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African Journal of Agricultural Research

Full Length Research Paper

Foodborne diseases, fish and the case of Aeromonas spp.

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Food intake is an imperative need of living beings in order to obtain the nutrients and energy necessary for their growth and development. All human beings worldwide have the right to access to nutritious and safe food. Food safety is a term that has become increasingly important in food production; is defined as the set of conditions and actions necessary along all phases of the food chain (from its primary production to the consumer's table) that guarantee that the food that is ingested does not represent a risk in the health of consumers. Food can be contaminated by different types of hazards (heavy metals, pesticides, microorganisms among others) compromising their safety and functioning as vehicles for various diseases. Foodborne diseases are considered globally a serious problem in public health, and a challenge in the production and commercialization of food. The purpose of this document is to provide a general overview of foodborne diseases, causative agents with emphasis on the genus *Aeromonas* spp., methods of detection, actions of prevention and control of foodborne diseases in addition to considering the phenomenon of antimicrobial resistance detected in these pathogens of relevance in public health and production of food mainly of aquatic origin.

Key words: *Aeromonas*, ascites, fish, food safety, processing, public health, tilapia.

INTRODUCTION

The health and quality of life of people depend to a great extent on the nutritional value of the foods that are consumed. This property in turn is related to the hygienic and sanitary quality to which they are subjected along the food chain (from the field to the consumer's table) (Kopper et al*.*, 2009; Badui, 2015). Foods play a key role

in the transmission of diseases through contamination due to lack of hygiene and sanitation from air, water, soil, animals, utensils, human beings, during primary production, transport, storage, processing, distribution and elaboration (Vásquez, 2003; Kopper et al*.,* 2009).

Everybody has the right to access safe food. Food safety is defined as the set of conditions and actions necessary along the food chain to ensure when ingested, they do not represent a risk to consumers' health; food safety is not negotiable and cannot be dispensed in any food within the context of quality (Tafur, 2009; WHO, 2018a).

Foodborne Diseases (FD) are a priority in public health issues around the world due to their incidence and mortality as well as the socioeconomic burden due to high levels of productivity loss, health services costs, implementation and monitoring of food safety policies (Kopper et al*.,* 2009; Olea et al*.,* 2012; Badui*,* 2015; Forero et al.,2017).

The FDs are derived from the consumption of water and food infected with contaminants of physical, chemical or biological origin in enough quantity to affect consumers' health (Vásquez, 2003; Kopper et al*.,* 2009; Soto et al*.,* 2016). Approximately 250 causal agents have been described, including bacteria, viruses, fungi, parasites, prions, toxins and metals (Barreto et al*.,* 2010; Olea et al*.,* 2012; Jorquera et al., 2015); being the most frequent FD caused by agents of biological origin mainly bacteria such as: *Salmonella* spp., *Campylobacter* spp*., Shigella* spp*., Clostridium perfringens, Escherichia coli, S. aureus, Bacillus cereus, Vibrio* spp*., Yersinia* spp*., Aeromonas* spp*.,* and *Listeria monocytogenes* (Daskalov,2006;Barreto et al*.,* 2010; Badui, 2015; Soto et al*.,* 2016; Fernandes et al*.,* 2018; PAHO, 2018).

The purpose of this review was to do a general presentation of FD and causative agents, in particular the genus *Aeromonas* spp., the relevant considerations in public health and food production mainly of aquatic origin, like bacterial detection and isolation methodologies from foods, as well as the actions of prevention and control of foodborne diseases; also included are the aspects of the antimicrobial resistance phenomenon, an issue of relevance for food safety and public health, which has been presented for several years in this microbial genus through various studies on foods reported around the world.

FOODBORNE DISEASES

Foodborne diseases generated by the ingestion of water and food are classified as 1. Food infections are established and multiplied in the consumer and have two aspects: a) invasive infections: where the involved microorganisms colonize tissues and organs of the affected. This group includes viruses, parasitic protozoa and bacteria such as *Salmonella* spp*., Aeromonas* spp., *Campylobacter* spp., *Shigella* spp., *Vibrio parahaemolyticus, Yersinia* spp., enteroinvasive *E. coli*, among others; b) Toxi-infections: caused by non-invasive bacteria, with the ability to colonize and multiply in the intestinal tract of the host, where they excrete their toxins, as examples: *Vibrio cholerae, Bacillus cereus* (enterotoxin producers), *Clostridium botulinum, Clostridium perfringens* and enterotoxigenic *E. coli*, and 2. Food poisoning caused by toxins produced by microorganisms that have multiplied to a certain concentration in the food, controlled by a mechanism called *quorum sensing*; some causative microorganisms are: *C. botulinum, Bacillus cereus* (emetic toxin) and *Staphylococcus aureus* (Rodriguez et al*.,* 2015).

These diseases mainly affect children, the elderly, pregnant women and people with a weak immune system. Usually damaging of the gastrointestinal system leads to symptoms such as nausea, vomiting, diarrhea, abdominal pain and fever. In some cases, there may be complications such as sepsis, meningitis, abortions, Reiter syndrome, Guillan Barré syndrome or death (Kopper et al., 2009; Soto et al*.,* 2016).

It has been determined that factors such as changes in the eating habits of a society, the consumption of packaged foods, fast foods, commercial globalization, demographic movements, emergence and adaptation of pathogens (acquisition of virulence factors, development of resistance to antimicrobials, ability to survive in adverse environments), changes in population groups at risk, climate change, complex food systems and changes in food production technology have contributed to the increase of these diseases (Vásquez, 2003; Arispe and Tapia, 2007; Olea et al*.,* 2012; Rodriguez et al*.,* 2015; Jorquera et al*.,* 2015).

Foodborne diseases and fish

Foodborne diseases are presented within a range of illnesses, in addition to having an incidence worldwide, being considered a serious public health problem. According to data from the World Health Organization (WHO), each year 420,000 deaths are generated specifically by children under 5 years of age (WHO, 2018).

Fish as food for human consumption has its source in capture fisheries and aquaculture. These activities are

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among the most important sources of food, nutrition, income and livelihoods for hundreds of millions of people around the world; only the global per capita fish supply for 2014 was 20 kg (FAO, 2016) and it is estimated that globally by 2025 consumption will reach 21.8 kg per capita per year (Fernandes et al*.,* 2018).

Fish as a food is considered a highly nutritious product since it is a source of water (66-81%), proteins (16-21%), lipids (0.2-25%), including polyunsaturated fatty acids, carbohydrates (<5%), minerals (1.2-1.5%) and vitamins of complex B, A and D (Huss, 1999) that make it part of a healthy diet and lifestyle (Huss, 1999; Fernandes et al*.,* 2018). However, this characteristic also makes it a food highly susceptible to deterioration and putrefaction due to the action of microorganisms, oxidation reactions and endogenous enzymes as well as being a target of contamination and transmission vehicles of physical, chemical or biological hazards throughout the food chain going from its primary production, processing, storage to pre-consumer manipulation, thus becoming a high-risk food for consumers' health (Huss, 1999; Ghaly et al*.,* 2010; Ramírez et al*.,* 2011; Fernandes et al*.,* 2018).

The European Food Safety Authority (EFSA) reported that the most related foods to foodborne diseases in Europe in 2016 were chicken (9%), cheese (8%) and fish (7%) (Fernándes et al*.,* 2018). On the other hand, in countries of the continent such as Spain, through the National Network of Epidemiological Surveillance (RENAVE), it was reported that in the period corresponding to 2008 to 2011, 2,342 outbreaks had been reported; the cases associated with these outbreaks were 30219, having 1763 hospitalizations and 24 deaths; the main causal agents involved were bacteria (79%), the main foods involved were egg, egg products and mayonnaise, being 24.6% of the total, followed by meat and meat products (8%), seafood (7.4%), fish and products (6.5%). The main contributing factors to these outbreaks were cross contamination (26.5%), inadequate time or temperature (20.8%) and contaminated food (18.7%) (Espinosa et al*.,* 2014).

In the American continent, and metropolitan region of Chile, from January 2005 to July 2010, 2434 outbreaks of FD were reported with 12,196 cases; the main types of food involved correspond by 15.4% to mollusks and 15.1% to fish being the biological causal agents frequently related as: *Salmonella* spp., (20.9%), *Shigella* of unspecified type (20.4%), *Shigella sonnei* (17.7%) *Vibrio parahaemolyticus*, among others (Alerte et al*.,* 2012). For the year 2013, 1,164 outbreaks were notified with 7,841 cases. Out of the total outbreaks reported in 10% of them, the causal agent was identified. *Salmonella* spp. was responsible for 54% of the outbreaks, followed by *V. parahaemolyticus* (27%). The main foods involved in the outbreaks were meals and dishes prepared by40%, followed by fish and fish products by 32% and eggs and by-products by 10% (Jorquera et al*.,* 2015).

In Mexico, gastrointestinal diseases are derived from the consumption of contaminated food and water, mainly affecting children and adults over 60 years of age; among the pathogens involved are: *Salmonella* spp*., Shigella* spp*., E. coli, Vibrio* spp*., Aeromonas* spp. The Ministry of Health (SSA) in 2001 reported that gastrointestinal diseases, caused by bacteria or parasites, were the fourteenth cause of deaths nationwide, being the states with higher incidence: Chiapas, Oaxaca, Guanajuato, Veracruz, Federal District and Puebla (Hernandez et al., 2011). On the other hand, in 2008, the Mexican Institute of Social Security (IMSS) carried out 2 million 188 hospital consultations due to gastrointestinal diseases, where the states with the highest incidence were: Chihuahua, Coahuila, Jalisco, Michoacán, Guerrero, and Oaxaca (Hernandez et al*.,* 2011).

In Caribbean countries such as Cuba in the context of diarrheal diseases associated with *Aeromonas* spp. from February 1985 to January 2005, case studies were conducted with 2322 children under 5 years of age with acute diarrheal disease. *Aeromonas* spp*.* was isolated in 166 cases (7.15%); the most frequently isolated species were *A. caviae, A. hydrophila,* and *A. veronii bv sobria* (Bravo et al*.,* 2012).

While, in South American countries such as Venezuela, in the State of Zulia, a zone marked by aquatic environments, species of *Aeromonas* spp., have been frequently reported in individuals with episodes of diarrhea, being the main enteric bacterial pathogens prevalent in the population and where *A. caviae* and *A. hydrophila* have repeatedly been recorded as the most frequent species in fecal samples analyzed in the region. In addition, these species have been isolated in fresh commercialized foods, mainly those of vegetable origin (Rincon et al*.,* 2016).

AEROMONAS' GENERALITIES

The genus *Aeromonas* spp., belongs to the family of *Aeromonadaceae* which has 24 species that include *A. hydrophila, A. caviae, A. veronii biovar sobria, A. piscicola, A. jandaei, A. schubertii* and *A. trota* frequently isolated and related to clinical affectations in human beings. These bacteria are halophilic, bacilli-shaped Gram negative of 0.3-1.0 x 1.0-3.5 µm, mobile (except *A. salmonicida* and *A. media*), non-spore forming, have an optimal growth temperature of 22 to 35°C but some species grow at intervals of 0 to 45°C; their optimum pH is 5.5 to 9, are oxidase and catalase positive, facultative anaerobes, reduce nitrates to nitrites, ferment D-glucose as a carbon source and energy in addition to maltose, D-galactose, and trehalose, can grow in media containing 0 to 4% NaCl (Castro et al*.,* 2002; Parker and Shaw, 2011; Beaz et al*.,* 2012; Suárez and Herrera, 2012; Stratev et al., 2012; PHE, 2015; Priyam et al*.,*2016; Bhunia, 2018).

The habitat of these microorganisms is generally aquatic being considered autochthonous (rivers, lakes, sea, ponds, estuaries, drinking water, chlorinated water, groundwater, and wastewater) as well as located in the intestinal tract of humans and animals (Castro et al*.,* 2002; Parker and Shaw, 2011; Beaz et al., 2012; Suarez and Herrera, 2012; PHE, 2015; Priyam et al., 2016; Bhunia, 2018; Fowoyo and Achimugu, 2019). The isolation of these bacteria in water samples depends on several factors: the season of the year, concentration of organic matter, available oxygen, levels of chlorine and salinity (Castro et al*.,* 2002).

These bacteria present a variety of virulence factors to generate disease, including structural components such as flagella, pili, capsule, S layer, lipopolysaccharide (LPS), outer membrane proteins and extracellular products such as adhesins, hemolysins, cytotoxic enterotoxins, proteases, lipases, deoxyribonucleases, ribonucleases, siderophores as well as biofilm formation capacity (Castro et al*.,* 2002; Suárez and Herrera, 2012; Priyam et al., 2016; Bhunia, 2018; Fowoyo and Achimugu, 2019).

Aeromonas spp*.,* present pathogenicity towards different living beings such as: amphibians, fish, reptiles and humans. The symptoms in humans related to infection caused by *Aeromonas* spp. are gastroenteritis (diarrhea), septicemia, infection of skin and tissues, pneumonias, ocular and urinary tract infection, as well as complications in some cases such as hemolytic uremic syndrome (HUS) (Pascual and Calderón, 1999;Parker and Shaw, 2011; Beaz et al., 2012; Bravo et al., 2012; Suarez and Herrera, 2012; Stratev et al., 2012; FDA, 2012; Priyam et al., 2016; Bhunia, 2018).The strains causing diseases in fish are *A. salmonicida* and *A. hydrophila* and in humans are: *A. hydrophila, A. sobria, A. caviae, A. veronii, A. jandae* and *A. schubertii.* They affect all population groups with emphasis on children, the elderly and immunosuppressed; the infectious dose is not completely known, with an estimated increase of 10 colony-forming unit (CFU) for *A. hydrophila* up to 10^7 CFU / g of food (Parker and Shaw, 2011; Beaz et al*.,* 2012; Bravo et al*.,* 2012; Pascual and Calderón, 1999; Suárez and Herrera, 2012; FDA, 2012; Priyam et al., 2016; Bhunia, 2018).

Presence and isolation of *Aeromonas* spp., have been reported in a variety of foods such as meat products, fish, seafood, prepared foods, confectionery products, vegetables, milk and dairy products that act as potential vehicles for infection (Castro et al*.,* 2002; Castro et al*.,* 2003; Daskalov, 2006; Stratev et al., 2012; FDA, 2012; Sanchez et al*.,* 2018; Fowoyo and Achimugu, 2019).

Aeromonas and fish

The genus *Aeromonas* spp*.,* in the products of fishing and aquaculture is of relevance since it is a generator of diseases such as Aeromonas (Ascites) in fish. It presents two different symptoms, one called maculosa with red cutaneous spots with different shapes and sizes and the other ascetic of greater severity with lesions, necrosis and tissue loss. Healthy and cured carriers do not present clinical symptoms, but can transmit the disease which can appear individually or epidemic, especially in pond culture where species such as: *A. salmonicida, A. hydrophila A. veronii, A. sobria, A. caviae,* and *A. bestiarum* have been related as causal agents of high mortalities and economic losses in food production (Acha and Szyfres, 2001; Balbuena et al*.,* 2011; Stratev et al.,2012; Pridgeon and Klesius, 2011; Samal et al*.,* 2014; Zepeda, 2015). The treatment for these cases is the application of antibiotics such as streptomycin and sulfonamides orally in the feed (500 mg / kg of fish). However, it is not very effective when the disease has infested a considerable percentage (> 10% of the organisms in the pond); where it is preferable to eliminate the lot, to avoid propagation (Balbuena et al*.,* 2011).

This bacterial genus has also been related to food spoilage due to its psychrotrophic growth properties during storage stages at refrigeration temperatures (Hernández, 2016; Bhunia, 2018). Psychrotrophic microorganisms are considered as microbial indicators of food quality. Noting storage conditions, sources of contamination and possible shelf life of food mainly in those that require refrigeration conditions (Hernandez, 2016). Psychrotrophs grow at temperatures below 7°C for 7 to 10 days of culture. The genus of microorganisms most frequently isolated in foods are *Pseudomonas, Alcaligenes, Flavobacterium, Enterobacter, Proteus, Aeromonas, Lactobacillus* among others. They are involved in the alteration and decomposition and synthesis of pigments of foods of a protein nature (chicken, meat and fish) (Hernandez, 2016).

Microbial spoilage is the main mechanism of deterioration in the quality of fresh and frozen fish (Parlapani et al., 2013). The microorganisms are located in the skin, gills and intestines of live and freshly caught fish. The microbiota in freshly caught fish depends more on the capture environment than on the species (Huss, 1999). In warm water fish, there is a variety of mainly gram-negative psychotropic bacteria such as *Pseudomonas* spp., *Acinetobacter* spp*., Moraxella* spp*., Flavobacterium* spp*., Shewanella* spp*., Vibrionaceae* spp*.* and *Aeromonas* spp., which are part of the initial fish microbiota; being only a small fraction of fish microbiota identified in deterioration processes such as: *Pseudomonas* spp*., Shewanella* spp*., Photobacterium* spp*., Moraxella* spp*., Acinetobacter* spp., and *Aeromonas* spp. (Huss, 1999; Parlapani et al., 2013; Buhnia, 2018).

In the deterioration of fish, microbial activity plays an important role in their shelf life, where it can be included that the fish may be contaminated with pathogenic

microorganisms that put the health of consumers at risk. Microorganisms of a pathogenic character can be species whose natural habitat is water and where the temperature has a selective effect, such as *Vibrio* spp*., Aeromonas* spp*., Plesiomonas* spp*., C. botulinum*, among others, and those microorganisms that are present in the water due to contamination of fecal origin and associated to the manipulation process to which the fish is later subjected as: *E. coli*, *Salmonella* spp., *Shigella* spp., *L. monocytogenes*, *S. aureus* (Huss, 1997; Ramírez et al*.,* 2011; Sánchez and Delgado, 2016; Soto et al*.,* 2016). It should be noted that factors such as the time of year of capture or harvest, the characteristics of the diet, geographical area, species of fish, sanitary quality of the water and the capture system influence qualitatively and quantitatively microbiological aspects present initially in fish; the handling and storage hygiene conditions can influence the microbiota responsible for deterioration and pathogenicity (Ramirez et al*.,* 2011; Sánchez and Delgado, 2016; Soto et al*.,* 2016, Fernandes et al*.,* 2018).

In the case of bacteria of the genus *Aeromonas* spp*.,* the detection of species such as *A. hydrophila, A. jandaei, A. veronii, A. popoffii, A. eucrenophila, A. caviae/A. media, A. schubertii, A. eucrenophila,* and *A. salmonicida* has been reported in the microbiological analysis of fish products (Suárez and Herrera, 2012; Soto et al*.,* 2016; Samal et al*.,* 2014; Praveen et al*.,* 2016). Being indigenous environmental microorganisms of water, pathogens of fish and humans, capable of tolerating low temperatures (-2 to 10°C), this genus has been considered important for animal health (fish) and public health (Balbuena et al*.,* 2011; Hernández et al*.,* 2011; Samal et al*.,* 2014; Zepeda, 2015; Praveen et al*.,* 2016).

MICROBIOLOGICAL ANALYSIS FOR THE ISOLATION AND DETECTION OF *AEROMONAS* SPP.

The isolation and identification of microorganisms in a traditional way is through different techniques such as the observation of macroscopic, microscopic morphology, and biochemical or phenotypic characteristics (Bou et al*.,* 2011; Sánchez et al*.,* 2017). Different procedures have been developed for the determination in meat foods of species of *Aeromonas* spp*.*, some of them standardized as it is developed by the Department of Agriculture of the United States of North America (USDA, 2018) or the Department of Public Health of England (PHE, 2015).

Aeromonas spp*.* are microorganisms that can grow in different common culture media including those differential and selective media used in the isolation of Gram-negative bacteria (Castro et al*.,* 2002; Sanchez et al*.,* 2017). In the isolation and detection in fecal matter, water and / or food, for example, the cefsulodin irgasan novobiocin agar, ampicillin blood agar, ampicillin-dextrin agar and *Aeromonas* agar which contain the selective agents such as bright green and irgasan, can be used. Direct plate count or previous enrichment in liquid medium followed by sowing in selective solid culture medium (Pascual and Calderon, 1999; Sharma et al*.,* 2010; Zepeda, 2015; Sánchez et al*.,* 2017) and subsequent identification by complementary biochemical tests of genus and species (Table 1). Figures 1 and 2 show the methods used for the isolation and identification of *A. hydrophila*; for food samples, a pre-enrichment broth is suggested, an option may be alkaline peptone water to pH 8.7, incubated at 37°C / 18-24 h and then sown on bile-irgasan-bright green agar (Castro et al*.,* 2002; Sharma et al*.,* 2010). While for the analysis of water samples, the use of the membrane filtration technique and subsequent culture in agar dextrin ampicillin agar, without pre-enrichment is effective and allows isolation (Castro et al*.,* 2002). Likewise, for the isolation and rapid identification of *Aeromonas* species, chromogenic culture media have been developed and used with good results (Viera et al*.,* 2016) (Table 2).

For the biochemical identification of isolates, in addition to traditional tests, commercial systems can be used, such as API, or automated systems such as Vitek, GNI-CARD, BBL Crystal, DD Enteric/Nonfermenter (Feng, 2001; Castro et al*.,* 2002; Priyam et al., 2016).

The profile of sensitivity to antibiotics of the bacterial isolates obtained can be made through the disc diffusion method using Mueller-Hinton agar and microbroth dilution method using cation-adjusted Mueller-Hinton Broth as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (Priyam et al., 2016). These tests in recent years have become a must as part of the characterization of pathogenic isolates of clinical, environmental and food origin.

In spite of having a variety of biochemical tests, it is sometimes necessary to have faster and more specific results. In addition, the absence of correlation between the observable morphological and / or phenotypic characteristics of the isolate under study and those corresponding to the strain of the type species can be presented in the obtaining of results, making the phenotypic methods perform the most probable identification and not definitive. To solve these cases genetic methods have been developed in the microbiological analysis, these being considered complementary or alternative procedures to the traditional procedure (Bou et al*.,* 2011; Sanchez et al*.,* 2018).

A variety of genes have been used as molecular targets in taxonomic studies or phylogeny in different genre and different bacterial species including *Aeromonas;* so the identification and genetic differentiation can be made by using markers such as: 16S rRNA, *gyr*B (subunit B DNA gyrase), *rpo*D (factor σ), *rpo*B (subunit β, DNA dependent on RNApolymerase) and DnaJ (head of protein shock 40) (Porteen et al., 2007; Sharma et al*.,* 2010; Zepeda, 2015; Yang et al*.*,

Table 1. Biochemical tests for the identification of microorganism of the genus *Aeromonas* spp. (Castro et al., 2002; Romero, 2007; PHE, 2015).

Test	Result
Gram stain	Negative Gram
Oxidase	(+)
Catalase	(+)
Growth in nutritious broth with 3 or 6% of NaCl	Difference of *Vibrio* spp., which are positive growth in 6% NaCl, *Aeromonas* spp., only grows at 3% NaCl
Growth on thiosulfate citrate bile sucrose agar (TCBS)	Positive growth yellow colonies.
Sensitivity to pteridine O129	Difference from *Vibrio* spp., where *Aeromonas* spp., are resistant.
Production of acid from inositol	Negative reaction for *Aeromonas* spp., difference of *Plesiomonas* spp., which give a positive reaction
Oxidation-fermentation in Hugh Leifson medium (O/F) with glucose	Difference of *Pseudomonas* spp., glucose fermentation is positive for *Aeromonas* spp.
Voges-Proskauer	Difference of *Vibrio* spp., *Aeromonas* spp., have positive reaction

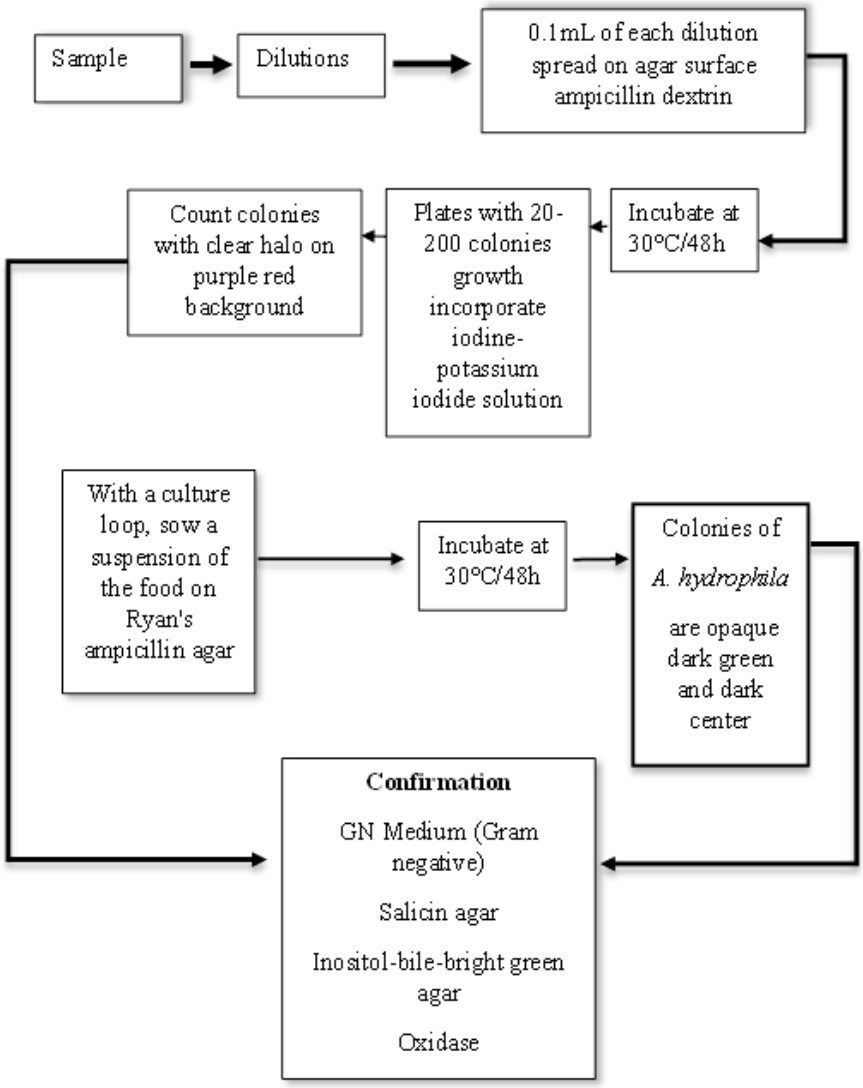

Figure 1. Direct plate count of *A. hydrophila* in food (Pascual and Calderon, 1999).

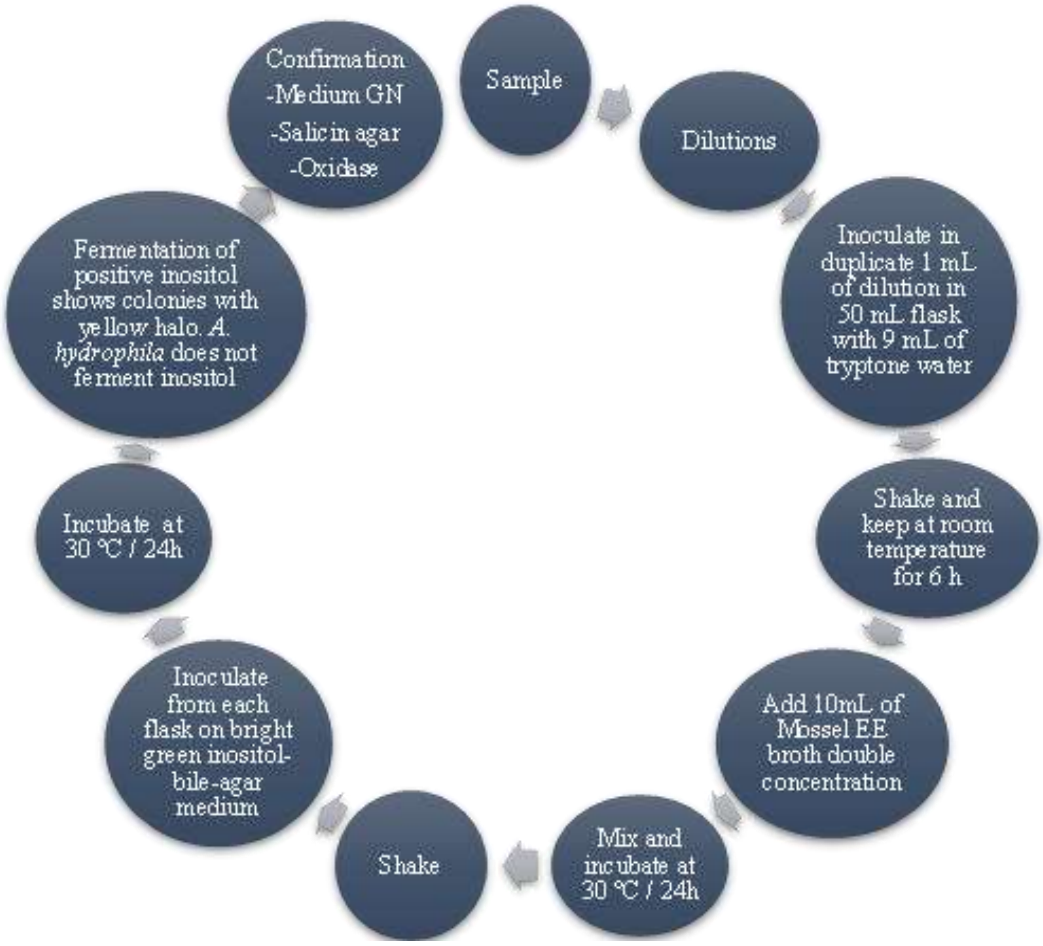

Figure 2. Presence-absence by selective enrichment of *A. hydrophila* in food (Pascual and Calderon, 1999).

2017; Sánchez et al*.,* 2018; Fowoyo and Achimugu, 2019). Also, molecular methods such as: Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), Multiple-Locus Variable Number Tandem Repeat Analysis (MVLA), Fluorescent Amplified Fragment Length Polymorphism (FAFLP) and Whole Genome Sequencing (WGS) have been used as epidemiological analysis tools for the strain typing (PHE, 2015; Teng et al., 2017). Also, through the study and characterization of the set of proteins expressed by a genome (proteomics) using the Mass Spectrometry by Matrix Assisted by Laser Desorption-Ionization of Flight Time (MALDI-TOF) is able to identify species of *Aeromonas* for clinical diagnosis and a rapid decision making (PHE, 2015; Sánchez et al*.,* 2017; Sanchez et al*.,* 2018).

Likewise, the analysis of *A. hydrophila* based on systems for rapid detection in culture media (<2 h) has been developed, with the use of an electronic device, detecting the changes of patterns of volatile organic compounds generated during growth. Considering the authors that this methodology has potential for the detection of microorganisms; where rapid detection of pathogens is essential for the diagnosis of associated infections and food safety (Fujioka et al., 2013).

Control and prevention of diseases by *Aeromonas* spp.

Agricultural production is considered a key point in the economy worldwide, mainly in developing countries, and food safety is of vital interest for its economic and social development. The safety of food has been established as a fundamental attribute of quality which is generated in primary production and mobilizes the other phases of the food chain such as processing, packaging, transport and preparation of the product (Tafur, 2009).

Food safety is currently one of the main challenges in the agri-food industry; producers and health regulatory

Table 2. Biochemical tests for the identification and selection between species of genus *Aeromonas* (Pascual and Calderon, 1999; Castro et al*.,* 2002; Priyam et al., 2016).

Test	*A. hydrophila*	*A. caviae*	*A. media*	*A. salmonicida*	*A. jandaei*	*A. schubertii*
Oxidase	+	+	+	+	+	+
Lysine decarboxylase	+	-	-	V	+	V
Ornithine decarboxylase	-	-	-	-	-	-
Voges-Proskauer	+	-	-	V	+	V
Arginine hydrolase	+	+	+	V	+	+
Glucose gas	+	-	-	V	+	-
D-mannitol	+	+	+	+	+	-
Sacharose	+	+	+	+	-	+
Indol	+	+	+	+	+	V

V, variable reaction; +, positive reaction ; -, negative reaction.

agencies are in charge of its surveillance, so within this context there is a continuous demand for the strengthening of regulatory frameworks, quality standards, inspection and control (Tafur, 2009; Gutiérrez et al*.,* 2017).

Around the world, regulations have been established on hygiene and sanitary quality of food. In Europe through Regulation (CE) 178/2002 of the European Parliament and the Council of the European Union, the principles and general requirements of food legislation were established, creating the European Food Safety Authority (EFSA) and procedures related to food safety subsequently through Regulation CE 853/2004 established the hygiene standards of foods of animal origin, including fish. Likewise, in Regulation CE 854/2004 the specific rules for the organization of official controls of products of animal origin destined for human consumption were presented. Moreover, Regulation CE 2074/2005 and Regulation CE 2406/96 set out the visual examinations and freshness criteria for fishery products and establish common marketing standards for certain fishery products, respectively.

Specifically, in pathogens such as *Aeromonas* spp., in the Netherlands, health authorities established maximum values for the density of these bacteria in drinking water, being for water in purification plants 20 CFU/100ml and for distribution water 200 CFU/100 ml (Acha and Szyfres, 2001).

In Mexico, the official Mexican standard “NOM-242-SSA1-2009”, establishes the sanitary requirements of mandatory observance in the national territory for producers engaged in the capture, extraction, processing, conservation, storage, distribution, transportation, sale or import of fishing products (NORMA Oficial Mexicana, 2009). It is mentioned that the sanitary microbiological specifications for microbiological indicators and pathogens such as fecal coliforms and / or *E. coli* must be as maximum limit of 400 MPN / g, *Salmonella* spp*.,* must be absent in 25 g, as well as *Vibrio cholerae* O:1 and No O:1 absent in 50 g. However, this regulation does not mention specifications regarding *Aeromonas* spp.

As part of the measures for the prevention of diseases by *Aeromonas* spp. and other pathogens specifically in primary production, basic and documentary operating conditions and practices are required, such as good agricultural, livestock and aquaculture practices in the latter, with an emphasis on avoiding overpopulation, adverse environmental factors (increased organic matter and decreased dissolved oxygen), change and monitor water quality, regulation of antibiotic use, adequate climate control and nutrition necessary for food production (Acha and Szyfres, 2001; Tafur, 2009; Balbuena et al*.,* 2011; Fernandes et al*.,* 2018). While in the food industry involving the stages of fish handling and processing, it is required to establish control and prevention procedures such as good manufacturing practices and hazard analysis and critical control point (HACCP) systems (Tafur, 2009; PAHO, 2016; Fernandes et al*.,* 2018). Likewise, different food safety management systems have been developed and implemented at a global level within the scope of food production and commercialization, including fish products such as: Global Food Safety Initiative (GFSI), British Retail Consortium (BRC), International Food Standards (IFS), ISO 22000, Quality Certification Services (QCS), FSSC 22000, Safety Quality Food (SQF), Global Aquaculture Alliance (GAA)/Aquaculture Certification Council (AAC), GlobalGAP or PrimusGFS (Racua, 2018).

Within the processes of control and prevention of diseases involve the continuous participation of government authorities, agro-industry, academia and consumers and should be considered a priority (Kopper et al*.,* 2009; Tafur, 2009; Arispe and Tapia, 2007). The foregoing should involve the adoption of practical

measures in the businesses themselves relating to the technical innovations of the processes, models of productive organization, administrative management, and investment for the improvement of the work infrastructure. In addition, health authorities and other related institutions should promote training and propaganda campaigns for the adoption and implementation of actions for the safe manipulation or processing at the commercial or household level of food (Kopper et al*.,* 2009).

In this sense, the prevention through health education of consumers and food handlers is key to preventing the occurrence of diseases by *Aeromonas* spp., so it is promoted to avoid the consumption of raw fish and shellfish, avoid cross contamination between foods raw and cooked, do not use seawater, use potable water, maintain cleanliness of food preparation area, food handler must maintain personal hygiene, complete cooking of vegetables and meats, keep food at safe temperatures, consume milk and pasteurized derivatives (Acha and Szyfres, 2001; WHO, 2007;Priyam et al.,2016; González et al*.,* 2018).

Resistance to antimicrobials by *Aeromonas* spp.

In general, diarrheal processes derived from infection by *Aeromonas* spp*.,* in healthy individuals are self-limiting and are cured in a few days with diet and oral rehydration; of requiring treatment with antibiotics, cephalosporins, quinolones, tetracyclines, chloramphenicol and aminoglycosides can be used in treatment of infections (Castro et al*.,* 2002; Romero, 2007; Priyam et al., 2016). However, the resistance of these microorganisms to several antibiotics such as beta-lactams, including penicillin, ampicillin, carbenicillin, ticarcillin and cephalothin, has been reported for several years (Castro et al*.,* 2002; Priyam et al., 2016).

Antimicrobial resistance is the ability of a microorganism to resist the inhibitory or killing activity of an antimicrobial growth beyond the normal susceptibility of specific bacterial species (Verraes et al*.,* 2013). At present, this phenomenon constitutes a threat and a global issue in human and animal health due to the increase in morbidity and mortality rates due to infections, dilation of the disease and increased health costs (Cabello, 2004; Puig et al*.,* 2011; FAO, 2018). This phenomenon affects food safety, food security, social and economic well-being (Verraes et al*.,* 2013; FAO, 2018).

It has been identified that resistance in bacteria to antimicrobials is generated by the combination of several factors such as the inappropriate use of antimicrobials in humans and animals; where in primary animal production these compounds are used for therapeutic, prophylactic and / or growth promotion purposes (Cabello, 2004; Puig et al., 2011; Verraes et al*.*, 2013; Stratev and Odeyemi, 2016). It has been reported that in livestock activities including aquaculture, the use of antibiotics as prophylactic is probably the main purpose; and it has also been shown that the prophylactic use of antibiotics is unnecessary and dispensable and can be replaced by good hygiene practices, without consequences for animal health and industrial economy (Cabello, 2004).

The transfer of resistance to antibiotics by microorganisms such as bacteria is through genetic material through plasmids, insertion sequences (IS), integrons, transposons (Tn) and bacteriophages through conjugation, transformation or transduction, which can occur in any environment from soil, water, food, human and animal digestive system (Sánchez et al*.,* 2012; Verraes et al*.,* 2013); it generates different mechanisms to resist the action of antibiotics such as the alteration of membrane permeability, ejection pumps, enzymatic inhibition and the modification of the attack target (Tafur et al*.,* 2008; Puig et al*.,* 2011).

Several studies have been conducted indicating the incidence of different pathogenic microorganisms with this characteristic from foods such as *Salmonella* spp*., E. coli, Campylobacter* spp., *Listeria* spp., *S. aureus, Aeromonas* spp., among others, highlighting the potential risk to health in the consumption of these foods generated under inadequate hygiene practices (Puig et al*.,* 2011; Sasidharan et al*.,* 2011; Nagar et al*.,* 2011; Rahimi et al*.,* 2012; Stratev and Odeyemi, 2016). Microbiological studies in foods around the world, in the particular case of fish, have reported the isolation of strains of *Aeromonas* spp., with resistance to different antimicrobials. Castro et al*.* (2003) in an analysis of samples of frozen Tilapia (*Oreochromis niloticus*) sold in markets in Mexico City, reported 82 strains of *Aeromonas* spp*.,* with *A. salmonicida* being the most frequent followed by *A. hydrophila, A. veronii bv. sobria, A. bestiarum, A. encheleia* and *A. caviae.* These strains showed resistance to antibiotics such as ampicillin, penicillin, cephalothin, and clindamycin. The studies concluded that the presence of these microorganisms in food, especially when consumed raw, represents an important risk to public health (Castro et al*.,* 2003). Ashiru et al*.* (2011) carried out the microbiological analysis and antibiotic profile of *Aeromonas* spp*.,* isolated from species in Tilapia and Bagre fish commercialized in markets of Makoko, Nigeri, for which they reported the presence of *A. caviae, A. hydrophila* and *A. sobria* where all of those were resistant to tetracycline, nitrofurantoin and augmentin but susceptible to pefloxacin, ofloxacin and ciprofloxacin.

On the other hand, in a study of 60 samples of live fish (common carp) and frozen fish collected from 15 local markets in the city of Baghdad, focused on the isolation of *A. hydrophila* to determine susceptibility to antibiotics, it was reported that 65% of samples were positive for the isolation of *A. hydrophila*, 76.6% were samples of live fish

and 53.3% in frozen fish, and all isolates were 100% resistant to penicillin, ampicillin, cloxacillin and bacitracin. Other antibiotics were oxytetracycline with 56.5%, tetracycline 33.4%, cefoxitin 30.8%, chloramphenicol, kanamycin 28.2%, and finally streptomycin and rifampicin in 23.1 and 15.4% respectively (Alzainy, 2011). On the other hand, Roy et al. (2013) collected samples of the fish *Lepidocephalicthys guntea* and water from the Lotchka River of Darjeeling District, West Bengal, India, with the purpose of isolating strains of *Aeromonas spp.* and analyzing their resistance to antimicrobials, reporting the isolation of 49 strains of *Aeromonas* spp., which showed high resistance to ampicillin, penicillin, cephalothin and erythromycin and minimal resistance to ciprofloxacin and tetracycline.

In all the previous studies, the authors agree on the potential risk to public health posed by the handling and consumption of these foods (mainly raw), which are contaminated with antibiotic-resistant pathogens and are marketed in different regions of the world. This involves the need to implement research actions and regular surveillance of the phenomenon of antimicrobial resistance in fish and aquatic production environments intended for human consumption by various social sectors such as government, nongovernmental and academic organizations.

CONCLUSION

Foodborne diseases are considered as serious and significant public health problem at a global level due to their incidence and mortality, mainly in children, together with the fact that different causative agents of biological origin, mainly bacteria, have shown resistance to different antibiotics used in the treatment of diseases.

The food safety is considered a preponderant factor in the production, processing, conservation, distribution and handling of food, which is put at risk due to the presence of various causative agents, which may be of physical, chemical or biological origin, the latter being the most frequently related to disease outbreaks.

Fish and products around the world are considered a source of food of good nutritional quality and economic development for human beings; however, due to its composition, fish is highly susceptible to deterioration and contamination along the food chain, making it a potential vehicle for disease transmission.

The biological agents that cause diseases through foods that are mostly related are bacteria; species of the genus *Aeromonas* spp., whose natural habitat is an aquatic environment, is considered important in terms of human health, animal health and food due to the different diseases generated, sources and forms of transmission.

In order to reduce, control and prevent the incidence of diseases through food by *Aeromonas* spp., recommendations and practices have been developed and proposed around the world by different international organizations in the field of health and food that involve the control and prevention of diseases along the food chain (from the farm to the consumer's table); they range from good aquaculture practices, good hygiene practices, traditional and molecular microbiological analysis methods in the laboratory to foods with a risk of contamination as well as specifications or microbiological limits of these microorganisms in food or water for human use or consumption. Likewise, actions focused on the handling and hygienic preparation of food at the household level have been developed and promoted in such a way that the final manipulators and consumers, along with governmental entities, academia and food industry, contribute to the protection of public health with a supply of nutritious and safe foods for people.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Application of nitrogen in different phenological states of the corn crop

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Corn is a crop of high productive potential, but to achieve this, it is essential to apply production technologies, especially fertilizers with a high level of nitrogen. The objective of this work was to evaluate the influence of nitrogen fertilizer in different phenological stages on corn production. The experiment was installed in the experimental plot of the Facultad de Ciencias Agrarias, Universidad Nacional de Concepción, Concepción Department, Paraguay. The experimental design was randomized blocks, with 5 treatments and 4 repetitions. The treatments consisted of the application of nitrogenous fertilizer in the phenological stages V0, V3, V5, V7 and V9. Number of leaves on and below the spike, number of seed lines per spike, plant height, spike height and yield were evaluated. The obtained data was subjected to the analysis of variance by Fisher's test at 5%, when significant differences was detected, it was proceeded to compare the means obtained by the effect of the treatments using the Tukey test at 5% of significance. The results indicate that the application of nitrogen in different phenological stages of corn cultivation influenced the height of the plant, height of insertion of the spike and yield; not so, for: number of leaves above and below the spike and number of seed lines per spike. It is concluded that the application of nitrogen in different phenological stages of corn, has influence on productive characteristics of the crop. It was recommended that it should be done in phenological stage V5, to achieve maximum efficiency.

Key words: *Zea mays* L., phenological stages, nitrogen, fertilization.

INTRODUCTION

Corn (*Zea mays* L.), is the cereal with the highest volume of production on the planet, with about 960 million tons; and of the 5 main world producers, 3 of them are in America: the United States, Argentina and Brazil (FAO, 2012).

It is a crop that requires adequate supply of nitrogen from the early stages of growth, as it is one of the most limiting nutrients for obtaining high productivity, and fertilization recommendations depend on various factors, such as plant support, extraction/export of nutrient by the crop,

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capacity of supply of nitrogen by the soil and index of use of nitrogen fertilizer by the plants (Sousa and Lobato, 2004).

The criteria to define the quantity of N to be supplied normally are based on the expectation of yield, the history of the area, soil type, organic matter content, N mineral content or potentially mineralized, previous crops and in the use or not of green fertilizers (Amado et al., 2002; Cantarella and Duarte, 2004).

The definition of time of application of N and the dose that coincide with the needs of corn in different environmental conditions and soil types are very important since these factors improve nutrients, absorption and decrease N losses (Androski et al., 2000).

The productive potential of corn cultivation is defined around the phenological stages V4 and V5, of four and five expanded leaves, respectively, due to the floral differentiation, in this period the plant originates the primordia of the banner and the spike, and differentiation of all leaves also occurs (Ritchie et al., 1993).

In state V3, the apex of the stem (point of growth) is still below the surface of the soil. In state V5, the apical meristem is still below the surface of the soil, which allows the plant to recover from the damage caused in the aerial part (Ritchie et al., 1993; Magalhães and Durães, 2006). In the V7 state, the adventitious roots are the main functional system, some tillers are visible. In the V9 state, the panicle develops rapidly and the stem continues a rapid elongation through the elongation of its internodes. Each internode will start the elongation before the one that is above it on the stem, similar to the initial development of the spike primordia (Magalhães and Durães, 2006).

The objective of this work was to evaluate the influence of nitrogenous fertilizer in different phenological stages.

MATERIALS AND METHODS

The experiment was installed in the experimental plot of the Faculty of Agricultural Sciences, National University of Concepción, Concepción Department, Paraguay, at the coordinates (230 40'13 "South, 570 41'85" West, elevated 160 m above sea level). The experiment was conducted between the months of March and July 2017.

The climate of the area is characterized by an average temperature of 26 and 14°C, depending on the season, with maximum that can reach 45°C in summer and minimum of up to 4°C in winter, with slight incidences of frost (DMH - DINAC, 2016).

According to the analysis performed, the soil of the experimental area, presents the following chemical and physical characteristics in the depth of 0 to 0.20 cm: pH (H_2O) 5.67; organic matter (Walkley Black): 1.67%; Ca + 2, Mg^{2+} and K^{+}: 5.06, 1.27 and 0.19 cmol/LS, respectively; P (Mehlich) and S: 28.94 and 11.73 mg/LS, respectively; Al + 3: 0.05; CIC: 9.71 cmol/LS; V: 67.21%. The soil texture is sandy loam (CETAPAR, 2017).

The experimental design used was randomized complete block with 5 treatments and 4 replications. The treatments consisted of five moments of application of nitrogen (N) in coverage (phenological stages V0, V3, V5, V7 and V9). The phenological stages V0, V3, V5, V7 and V9 correspond to the periods in which the plant presents 0, 3, 5, 7 and 9 fully expanded leaves, respectively (Magalhães and Durães, 2006).

The soil preparation was carried out prior to planting, with a light harrow. Subsequently, the experimental area was delimited, each unit had 14.4 m^2 (4 m × 3.6 m), and it composed of four 4 m long rows and the spacing of 0.90 m between rows and 0.4 between plants. The seed used was the hybrid DKB 7910 VT3 PRO (DEKALPAR, 2017).

The dose used was 77 kg ha^{-1} of N for all treatments, according to soil analysis, using urea as a source of the nutrient. The fertilizer application was made by incorporating it in a localized way at a distance of 0.10 m from the plant. The crop was kept free from weeds by an herbicide application. Glyphosate was used according to recommendations of the Technical Guide DEKALB (2012).

For data collection, the two central rows of each experimental unit were considered. The following variables were evaluated:

(1) Number of leaves above the spike and number of leaves below the spike: the corn leaves were counted at the end of the crop cycle, in 6 plants randomly chosen from the selected area; according to the methodology used by Ángeles et al. (2010).
(2) Number of seed lines per spike: 10 spikes of each experimental unit were selected and the lines of each spike counted; according to the methodology used by Gott et al. (2014).
(3) Height of plant: This was obtained with a metric belt, whose measurements were made in 6 plants randomly chosen from the selected area; according to the proposal of Mar et al. (2003).
(4) Spike height: This was determined in the final cycle of the crop, measuring from the base of the plant until the insertion of the first spike, obtaining the value in cm according to Mar et al. (2003).
(5) Yield: This was obtained by means of weighing the harvested grains in the selected area, with humidity of 13.0% and the data were expressed in kg ha^{-1}; based on the methodology employed by Gott et al. (2014).

The obtained data were subjected to analysis of variance by the Fisher test at 5% of significance, when significant differences were detected, it was proceeded to compare the means obtained by the effect of the treatments using the Tukey test at 5% of significance.

For the statistical analysis, the AgroEstat System was used (Barbosa and Maldonado, 2015).

RESULTS AND DISCUSSION

Table 1 shows the average values of the analyzed variables whose data indicate that there were no statistical differences in any of the evaluated characteristics. The highest values of number of leaves above the spike occurred with the application of N in stage V3; for the variable of number of leaves underneath the spike was observed more leaves with the application of nitrogen fertilizer in the V5 phenological stage, with averages of 5.90. In the variable, number of seed lines per spike was obtained. The highest result with 15.85 grain lines per spike, with nitrogen fertilization in the V0 stage.

Gott et al. (2014), evaluating the sources and times of application of nitrogen in late summer - early fall corn production, observing that the number of seed lines per spike was not affected by the factors, reaching an average of 15.5 with similar results obtained in the present work with an average of 15.52. In contrast, Kappes et al. (2009), worked with corn in late summer - early fall production, in soybean succession, evaluated

Table 1. Means of the characteristics of number of leaves above the spike, number of leaves underneath the spike and number of seed lines per spike of the evaluated corn crop.

Phenological stage	Characteristics		
	Number of leaves above the spike(ns)	Number of leaves underneath the spike(ns)	Number of seed lines per spike(ns)
V0	5,75	5,65	15,85
V3	5,85	5,70	15,05
V5	5,80	5,90	15,80
V7	5,55	5,35	15,50
V9	6	5,10	15,40
GM	5,79	5,54	15,52
CV	7,05	7,73	5,71
LSD	0,52	0,96	1,99

(ns): Not significantly by Fisher test at 5% of significance; GM: general mean; CV: coefficient of variation; LSD: least significant difference by Tukey test at 5% of significance.

Table 2. Means of the characteristics of plants height, spike insertion height and yield, of corn crop submitted to the application of nitrogen fertilizer in different phenological stages.

Phenological stage	Characteristics		
	Plant height (**)	Spike insertion height (**)	Yield(**)
	cm	cm	kg ha^{-1}
V0	153.10^{b}	65.5^{b}	5885.38^{c}
V3	160.10^{ab}	71.15^{b}	6557.48^{b}
V5	177.46^{a}	86.15^{a}	7359.26^{a}
V7	165.90^{ab}	73.75^{b}	6448.24^{bc}
V9	157.05^{b}	68.15^{b}	6097.44^{bc}
GM	162.72	72.94	6469.56
CV	5.28	5.92	3.89
LSD	19.38	9.73	568.24

(**)Significant by Fisher test at 5% of significance; GM: general mean; CV: coefficient of variation; LSD: least significant difference by Tukey test at 5% of significance.

the performance of the crop in the function of different times of nitrogen application when the plants have three, seven and ten fully expanded leaves and sources of nitrogen (urea, ammonium sulfate and Entec®) in cover, whose results influenced, in terms of time, on the number of seed lines per spike.

As shown in Table 2, there are differences in the function of nitrogen fertilizer in different phenological stages, both for the plant height, spike insertion height and yield of corn.

As shown in Table 2, based on the determinations evaluated in the V5 phenological stage, the best behavior was obtained in relation to the other phenological stages. In the plant height characteristic, the V3, V5 and V7 stages are statistically equal and superior to V0 and V9 stages; while V0 stage presented the lowest value.

Cazetta (2010), evaluating the influence of the times of application of N on the agronomic characteristics and efficiency of nutrient use in the corn crop, concludes that V4-V5 stages with 120 kg ha^{-1} of nitrogen application was the best for plant height, as well as spike insertion height.

Marschner (1995), reports that the application of ideal doses of N in the initial stages of development (2 to 4 leaves), in cereal crops, it increase the production of growth promoting phytohormones and responsible for the development by the processes of division and cellular expansion (gibberellins, auxins and cytokinins), increasing the size of the stem and consequently, the height of the plants.

The best performances were obtained in V3, V5 and V7 stages; these results differ from those found by Mar et al. (2003), who evaluating some components of corn crop production (AG 3010) as a function of doses of N (30, 60, 120 and 150 kg ha^{-1}) in Urea form and application times (1/3 at planting and the remaining 2/3 when the crop presented four, eight and ten fully expanded leaves,

respectively). They demonstrated that the best period of application of N in coverage was in the state with four to eight fully expanded leaves with doses of 90 to 120 kg ha^{-1} of N for the variables of number of rows per spike and grain productivity.

According to Gott et al. (2014), evaluating the sources and times of application of nitrogen in late summer - early fall corn, obtained the best results in V10 phenological stage with 6,183.9 kg ha^{-1}; however, in this investigation it was found that 7359, 26 kg ha^{-1} in V5 stage. Ritchie et al. (2002), mention that the formation of the total number of grains per corn cob is defined during vegetative V6 and V12 stages. The nutritional level, particularly of N, that is present during this period is an important regulator of the total number of grains and consequently of the total accumulation of yield.

Sangoi et al. (2007), investigating corn response at the time of application of nitrogen fertilizer in two systems of cultivation in a soil with high content of organic matter, achieved an increase in corn yield with the treatment where it was applied in V5 stage, coinciding with the results obtained in this work.

CONCLUSIONS AND RECOMMENDATIONS

The application of nitrogen in different phenological stages of corn has influence on productive characteristics of the crop. It is recommended to do it in phenological stage V5, to achieve maximum efficiency. Work is currently being done on organic and mineral fertilization in the corn crop, which will be subject to review and evaluation in a short time.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effects of biogas slurry and conventional fertilizer on the abundance, diversity, and function of the soil microbe community in continuously cropped Chinese chives

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The effects of biogass slurry and conventional chemical fertilizer as topdressings on relative abundance and community diversity of soil microbes in fields under continuous cropping of Chinese chives were investigated. It was found that topdressing treatment altered soil bacteria community and diversity, where relative abundance and taxonomic diversity were greater in the biogas slurry treatment. Although average well color development was higher in the conventional fertilizer treatment than in the biogas slurry treatment and the control, indicating improved functional diversity of the carbon-using soil microorganism community, taxonomic diversity decreased (Shannon diversity and Chao 1 indexes). Of 24 bacterial genera that had a relative abundance greater than 1%, it was found that relative abundance of *Saccharibacteria_genera_incertae_sedis* and Gp6 was correlated with soil organic matter content (r = 0.804, *P* < 0.01 and r = -0.85, *P* < 0.01, respectively). The organic matter content in biogas slurry is richer than fertilizer. The result showed that biogas slurry as topdressings may be a useful biological fertilizer in sustainable continuous cropping systems.

Key words: Bacterial community, biogas slurry, Chinese chives, continuous cropping.

INTRODUCTION

Chinese chives (*Allium tuberosum* Rottler ex Spreng.) is a vegetable traditionally favored by consumers in China due to its unique flavor and high nutritional value (Zhang et al., 2013). Continuous cultivation of Chinese chives has resulted in decreased yield and quality (Wang et al., 2006) and while methods for their improvement have received little attention, it has been shown that biogas slurry applied as a topdressing increases chive quality through greater leaf uniformity and length, reduced number of dead leaf-tips, enhanced resistance to

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Table 1. Biogas slurry physicochemical properties.

Property	Contents (mg/L)	Property	Contents (mg/L)
Organic carbon	162	Total nitrogen	281
Ammonium nitrogen	269	Nitrate nitrogen	0.32
Total phosphorus	54	Total potassium	188

disease, and lower abundance of insect pests (Sun et al., 2012).

Biogas slurry, which is rich in soluble inorganic salts and biochemical products of anaerobic fermentation (Arthurson, 2009), has been shown to have positive effects on plant yield and soil chemical, physical and soil microbial biomass characteristics in artificial incubation (Sänger et al., 2011), greenhouse pot (Andruschkewitsch et al., 2013), and short-term field (Bachmann et al., 2011; Johansen et al., 2013) experiments.

Since effects on soil microbial community composition and diversity of biogas slurry applications to Chinese chives are unclear, the objectives of this study were to quantify microbial activity and soil organic carbon (C), total nitrogen (N), and soil pH from field plots of Chinese chives treated with either biogas or chemical fertilizer.

MATERIALS AND METHODS

Experimental design

The field experiment was carried out in Dongqiao, Hubei province, China (31°15′N, 113°41′E; 48 m asl). This region has a tropical monsoon climate, with an average annual precipitation of 1100 mm and an average temperature of 16°C and soils at the study site were a sandy loam.

Previously, the field had been cultivated as a paddy with moderate levels of fertility. We arranged three replicates of nine 5.0 × 10.0 m plots in a completely randomized block design. On April 28 2014, Chinese chives seedlings were planted along raised ridges (15 cm high and 160 cm wide) at 10 cm spacing in double rows that were separated by 25 cm. Blended fertilizer (750 kg·ha^{-1}) was applied before seeding, with a ratio of 15:15:15 $(NH4)_2SO_4$:P_2O_5:K_2O and topdressing treatments were applied when the Chinese chives were harvested after two days. The treatments comprised applications of urea at 150 kg·ha^{-1} with 46% the nitrogen content plus blended fertilizer at 150 kg ha^{-1} (CP), biogas slurry (BS), and an unfertilized control (CK). CP and BS received the same amount of total N, while fertilizer supplemented P_2O_5 and K_2O in the BS treatment. Pig dung and urine was the raw material for biogas slurry and was fermented for more than 3 months until it had become transparent with no obvious fecal odor. Slurry physicochemical properties are shown in Table 1; pH was found to be 7.67.

On October 20 2017, 8 to 15 soil samples were randomly collected from each plot at a depth of 5 to 25 cm and combined to form single composite samples per plot, where loose soil was removed from the roots of the Chinese chives and any that remained strongly adhered to the roots was recovered as rhizosphere soil. Soil samples were divided into three subsamples that were stored at 4°C for determination of microbial abundance, -80°C for microbe DNA analysis or air-dried, ground, and passed through 1- and 2-mm mesh sieves for physicochemical analysis.

The physicochemical properties of the rhizosphere soil are tested. The pH of soil was measured by preparing slurry of 1:2.5 fresh soils to water (v/v) and using a pH meter (OHAUS, Starter 3C). Soil organic matter (SOM) was determined using the standard Walkley-Black potassium dichromate oxidation method (Nelson and Sommers, 1982). Available N (AN) was measured using the alkali-hydrolysis and diffusion method (Cornfield, 1960), available P (AP) was extracted with 0.5 M $NaHCO_3$ using the Olsen method (Blakemore et al., 1972), and available K (AK) was extracted with 1 M NH_4OAc (1:10 soil:solution ratio for 1 h) and analyzed using atomic absorption spectrophotometry (Lanyon and Heald, 1982).

DNA extraction

For each soil sample, three samples of total microbial genomic DNA were extracted from 0.5 g of the frozen soil using a PowerSoilDNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA), according to the manufacturer's instructions, and the three samples were pooled to reduce DNA extraction bias. Extracted DNA was evaluated on 1% agarose gel, where the quality and quantity of the extracts were determined using a NanoDrop ND-2000 spectrophotometer (ThermoScientic, DE, USA). All DNA samples were diluted to 10 ng μL^{-1} and stored at -20°C until further use.

Biolog ecoplate analyses

Fresh soils (5 g) were shaken for 20 min at 200 rpm with 100 ml 0.85% NaCl and then allowed to settle for 15 to 30 min. Ten-fold dilutions were performed until the desired (10^{-3}) dilution was reached and then an aliquot (125 mL) of the diluted suspension was placed in each well of the Biolog Ecoplate using a multi-channel repetitive-dispensing pipette. The plates were incubated at 28°C, and absorbance at 590 and 750 nm was recorded at 24 h intervals for 7 days using a Biolog GEN III MicroStationTM (USA) to assess average well color development (AWCD). Three replicates per treatment and sampling time were performed. Each well of the Biolog Ecoplate was loaded with one of 31 single carbon sources that belonged to classes defined as carbohydrate (12), amino acid (6), carboxylic acid (5), amine (2), polymer (4) or phenolic acid (2). The well absorbance values were adjusted by subtracting the absorbance of the control well (water only) before data analysis, and substrates with an optical density (OD) < 0 were excluded from further analysis.

Statistical analysis

Treatment differences ($P < 0.05$) between means were determined using t-test with LSDs in SPSS (v. 14.0 for Windows, Chicago, USA). The False Discovery Rate (FDR) of p-values was assessed using the BH method with the mt.rawp2adjp function in R. Redundancy analysis (RDA) was run in excel using the XLSTAT.

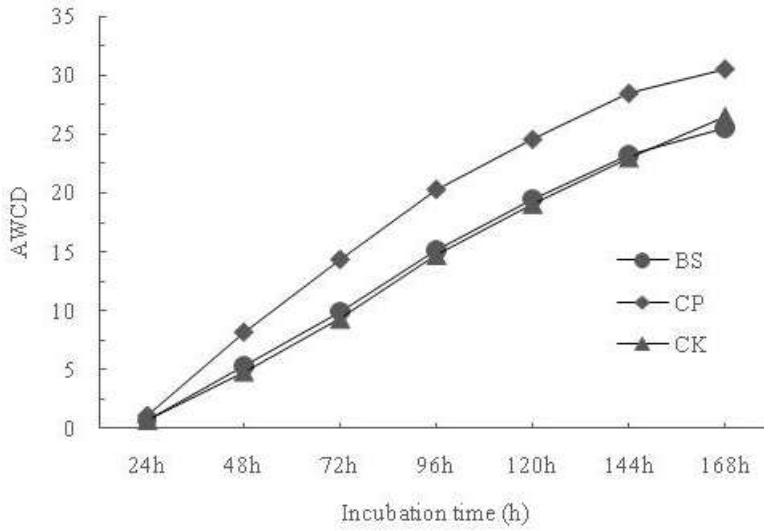

Figure 1. Treatment effects on changes in average well color development (AWCD) of 31 carbon sources.

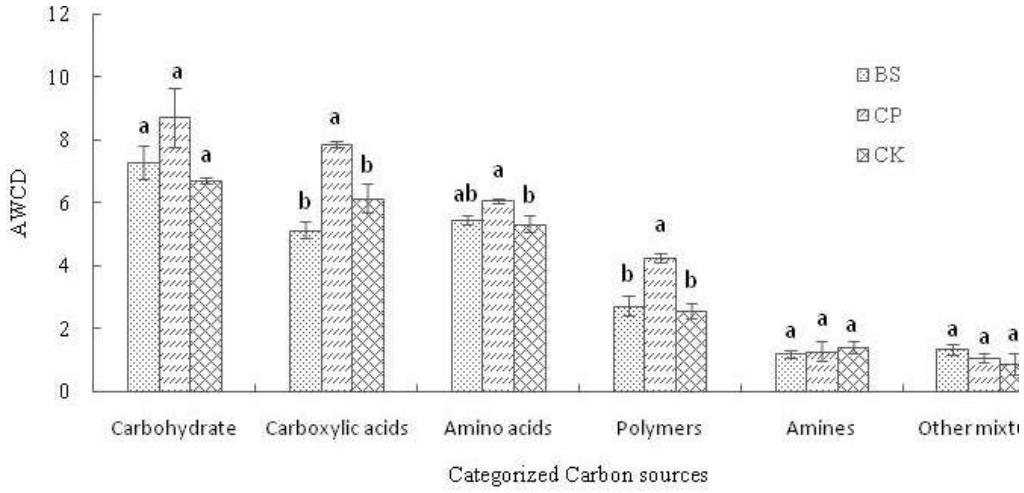

Figure 2. Rhizosphere microorganism use of six major carbon sources.

add-on statistical software

RESULTS

Carbon substrate metabolic profiles of soil microbial communities

It was found that AWCD gradually increased with the cultivation, but there was no treatment effect on carbon utilization the first 24 h (Figure 1). Increase in soil microorganisms was logarithmic from 24 to 144 h, when AWCD of all soil samples increased to approximately 25, whereas rate of AWCD decreased after 144 h. The AWCD was higher in CP than in BS and the control.

Specific substrate utilization of soil microbial communities

The relative absorbance of carbohydrates, carboxylic acids, amino acids and polymers was highest in CP and similar between the BS treatment and control (Figure 2).

Bacteria alpha-diversity

There were treatment effects on the abundance and

Table 2. Treatment effects on diversity index of 16S rRNA.

Sample	Chao1	ACE	Shannon	Simpson
CK	3853.33±179.15[a]	3802.54±170.99[a]	6.5585±0.0959[a]	0.0040±0.0004[a]
CP	3302.10±485.99[a]	3238.10±499.29[a]	5.7643±0.5601[a]	0.0082±0.0083[a]
BS	3754.19±244.24[a]	3714.24±227.08[a]	6.3469±0.2276[a]	0.0059±0.0021[a]

Data are means ±SD (n = 3). Different letters within a column indicate treatment differences at $P < 0.05$.

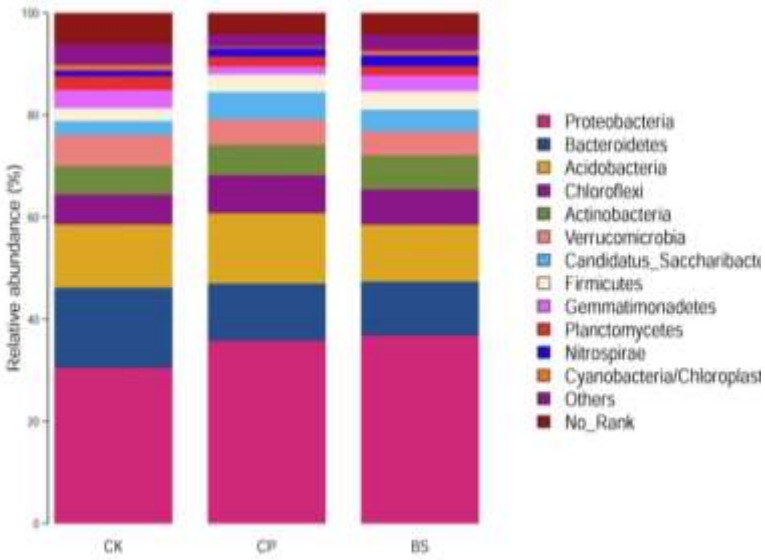

Figure 3. Treatment effects on the relative abundance of dominant phyla. Relative abundances are based on the proportional frequencies of those DNA sequences that could be classified.

Table 3. Treatment effects on phylum abundance.

Phylum	Comparison	Relative fold-change	*P* value
Gemmatimonadetes	CK/CP	2.31	0.030
BRC1	CK/BS	2.14	0.03*
Gemmatimonadetes	CP/BS	0.51	0.03*

diversity of bacteria, where they are higher in the BS treatment and control than in the CP treatment (Chao1 and Shannon index; Table 2).

Bacterial community composition in the soil

At the phylum level, a total of 30 phyla were shared across the three treatments, where the *Proteobacteria, Bacteroidetes, Acidobacteria, Chloroflexi, Actinobacteria, Verrucomicrobia, Candidatus_Saccharibacteria*, and *Firmicutes* were dominant accounting for 80.9 to 94.6% of DNA sequences (Figure 3). The relative abundance of the *Gemmatimonadetes* and *BRC1* differed among the treatments ($P < 0.05$; Table 3). *Proteobacteria* was consistently the most dominant phylum, representing 29.1 to 41.1% of the phyla, where relative abundance was greater in the BS and CP treatments than in the control. *Bacteroidetes* was the second most dominant phylum (6.0-17.0%), where relative abundance was lower in the BS and CP treatments than in the control.

At the genus level, there are 24 genera with a relative abundance greater than 1%, including *Saccharibacteria_genera_incertae_sedis, Gp6, Gemmatimonas, Gp4, Subdivision3_genera_incertae_sedis, Terrimonas, Chryseolinea, Sphingomonas*, etc. (Figure 4). The relative abundance of some genera was significantly affected by the different topdressing (Table S1).

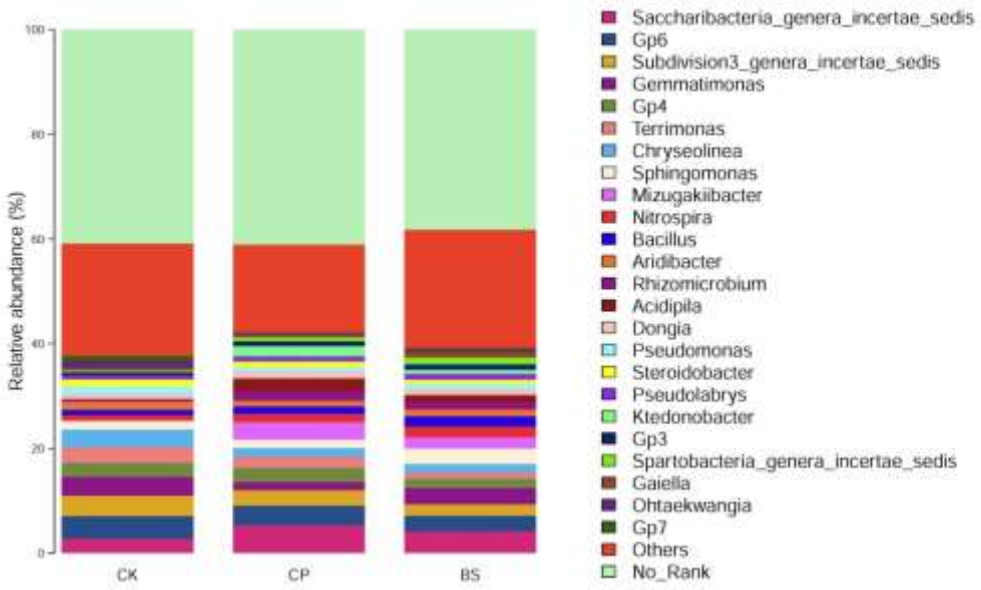

Figure 4. Treatment effects on relative abundance of genera. Relative abundances are based on the proportional frequencies of those DNA sequences that could be classified.

Table 4. Treatment effects on key soil physicochemical properties.

Treatment	pH	Organic matter (g·kg^{-1})	Available N (mg·kg^{-1})	Available P (mg·kg^{-1})	Available K (mg·kg^{-1})
BS	6.21±0.00^{c}	2.73±0.16^{a}	77.93±3.22^{a}	245.04±13.22^{b}	272.67±4.18^{b}
CP	5.70±0.05^{b}	2.51±0.86^{a}	76.30±1.65^{a}	199.36±4.02^{b}	176.00±0.00^{a}
CK	4.94±0.02^{a}	1.76±0.15^{a}	87.27±12.45^{a}	94.20±2.98^{a}	173.00±3.51^{a}

Different letters within a column indicate treatment differences at $P < 0.05$.

Cystobacter, Sphingobium and *Anaerosalibacter* dramatically differed ($p < 0.01$) between CK and BS. *Desulfosporosinus, Pontibacter* and *Solibacillus* dramatically differed ($p < 0.01$) between CK and CP. *Arthrobacter, Gp2* and *Niastella* dramatically differed ($p < 0.01$) between CP and BS.

Relationship between bacterial community and environmental factors

There were treatment effects on soil physicochemical properties (Table 4), where pH was the highest in the BS treatment and lowest in the control; AP was higher in the BS and CP treatments than in the control; and AK was the highest in the BS treatment. There were no treatment effects on SOM content. Redundancy analysis (RDA) of the 17 most abundant genera and their associations with SOM, pH, and AN, AK, and AP content showed that the decreasing rank order of influence on genus abundance was SOM, AK, AN, pH, and AP (Figure 5). Genus abundance in the BS treatment was positively associated with AK, SOM, AP, and AN, and negatively related to pH, while in the CP treatment and control, genus abundance was negatively associated with AK, OM, AP, and AN, and positively associated with pH.

Of the 24 genera with a relative abundance greater than 1%, the abundance of nine was associated with SOM; there were no associations between the other physicochemical properties and abundance of genera. Abundance of *Saccharibacteria_genera_incertae_sedis* was positively correlated ($r = 0.804$, $P < 0.01$) and *Gp6* was negatively correlated ($r = -0.850$, $P < 0.01$) with the SOM content (Table 5).

DISCUSSION

Effects on bacterial community composition

The depletion of soil organic matter is a major factor in the degradation of ecosystem services and ecosystem resilience to perturbations (Feller et al., 2012) and studies have suggested that amendment of organic soil may be an approach to improve the economics of viable crop production whilst minimizing the impacts of environmental

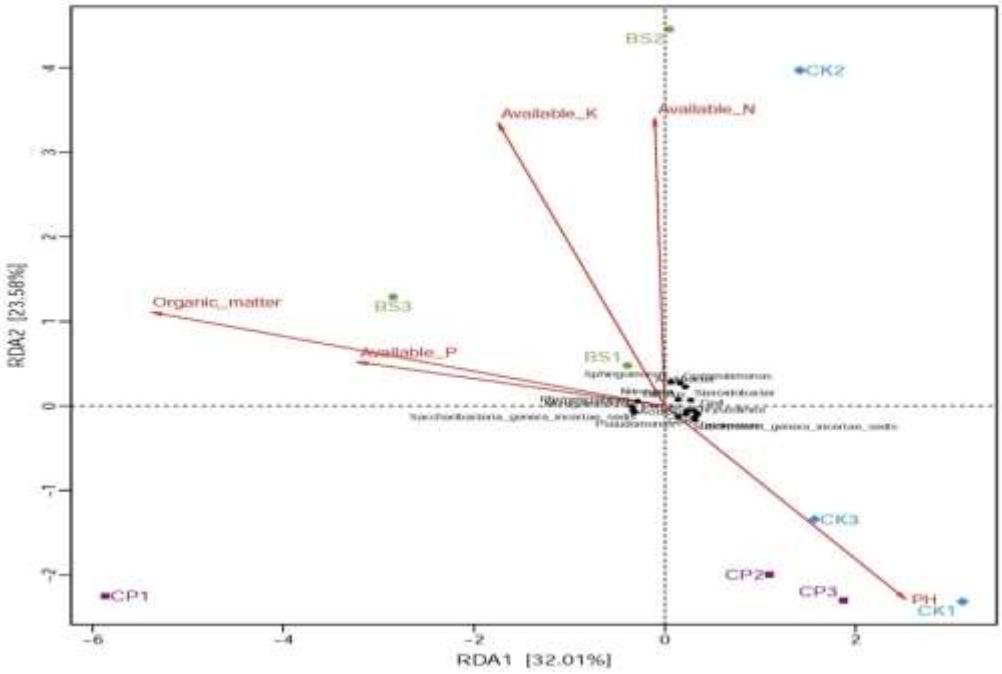

Figure 5. RDA correlation analysis of the soil bacteria communities and physicochemical properties in the treatments.

Table 5. Association between genus relative abundance and physicochemical properties (n=20).

Genus	Pearson's correlation coefficient				
	Available K	Available N	Available P	Organic matter	pH
Acidipila	0.008	0.003	0.315	0.767*	-0.148
Gp6	-0.279	-0.308	-0.294	-0.850**	0.28
Steroidobacter	-0.301	0.4	-0.332	-0.741*	0.291
Nitrospira	0.456	-0.333	0.49	-0.299	-0.553
Saccharibacteria_genera_incertae_sedis	0.019	0.073	0.197	0.804**	-0.08
Dongia	-0.034	-0.219	0.165	-0.785*	-0.085
Aridibacter	-0.072	0.648	-0.269	-0.489	0.12
Pseudomonas	-0.095	-0.309	-0.165	-0.472	0.17
Terrimonas	-0.473	-0.226	-0.499	-0.784*	0.51
Bacillus	0.414	0.089	0.472	0.635	-0.444
Chryseolinea	-0.317	0.01	-0.507	-0.641	0.448
Sphingomonas	0.615	0.272	0.413	-0.097	-0.599
Mizugakiibacter	0.078	0.003	0.405	0.709*	-0.235
Gp4	-0.264	-0.316	-0.266	-0.632	0.236
Gemmatimonas	0.198	0.516	-0.401	-0.274	0.097
Rhizomicrobium	0.114	0.118	0.343	0.850**	-0.226
Subdivision3_genera_incertae_sedis	-0.481	-0.268	-0.553	-0.835**	0.56

pollution. Biogas slurry, which is rich in organic matter, has high levels of bioactivity and associated nutrient utilization efficiency and is known to reduce disease incidence and improve stress tolerance that lead to increased production and product quality (Gao et al., 2011). Soil microorganisms play an important role in nutrient cycling and decomposition (Kennedy and Smith, 1995) and are affected by the application of fertilizer (Ge et al., 2015); understanding the soil microbe community and its response to various agricultural management

practices will allow the selection of a suitable management strategy for more stable and sustainable agroecosystems (Li et al., 2012; Zhao et al., 2014). Ai et al. (2018) reported that microbe functional diversity was high in paddy soils subjected to long-term, high levels of fertilizer application. Here, AWCD was found to be higher in the CP treatment than in the BS treatment and the control, indicating that fertilizer topdressing improved the functional diversity of the carbon source-using soil microorganism community, as reflected by increases in utilization of the carboxylic acids, amino acids, and polymers (Figure 2).

Effects on bacterial diversity

Soil health is one of the most vital requirements for crop production in agricultural systems, where soil microorganisms play a major role in its development and maintenance. Yu et al. (2017) found long-term application of inorganic nitrogen fertilizer reduced the diversity of soil bacteria and here, it was found that biogas slurry increased soil microbe taxonomic diversity (Table 2). Soil organic matter is essential in the maintenance of soil structural stability and improvement of physical, chemical, and biological properties (Oo et al., 2015) and the addition of organic matter in soil remediation is considered essential for sustainable land use and crop productivity. In the present study, the dominant phyla Proteobacteria, Bacteroidetes and Acidobacteria accounted for more than 50% of bacteria abundance, which was consistent with Lauber et al. (2009) and Chu et al. (2010) (Figure 3). Nine genuses had significant differences with SOM, but none of them had significant differences with the other physicochemical properties (Table 5). Thus, the application of biogas slurry to vegetable, fruit tree, flower and field crops may provide multiple benefits, including increases in SOM content and microbial diversity, as a biological fertilizer.

Conclusions

It was found that biogas slurry as topdressing is conducive to increase relative abundance and taxonomic diversity of the soil bacteria community in continuous cropping. Of the 24 genera with a relative abundance greater than 1%, the abundance of nine was positively associated with SOM content. Relative abundance of *Saccharibacteria_genera_incertae_sedis* and *Gp6* was positively and negatively correlated with SOM, respectively, and RDA indicated that SOM was a key driver of the composition of the soil bacteria community and physicochemical properties. Biogas slurry contains the essential nutrients for plant growth (NPK), improves SOM content, and enhanced the soil microbe community, so we suggest its application in sustainable vegetable production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Table S1. Treatment effects on relative abundance of bacteria genera.

Genus	Relative fold-change	p value (*p < 0.05,**p < 0.01)
	CK/CP	
Desulfosporosinus	0.27	0.003**
Pontibacter	33	0.005**
Solibacillus	0.29	0.008**
Chlorophyta	2.35	0.01*
Gp2	5.09	0.013*
Geminicoccus	3.96	0.016*
Ignavibacterium	4.26	0.018*
Geothermomicrobium	0.54	0.021*
Albidovulum	7.5	0.023*
Nannocystis	3	0.026*
Blastococcus	3.1	0.029*
Paenisporosarcina	2.92	0.031*
Taibaiella	0.17	0.034*
Curvibacter	2.75	0.036*
Microvirga	2.85	0.039*
Clostridium_XlVa	0.42	0.042*
Gemmatimonas	2.31	0.044*
Solirubrobacter	3.74	0.047*
Desulfobacca	0.32	0.049*
	CK/BS	**p value (*p<0.05, **p<0.01)**
Cystobacter	3.98	0.003**
Sphingobium	0.08	0.005**
Anaerosalibacter	0.08	0.008**
Singulisphaera	0.29	0.010*
Albidovulum	6.00	0.013*
Cupriavidus	0.31	0.015*
Thiobacillus	0.00	0.018*
Chlorophyta	2.27	0.020*
Blastococcus	3.56	0.023*
Gaiella	0.52	0.026*
Skermanella	3.37	0.028*
Arthrobacter	0.29	0.031*
Sporacetigenium	1.50	0.033*
Microvirga	2.22	0.036*
BRC1_genera_incertae_sedis	2.14	0.038*
Oryzihumus	3.33	0.041*
Azoarcus	0.04	0.043*
Pirellula	1.78	0.046*
Brevibacterium	0.18	0.049*
	CP/BS	**p value (*p <0.05, **p <0.01)**
Arthrobacter	0.11	0.003**
Gp2	0.14	0.005**
Niastella	0.08	0.008**
Bdellovibrio	0.34	0.010*
Armatimonadetes_gp4	0.19	0.013*
Turicibacter	0.12	0.016*
Gaiella	0.47	0.018*
Methanocella	2.52	0.021*

Table S1. Contd.

Thermobifida	0.08	0.023*
Fervidicella	3.00	0.026*
Azoarcus	0.04	0.029*
Armatimonadetes_gp5	0.18	0.031*
Aggregicoccus	0.24	0.034*
Solibacillus	2.38	0.036*
Schlesneria	0.24	0.039*
Gp13	0.11	0.042*
Amycolatopsis	0.07	0.044*
Gemmatimonas	0.51	0.047*
Stenotrophomonas	0.21	0.049*

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