bacteria.

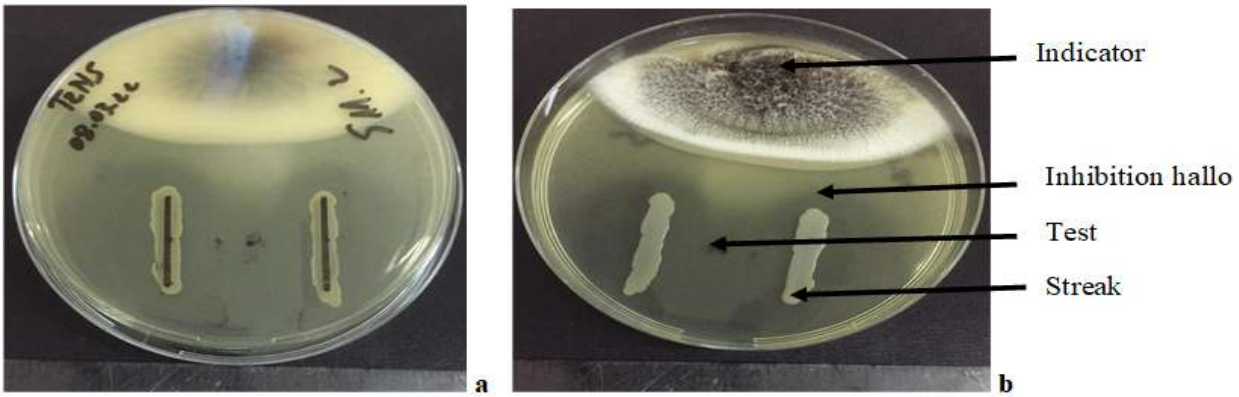

Figure 3:- Growth inhibition of Aspergillus spp strains by acetic acid bacteria.

For the illustrations; **a:** Reverse and **b:** Obverse

Discussion:-

This study was carried out to evaluate antimicrobial capacity of acetic acid bacteria strains isolated from cocoa beans and palm wine during fermenting process in the Agri-food Biotechnology Laboratory of Felix Houphouët-Boigny University against potentially pathogenic germs coming from litter of poultry farms in Abidjan. These include Escherichia coli, Salmonella spp. and Aspergillus spp.

Results obtained showed that all 16 strains of acetic acid bacteria tested have acidifying power and antimicrobial activity against pathogens isolated from farm chicken litter. The percentage of titratable acidity ranged from 0.200±0.00 to 1.010±0.07%. These acetic acid bacteria strains had inhibitory activity against Escherichia coli with inhibition diameters ranging from 15.800±1.21 to 23.533±1.15 mm, against Salmonella spp with inhibition diameters ranging from 13.667±3.05 to 28.667±1.15 mm and against Aspergillus spp with inhibition rates ranging from 77.137±1.82 to 91.037±4.24 %. Acetic acid bacteria (AAB) are known to be highly versatile microorganisms of great biotechnological relevance. They are Gram-negative or Gram-variable, have ellipsoidal or rod-shaped cells and have obligatory aerobic metabolism with oxygen as the terminal electron **(Gomes et al., 2018)**. Acetic acid bacteria are microorganisms found in nature, on the surface of flowers and fruits, in sweet substances or in alcoholic beverages **(Mamlouk and Gullo, 2013; Saichana et al., 2015)**. They are well known for their ability to oxidize alcohols and sugars to produce bioacids **(Cepec and Trcek, 2022)**. Acetic acid bacteria can oxidize ethanol to acetic acid through the combined action of alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH), with pyrroloquinolinequinone (PQQ) as a coenzyme **(Lynch et al., 2019)**. They are also important in the production of industrial vinegar **(Ndoye et al., 2006)**. The acetic acid contained in vinegar is known for its antibacterial activity at low concentrations, and its ability to kill Gram-positive and Gram-negative opportunistic pathogens living as mono-specific biofilms **(Boban et al., 2010; Halstead et al., 2015).**

As indicated above, the 16 strains studied did not have same acid production capacities with percentage of titratable acidity varying from 0.200±0.00 to 1.010±0.07%. Acetic acid bacteria are known for their high acid production in a medium through the use of ethanol or sugar **(Stasiak and Błażejak, 2009; Tanamool et al., 2020; Qui et al., 2021; El-Askrit et al., 2022; Song et al., 2022)**. Studies carried out elsewhere have even demonstrated this high acid production capacity of acetic acid bacteria, with titratable acidity percentages in excess of 4% **(Romero et al., 2012; Kim et al., 2023; Kong et al., 2023)**. While in our study the percentage of titratable acidity obtained is lower than that obtained in the work of these authors. Yet, these 16 strains of acetic acid bacteria have high capacity to inhibit growth of potentially pathogenic germs such as Escherichia coli, Salmonella spp and Aspergillus spp found in litter of poultry farms in the District of Abidjan. Other researchers have also shown on both sides inhibition capacities of acetic acid bacteria strains against Escherichia coli and Salmonella typhimurium with inhibition diameters ranging from 12.3±0.3 mm to 23.2±0.1 mm and from 12.0 ± 1.0 to 22.6 ± 0.1 mm respectively **(Haghshenas et al., 2015; Kim et al., 2023)**.

Some scientists have investigated studies on the antimicrobial activity of vinegar against pathogenic microorganisms. Their results revealed inhibitory capacity of vinegar against Escherichia coli and Salmonella

typhimurium, with inhibition diameters ranging from 15.0±0.1 to 30.0±0.1 mm **(El aid Ridha et al., 2022)**; these results are quite similar to those obtained in our study. Work undertaken by certain authors has shown that some vinegars presented antibacterial activity exceeding that predicted by their acetic acid content alone, meaning this depends also on the bacterial species being investigated and the growth conditions **(Harrison et al., 2023)**. Other researchers have shown that vinegar has an inhibitory effect on the growth of Penicillium chrysogenum but not on Aspergillus fumigatus **(Rogawansamy et al., 2015)**; whereas in present study, all of the 16 acetic acid bacteria strains tested had strong inhibitory capacity on Aspergillus spp with high inhibition diameters.

Several authors have also demonstrated the antimicrobial capacity of acetic acid at different concentrations against germs such as Escherichia coli, Salmonella spp and Aspergillus spp **(Kim and Kim, 2007; Peláeza et al., 2012; Olaimat et al., 2018; Wali and Abel, 2019; Zinn and Bockmuhl, 2020)**. In the work of these authors, they were unable to demonstrate the antimicrobial effect of acetic acid against the germs targeted in our study at the same time. While our results showed antimicrobial capacity of the 16 strains of acetic acid bacteria used against such germs as Escherichia coli, Salmonella spp and Aspergillus spp with high inhibition diameters. Previous study made by **Pangprasit et al (2020)** showed that acetic acid had the highest zone of inhibition against all pathogens except Escherichia coli, compared to lauric acid and caprylic acid. In contrast, in our study, inhibition diameters (mm) of the 16 acetic acid bacteria strains ranged from 15.800±1.21 to 23.533±1.15 mm, showing their antagonistic activities against E. coli. These authors concluded that acetic acid had antimicrobial activities against most mastitis pathogens compared to other acids. Still on the subject of acetic acids antimicrobial effect, studies have shown that acetic acid can be the most effective antimicrobial agent, with an excellent bactericidal effect and a disinfectant effect against other species **(Ryssel et al., 2009; Zinn and Bockmuhl, 2020; Park et al., 2021).**

In view of the above, we are convinced that our 16 strains of acetic acid bacteria, with their high inhibitory activities, could be used in other industrial fields, in addition to poultry litter sanitation; that would avoid the usage of chemically produced preservatives. Indeed, among the organic acids responsible for vinegar total acidity, acetic acid is the major compound of this beverage (**Moussa et al., 2015**).

Conclusion:-

All the 16 acetic acid bacteria strains tested in this study presented good acidifying power and strong antimicrobial activity against potential pathogenic germs such as Escherichia coli, Salmonella spp and Aspergillus spp isolated from litter of chicken farms in the District of Abidjan. Therefore, these acetic acid bacteria strains could be used in poultry farming for the safety of litter in order to promote biological control against the excessive use of antibiotics in this area, or even in other areas of the agri-food industry.

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Conflict of interest
The authors declare that there is no conflict of interest.

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