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Table of Contents: Volume 11 Number 26 14 July, 2017

ARTICLES

Antibiofilm activity of natural substances derived from plants 1051
José Walter Araújo Nogueira, Renata Albuquerque Costa, Magda Turini da Cunha and Theodora Thays Arruda Cavalcante

Evaluation of semi-nested polymerase chain reaction (PCR) and mannan antigen detection compared to blood culture for diagnosis of candidemia 1061
Nashwa M. Al-Kasaby, Nermein Abou El Kheir, Mohammed Mefreh and Maysaa El Sayed Zaki

Bacteriological quality of drinking water from source to point of use among rural communities of Farta Woreda in North West, Ethiopia 1069
Genet Gedamu Kassie and Desta Haftu Hayelom

Effect of the fermentation on the microbial population occurring during the processing of zoom-koom, a traditional beverage in Burkina Faso 1075
Fidèle Wend-bénédo TAPSOBA, Hagrétou SAWADOGO-LINGANI, Donatien KABORE, Diarra COMPAORE-SEREME, and Mamoudou Hama DICKO

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Review

Antibiofilm activity of natural substances derived from plants

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The main objective of this study was to conduct a review of bioactive substances derived from plants which show antimicrobial/antibiofilm properties. Vegetable species were widely employed as ingredients in medicine based on traditional knowledge. Various secondary metabolites have been proven to inhibit bacterial growth. Bacterial resistance mechanisms have increased over the years. Biofilms are types of bacterial association which gives the communities a higher resistance to drugs. The formation process of biofilms, the problems caused by them and the natural substances, as well as their main chemical components and action mechanisms, have been described according to existing literature.

Key words: Natural product, antimicrobial activity, biofilm.

INTRODUCTION

Pathogenic bacteria present a number of defense mechanism against antimicrobial agents, moreover, their resistance to drugs presently available in the market is increasing. Among the bacterial defense mechanisms, formation of bio-films is one that is responsible for a fair amount of chronic diseases and show extreme resistance to antibiotics and to the host's defense system (Lewis, 2001; Fux et al., 2003; Rozalski et al., 2013).

Biofilms are a more resistant form of bacterial life when compared with free-living planktonic form. Its resistance is directly related to the natural survival characteristics of microbial cells living in such communities, among which we highlight the slower growth of cells associated with biofilm, as opposed to the free-living microbial cells, and low regulation of cellular process, mainly caused by the more restricted contact of the cells in the interior of the biofilm with external nutrients. Besides that, these bacteria produce an extracellular polysaccharidic matrix that hinders the action of antimicrobial agents, collaborating even further to its resistance, since this matrix acts like a diffusion barrier against small molecules (Anderson and O'Toole, 2008; Hall-Stoodley and Stoodley, 2009).

Currently, natural compounds have emerged as potential candidates given the biotechnological focus in the search for antimicrobial and antibiofilm drugs

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(Schachter, 2003). The relevance of these researches is due to the large role played by biofilms in the etiology of a vast amount of persistent and chronic human diseases (Simões et al., 2010).

The observation of microbial worlds by different techniques of microscopy has made possible to researchers, throughout the years, to observe micro-organisms arranged in communities, sharing nutrients, metabolites, genetic elements and, thus, becoming able to resist environmental turbulence, causing diseases difficult to eradicate. Biofilms impacts men in a variety of ways, being formed in natural environments, medical instruments and industrial machines (Al-Bakri et al., 2010; Lopez et al., 2010).

In 1847, Leuwenhoek used a primitive microscope and described animalcules on a sample scrapped from human teeth. Almost 100 years later, in 1934, Claude Zobell, examining marine populations directly under the microscope, concluded that these bacteria were adhered to surfaces, forming sessile populations (Zobell, 1943). Between 1935 and 1978, microbiologists Ron Gibbons and Van Houte of the Forsyth Dental Center examined microbial biofilms that constitute dental plaque. The first stage of a biofilm formation in pure culture was observed in 1964, when it was established that the state of irreversible adhesion of microorganisms to a surface is the first in the formation of these microbial communities (Costerton, 1999).

More than seventy years after the first account of biofilm (Zobell, 1943), they continue to be a source of preoccupation to a wide array of activities, specifically food industry, environment and biomedics (Flint et al., 1997; Maukonen et al., 2003; Sihorkar and Vyas, 2001; Veran, 2002). Based on observations of dental plaque and sessile communities in mountain streams, Costerton and collaborators presented in 1987 a theory of biofilms that explained the mechanisms through which the microorganisms adhere to living or inert surfaces, and what benefits they receive from living in these communities.

According to Hoiby et al. (2010), a biofilm is a structured consortium of bacteria capable of producing a polymeric matrix which consists of polysaccharides, proteins and DNA. These communities can be established on a wide variety of surfaces (Abee et al., 2011). Aside from the ability to produce extracellular biopolymers, cells in community show, in a high or lower degree, a decreased growth pattern and specifically regulated genes. The organization of microorganisms in biofilms occurs naturally, for living in these communities considerably raises the chances of survival for these microscopic beings. The production of extracellular polymeric substances by microorganisms is accepted as a key mechanism in making irreversible the cellular adhesion to inanimate surfaces in watery environments, resulting in the development of a biofilm (Beech et al., 2005).

Bacterial biofilms are closely related to human health problems; they are responsible for many infectious diseases acquired from inert surfaces, including medical instruments for internal and external use. They might also be present in the water tubing of hospitals, leading to infections after internment (Bordi and de Bentzmann, 2011). It is important to highlight that the formation of biofilm in medical material, such as catheters or implants, result in chronic infections difficult to treat (Donlan, 2008; Hall-Stoodley et al., 2004; Hatt and Rather, 2008).

Since the first observations using confocal microscopy, it became evident that living mature biofilms are not single structured layers of microbial cells on a surface. On the contrary, they appear as heterogeneous entities in time and space, constantly changing as a result of external and internal processes (Dolan and Costerton, 2002). A biofilm might be formed of a single species of bacteria or fungus; however, it might consist of various bacterial, fungal species and even algae and protozoans (Batoni et al., 2003). An example of monospecific biofilm is those formed in cardiac valves of patients with infectious endocarditis, composed of *Staphylococcus epidermidis* (Butany et al., 2002). Besides that, infections have been associated with the formation of biofilms in human organic surfaces such as teeth, skin and urinary tract (Hatt and Rather, 2008). This organization in communities offers the microorganisms resistance to many antimicrobials, protection from protozoan attacks and hosts defenses (Anderson and O'Toole, 2008; Matz and Kjelleberg, 2005).

Currently, it is known that in natural environments, 95-99% of microorganisms exist in biofilm form (Nikolaev and Plakunov, 2007). These communities protect their microbial inhabitants not only from oxygen, but also from consequences of other damaging environmental factors (Paerl and Pinckney, 1996). Bacteria in biofilm may cause chronic infections (Costerton et al., 2003) that are characterized by persistent inflammation and tissue damage (Bjarnsholt et al., 2009). Chronic infections, including foreign body infections, are persistent despite antibiotic therapy, innate and adaptive immune system and inflammatory response of the host. In contrast to colonies, they show an immune response and pathological persistence (Hoiby et al., 2010).

These communities present an unique profile also because they shelter different species in a structure in which they are able to cooperate preferentially rather than compete (Bordi and de Bentzmann, 2011). They constitute microbial societies with their own set of social rules and behavior patterns, including altruism and cooperation, favoring group success (Parsek and Greenberg, 2005; Shapiro, 1998) by replicating behaviors on one side and competition (Velicer, 2003) on another. Certain subpopulations may show specialization; these behavioral patterns are orchestrated by chemical

communications (Weigel et al., 2007). Thus, they constitute a unique form of interaction between species, inducing drastic changes in symbiotic relations between their components (Hansen et al., 2007).

MECHANISMS OF BIOFILM FORMATION

The understanding of molecular bases in the creation of biofilms has been favored by improvements in the genetic and genomic methods and the development of visualization techniques that reveal the processes involved in the growth, physiology and adaptation of microorganisms to this life condition. A plethora of systems allows bacteria to identify and anchor to proper surfaces, and adhere one to another in order to form multicellular communities (Bordi and de Bentzmann, 2011).

Bacterial growth in pure cultures has been the main approach to microbiological culture, from pasteur to the present day. This kind of experiments are efficient in furnishing knowledge and understanding of the prokaryotic genetics and metabolism, and have simplified the isolation and identification of pathogens of a number of diseases (Costerton et al., 1987).

When it became evident that the behavior of bacteria associated on surfaces could not be predicted from microorganisms cultured in suspension, in their planktonic form, a new term to describe sessile microbial populations was introduced in the researches with biofilm (Jakubovics and Kolenbrander, 2010).

The formation of biofilm can be considered a protection mechanism for the bacterial community against external injury, thus, it seems reasonable that specific extracellular signals regulate the activation of metabolic patterns which set off the establishment of biofilms. This external signalization may come from diverse sources: they can be reproduced and secreted by the community itself, where there are molecules designed as autoinducers, which accumulate in the extracellular medium, their concentration correlated to the population density (Lopez et al., 2010), and that in high concentration might set off chains of signalization that lead to multicellular responses in bacterial population. This mechanism of cell-cell communication (called quorum sensing) controls a large number of processes, including those related to the formation of biofilm (Camilli and Basssler, 2006).

Each bacterial species has its own set of tools to perform adhesion, and there is a large number of different molecules for each species that can be used antagonistically or synergically, depending on the situation (Hagan et al., 2010).

The formation process of biofilms has been extensively described (Dolan and Costerton, 2002; Costerton et al., 1995; Habash and Reid, 1999). It is a process that follows many stages: the initial reversible adhesion of planktonic cells to a surface, followed by a maturation phase. The initial adhesion is based on attraction and repulsion forces between the cells and the surface. These forces include electrostatic and hydrophobic interactions, Van der Waals forces and hydrodynamic forces, in adequate temperatures (Agarwal et al., 2010). After the adhesion to the surface, the bacteria grow and divide, forming dense agglomerates which are characteristic of biofilm. This phase is associated with the production of polysaccharides by the bacterial cells, that become irreversibly adhered to the substrate. After a while, microcolonies develop in a mature biofilm, acquiring a typical architecture, with mushroom-shaped projections, separated by channels filled with fluids. The final stage (dispersion) consists of the detachment of unitary cells or cellular groups from the mature biofilm, it is considered an essential stage in bacterial dissemination (Batoni et al., 2003).

Considering cellular hydrophobicity and the presence of frimbriae and flagella, the production of EPS (extracellular polymeric substance) is one of the main factors that influences the rate and degree of adhesion of microbial cells to different surfaces, aside from protecting against environmental stress and dehydration (Vu et al., 2009). Thus, EPS production has been the topic of many researches to prevent the process of formation and maturation of these microbial communities (Murray et al., 2009; Nagorska et al., 2010).

The initial colonizers interact with the surface through weak interactions, mostly Van de Waals-type forces. If these microorganisms are not removed from the surface by mechanical or chemical action, they might anchor permanently through cellular adhesion molecules, such as pili and flagella (Boks et al., 2008; Hermansson, 1999). The adhesion of microorganisms to adjacent soft tissues is determined by the existence of adhesion molecules (adhesins), fixed by specific receptors, commonly simple sugars (Pereira et al., 2010).

The first microcolonies create a substratum and propitious environment for the arrival of other cells through adhesion sites and start to build the matrix which will form the biofilm. Only some species are able to adhere to a surface itself, others may anchor to the matrix or to preexisting colonies. Once the colonization has started, the biofilm develops in a combination of cellular division and recruitment of other cells (Carneiro et al., 2010).

BIOFILM RESISTANCE AND ANTIMICROBIAL MEASURES

Many human diseases are related to bacterial biofilms, including cavities, periodontitis, endocarditis and prostatitis. Biofilms have been described as bacterial mechanisms of persistence and resistance to anti-

microbial agents, unlike those found in free-living cells (Namasivayam and Roy, 2013). Various streptococcus infections, specially chronic infection, might be related to the formation of bacteria in biofilms (Al-Dhabi et al., 2012). The development of biofilms grants high resistance to the microorganisms, due to their involution in a matrix of extracellular polymeric substances, forming a barrier which stops or hampers the diffusion of antimicrobial substances through the colony (Trentin et al., 2011a, b). The ideal form of avoiding their action in pathogenic processes is to stop their development (Rozalski et al., 2013) in order to do that, the investment in the search of products and methods that interfere with the bacterial accumulation through adhesion control, inhibition of interbacterial communication or the establishment of the polysaccharidic matrix is needed (Jakubovics and Kolenbrander, 2010).

Among the mechanisms that grant resistance to antimicrobial measures are the bacterial quorum sensing, a form of communication and cooperation for the execution of different behaviors, including the production of toxins and the biofilm formation or the efflux pumps, a mechanism of active pumping of biomolecules to the extracellular medium, responsible for the resistance of Gram-negative bacteria to the majority of natural products (Savoia, 2012).

The oral cavity is colonized by a rich collection of beneficial microorganisms that live in harmony with the host, an advantageous situation to both parties. In this context, products designed for oral heath should preferentially aim to control the level of plaque instead of eliminating it, so that the beneficial properties of the resident microflora are maintained (Marsh, 1992, 2010).

Some measures taken to prevent the adherence of bacteria to biotic and abiotic surfaces and, consequentially, the formation of biofilm have been studied, these antimicrobial measures comprise experiments on the inhibition or death of the aim-bacteria driven by tests for the determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). These techniques are applied in tests with bacteria in their planktonic form. Nevertheless, for clinical use, the realization of tests using biofilm formation techniques or even evaluation of mature biofilm is more predictive.

Biofilm control mechanisms include the use of adhesin analogs, antibody for key epitopes and peptides that block specific sites. The interruption of the quorum sensing mechanisms is another possible goal for biofilm therapy (Njoroge and Sperandio, 2009). Such measures might turn bacteria present in biofilm more susceptible to antimicrobials or lessen its pathogenicity (Bjarnsholt et al., 2005).

A large variety of agents has been formulated so as to increase the potential control of biofilm such as: antibiotics, quaternary ammonium compounds, and chlorhexidine gluconate and acetate (Baehni and Takeuchi, 2003; Oliveira et al., 1998). Chlorhexidine shows good substantivity, with an ample spectrum of activity against Gram-positive, negative bacteria and yeast (Kleerebezem et al., 1997; Mukamolova et al., 1998).

Chlorhexidine found application in periodontal treatments, dermatological infections, skin wounds, eye and throat infections and endodontics (Teixeira and Cortes, 2005). Its frequent and prolonged use presents many collateral effects, such as changes in the taste of food and a burning feeling on the tip of the tongue (Greenberg et al., 2008; More et al., 2008; Porto et al., 2009).

Some factors have to be considered while choosing an antimicrobial substance, such as toxicity, low permeability, retentivity and the capacity of maintaining the balance of the microbiota in the mouth cavity (Cury, 2003). Another important consideration is the administration method of the anti-plaque agents. Their liberation in the oral cavity can be made by mouth washing, sprays, toothpaste, gel or vehicles of prolonged liberation, such as varnishes (Scheie, 2007).

CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY OF NATURAL SUBSTANCES

Considering the previous observations, the use of natural products to promote health is as old as human civilization and, for a long time, mineral, vegetable and animal products constituted the therapeutic arsenal at hand (Eisenberg et al., 1998). Although, the presence of antimicrobial substances in higher plants is not a recent fact, only from the discovery of penicillin onwards the search has received a greater impulse (Coelho et al., 2004; Tavares, 1996).

Plants have many secondary metabolic pathways that originate various compounds, such as alkaloids, flavonoids, isoflavonoids, tannins, coumarins, glycosides, terpenes and polyacetylenes, that are often specific to certain families, genera or species and whose functions, until not long ago, were unknown (Cowan, 1999; Savoia, 2012; Simões et al., 2007). As researches progressed, these substances were found to be important in the defense mechanism of plants against their predators: fungi, bacteria, viruses, parasites, insects, mollusks or higher animals (de Lima et al, 2006).

Researches concerning the biodiversity of the Brazilian flora appear to be extremely promising sources for the discovery of new substances that might be used in the treatment of diseases. Even if the accounts in literature are few (Aburjal et al., 2001; Aqil et al., 2005; Nascimento et al., 2000), the evaluation of the synergic action between natural products and antibiotics currently used in medical clinic show auspicious signs in the attempt of

minimizing the effect of resistant strains or even the ability of microorganisms organized in biofilms resisting antimicrobial measures.

Natural products derived from medicinal plants have been found to be abundant sources of biologically active compounds, many of which became the base in the development of new chemical products which may lead to a later pharmaceutical output. With respect to diseases caused by microorganisms, the increase in the resistance of many common pathogens to therapeutic agents commonly used, such as antibacterials and antivirals, has lead to a renewed interest in the discovery of new anti-infection compounds. Since there are approximately 500,000 species of plants, distributed all over the planet, and only 1% of which being phytochemically investigated, there is a great potential for finding new bioactive compounds (Palombo, 2011).

In the last years, a great scientific progress has occurred concerning chemical and pharmacological studies of plants aimed at obtaining new compounds with therapeutic properties (Cechinel-Filho and Yunes, 1998). Among the therapeutic agents derived from plants, essential oils - also called volatile or ethereal- occupy a preponderant position.

From a chemical point of view, essential oils are complex mixtures of volatile, lipophilic, generally odorific and liquid substances. They are extracted from various plant parts (flowers, inflorescences, seeds, leafs, sticks, shells, fruits and roots) by specific processes; they possess a frequently pleasant odor and are colorless or lightly yellow when recently extracted, with an oily appearance. Their main characteristic is their volatibility, which distinguish them from the fixed oils, mixtures of lipidic substances, generally extracted from seeds (Simões et al., 2003). These denominations derive from some of their physical-chemical properties, as example, are generally liquid, of an oily aspect at room temperature- hence, oil.

Volatile oils are defined as substances obtained from organs of vegetable species through steam distillation, as well as substances obtained by pressing the pericarps of citric fruits. They might also be called essential oils, ethereal oils or essences.

Another important characteristic is the pleasant and intense odor in most of the volatile oils, thereby named essences. They are also soluble in apolar organic solvents, as aether, hence their name ethereal oils, *aetheroleum* in latin. In water, the volatile oils display limited solubility, but enough to aromatize aquose solutions, named hydrolates.

Essential oils are complex mixtures of mainly terpenes (Edries, 2007) compounds, but they might include terpene hydrocarbons, simple and terpene alcohols, aldehydes, ketones, phenols, esters, ethers, oxides, peroxides, furanes, organic acids, lactones and coumarins (Simões et al., 2007). Strong *in vitro* evidences indicate that essential oils can act as antibacterial agents against a large spectrum of pathogenic bacterial strains (Burt, 2004; Nguefack et al., 2004; Schmidt et al., 2005).

The antimicrobial activity of some essential oils and isolated components was reviewed. It was observed that eugenol showed good antibacterial effectiveness in a 0.5% concentration. In the present study, after 24 h of incubation eugenol showed a statistically significant decrease in bacterial growth in concentrations of 2.5 to 0.078% and there was no significant difference in comparison of all concentrations to tests with chlorhexidine (Burt, 2004).

Carvacrol, a phenolic monoterpene and a potent antimicrobial, was effective against a biofilm formed by *Staphylococcus aureus* and *Salmonella enterica*. Non-biocidal concentrations prevent the accumulation of protein mass and interrupt the normal development of biofilm. This molecule, along with thymol, constitute the phenolic components that enable antimicrobial activities in the oregano oil (Savoia, 2012).

In 2005, Alviano and collaborators studied the antimicrobial and antibiofilm activity of the essential oil of the *Croton cajucara* and its major component linalol, observing that the effect of the oil was more potent than that of the isolated component (Alviano et al., 2005).

Jeon et al. (2011), while testing the capacity of tt-farnesol, a terpenoid that can constitute essential oils, observed its potential to affect biofilms of *S. mutans* through alteration in the proton-motive force, possibly by the interaction of lipophilic domains with the bacterial membrane, as is supposed to happen in the action against bacteria in the planktonic form; damaging the cellular functions of the membrane, and compromising the ability of *S. mutans* to produce and tolerate acids and to synthesize intra/extracellular polysaccharides (Koo et al., 2002, 2003). Data from this study suggest that the treatment with t-farnesol can later make the bacterial accumulation impossible in biofilms subjected to nutritional depletion (Jeon et al., 2011).

Recent studies reported complementary and alternative treatment options to combat *P. aeruginosa* infections. Quorum sensing inhibitors, phages, probiotics, anti-microbial peptides, vaccine antigens and antimicrobial nanoparticles have the potential to act against drug resistant strains. Unfortunately, most studies considering alternative treatment options are still confined in the pre-clinical stages, although some of these findings have tremendous potential to be turned into valuable therapeutics (Biswas et al., 2015).

It is relevant to observe that compounds derived from plants have received larger attention in the research of alternatives in the control of infections, especially those related to the formation of biofilm, which present a recalcitrant character. It is well known that there are two main reasons for the essential oils to restrain the development of resistant bacterial strains: they are

complex and constituted by a good number of compounds in varied proportions, depending on the chemotype of the plant. Therefore, even if the bacteria are able to resist the effect of one component, there are others, possibly, with different action goals to complete the global antimicrobial activity of the essential oil (Bakkali et al., 2008; Reichling et al., 2009).

ANTIBIOFILM ACTIVITY OF NATURAL SUBSTANCES

In the last years, medicinal plants have raised the attention of researchers because of their promising potential as a source of antimicrobials. In many communities around the world, the traditional medicine systems are the only means available for the treatment of infections (Savoia, 2012). Many of these agents have their activity tested in planktonic cultures. However, the activity on sessile forms of life, responsible for the gravest problems, is still understudied. Studies that evaluate antibiofilm activity should deal with the activity against consolidated biofilms and anti-adherent properties as a prophylactic measure against the formation of biofilm (Al-Bakri et al., 2010).

Efforts in the discovery of new medicine with antimicrobial properties are the basis to overcome the worldwide problem of microbial resistance. Extracts and oils obtained from plants have been used for a great variety of objectives for centuries (Jones, 1996). These objectives vary from the use of rosewood and ceder in perfume making, flavoring of beverages with lemon oil (Lawless, 1995) and the preservation of stored food (Mishra and Dubey, 1994). Particularly, the antimicrobial activity of plant oils and extracts have been in the roots of several applications, including conservation of raw and processed food, pharmaceutical products, alternative medicine and natural therapies (Lis-Balchin and Deans, 1997; Reynolds, 1996).

The search for natural products with anti-incrustation properties has been strongly encouraged for the reason that these components are not toxic to the environment. The larger part of these components was identified as terpenes, steroids, carotenoids, phenols, furanones, alkaloids, peptides and lactones. Isolates from a vast array of organisms, including sponges, coral, algae and microbes, have been studied worldwide (Viju et al., 2013).

A review of literature made by Agra et al. (2007) listed the popular use of 483 plants with bioactive potential in the Brazilian Northeastern region. Many are yet to be studied in relation to their chemical constituents and/or biological activity, but the number demonstrates the potential of the region as a source for future studies.

A study made with 24 plants empirically used by the Brazilian semiarid community for the treatment of a variety of diseases, such as skin infections, gastrointestinal disturbs, tuberculosis and urinary tract infections, showed that the trunk bark extract of *Commiphora leptophloeos*, known as Imburana in the region, showed inhibitory effect over *Staphylococcus epidermidis* in a minimal concentration of 1.0 mg/mL and decreased the formation of biofilm in 80%. Extracts of *Bauhinia acuruana* and *Pityrocarpa moniliformis* presented biofilm inhibitory activity for the same microorganism, without causing bacterial death (Trentin et al., 2011a, b).

The elimination of mature biofilm is still a very difficult task. Thus, the inhibition of the adherence of microorganisms in a way that does not involve bacterial death constitutes a new concept of antivirulence therapy. Substances that hamper the fixation of organisms, without affecting their growth, maintaining the cell in planktonic stage, turn them more susceptible to other antimicrobial agents and to the hosts immune system.

Rubus ulmifolius extracts studies using confocal microscopy revealed ellagic acid derivatives with biofilm formation inhibitory properties without bacterial growth inhibition in methicillin-sensitive *S. aureus* isolates (Fontaine et al., 2017). In 2008, Silva and collaborators investigated the *in vitro* antimicrobial action and adherence inhibition of the hydroalcoholic extract of *Rosmarinus officinalis* Linn. (rosemary) on standard strains of *Streptococcus mitis* ATCC 98811, *Streptococcus sanguinis* ATCC 10556, *Streptococcus mutans* ATCC 25175, *Streptococcus sobrinus* ATCC 27609 and *Lactobacillus casei* ATCC 7469. In this study, the extract of *Rosmarinus officinalis* Linn. was effective in the adherence inhibition of *S. mitis* ATCC 98811, *S. mutans* ATCC 25175 and *S. sobrinus* ATCC 27609 and might be used in future tests on the inhibition of biofilm formation (Silva et al., 2008).

A research carried out by the University of Barcelona, Spain Department of Genetics, in partnership with the MEDINA Foundation examined a set of 1120 natural product extracts identifying different activities, among them: inhibition of biofilm formation, detachment of mature biofilms, antimicrobial activity against planktonic cells and on biofilm component cells, using high-throughput screening (HTS), automated technology involving robotic and bioinformatics instruments to perform the pharmacologically important substances scanning. Of the 1120 extracts tested, 40 presented metabolites with potential antimicrobial action and antibiofilm against *Salmonella enteritidis* (Paytubi et al., 2017).

The casbane diterpene, isolated from the ethanolic extract of *Croton nepetaefolius,* can interact in a non-specific form with the bacterial membrane, destabilizing interactions and interfering in the cellular development. Experiments with microdilution in polystyrene plates have shown a promising activity of this compound over streptococcus species, achieving efficiency similar to that of chlorhexidine (Sá et al., 2012).

The essential oil and extracts of *Cupressus*

sempervirens were tested in their capacity of inhibiting biofilm formation. Among the studied microorganisms, *K. pneumoniae* was the most sensible strain. The methanolic extract and the essential oil showed a significant decrease in the fixation of *K. pneumoniae* in polyvinyl chloride (PVC), material used in the confection of medical catheters. The antimicrobial activity of these compounds can be mostly attributed to their phenolic constituents, α-pinene and cedrol (Selim et al., 2014).

Extracts of coconut shell fibers (*Cocos nucifera*) increased the hydrophobicity of *Pseudomonas* sp. and *Alteromonas* sp. isolated from marine biofilms, which, according to the physical-chemical theory of bacterial adhesion, decreases the adherence rate of micro-organisms (Viju et al., 2013). Silva et al. (2012) made a study of bioprospection of medicial plants in the Brazilian semi-arid region. The most promising results were obtained from extracts of *Schinopsis brasiliensis* which inhibited the formation of biofilm both in Gram-negative (*Pseudomonas aeruginosa*) as well as in Gram-positive (*Sthaphylococcus aureus*) bacteria, however, it was toxic to *Artemia salina*.

Extracts of *Humulus lupulus* (Cannabaceae), which contains xanthohumol as a major component, inhibited biofilm formation in *S. aureus* in 99.9%. Synergetic studies have shown that the addition of hop compounds decreased the value of MIC for commercial antibiotics oxacillin and linezolide from 0.125 and 0.5 to 0.094 and 0.38 µg/mL, respectively (Rozalski et al., 2013).

Trentin et al. (2011a, b) conducted a study on the effect of the filtrate of a *Cobetia marina* colony, a Gram-negative marine bacterium, on the formation of biofilm in *S. epidermidis*, an important agent in hospital-acquired infections. The filtrate does not possess the capacity of destructing consolidated biofilm; however, it inhibited its formation in 84.7%. It is possible that the action mechanism of this substance is related to its ability to alter the bacterial quorum sensing, avoiding the production of extracellular polymers and, consequently, biofilm formation.

Antibacterial agents used in the prevention and treatment of oral diseases, including cetylpyridinium chloride, chlorhexidine, fluorinated amines or products containing such agents might show undesired effects such as dental staining or, in the case of ethanol commonly found in mouthwash, in relation to the development of oral cancer (Knoll-Kohler and Stiebel, 2002; Lachenmeier, 2008; Mccullough and Farah, 2008; Neumegen et al., 2005; Rodrigues et al., 2007). Thus, the search for alternative products and phytochemical isolates in plants used in traditional medicine is considered a good alternative over synthetic drugs (Prabu et al., 2006).

The use of essential oils as antimicrobial agents is attested from a long time ago (Abee et al., 2011) and, in the specific case of oral microorganisms, mouthwashes containing essential oils have been shown beneficial and safe for daily use in oral hygiene (Claffey, 2003), depending on further studies to understand the spectrum of its action against these microorganism (Abee et al., 2011).

There are few mentions in literature about the antibiofilm activity of essential oil or their isolates on pathogenic biofilms related to oral diseases. Millezi et al. (2010) reported on the activity of cleaning detergents made of *C. citratus* and *T. vulgaris* over biofilms of *Aeromonas hydrophila*, a microorganism related to food contamination, showing decrease in the number of viable cells present in the communities subject to the substances.

Several screenings have shown that natural products, particularly phytochemicals, are an interesting source of quorum sense inhibitor (QSI) (Castillo-Juárez et al., 2015). They have been recognized as a large and attractive repository of QSI, offering a vast chemical diversity with structural complexity and biological activity (Borges et al., 2015). In fact, they resemble what is considered an “ideal” QSI, which includes being chemically stable, highly effective, low-molecular-mass molecules, and being harmless to health (Qian et al., 2013). Therefore, phytochemicals with QS inhibition activity can be promising tools to help the treatment of bacterial infections, including those that are biofilm-related, in an era where the availability of effective antibiotics is no longer guaranteed.

CONCLUSION

The rescue of the historical use of medicinal plants is inestimable in order to direct the investigations on bioprospection. A large number of plants have already been studied with regards to their antimicrobial properties. Brazil possesses a large diversity of plants mentioned with the popular use in the treatment of numerous infirmities that remain yet to be subject to a deeper scientific investigation, in order to prove their applicability, determine their bioactive substances and action mechanisms.

The focus of the study of antibiofilm properties is necessary, considering the problems these bacterial communities have caused in diverse clinical, environmental and industrial contexts. Brazil has a great potential for developing applied natural products. Thus, the use of medicinal plants reported in traditional knowledge, intertwining technology to scientifically validate it, and might become an efficient option in the treatment of various infectious diseases (Borges et al., 2016).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of semi-nested polymerase chain reaction (PCR) and mannan antigen detection compared to blood culture for diagnosis of candidemia

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Invasive *Candida* infections have emerged as an important pathogen in the last decade, especially in immunocompromized patients. The aim of the present study was to evaluate the detection of *Candida* species in blood samples from pediatric patients with sepsis by blood culture method versus antigen detection method by enzyme linked immunosorbent assay (ELISA) and molecular method by polymerase chain reaction (PCR). This cross-sectional study was carried out on children at Mansoura University Children Hospital (MUCH) with the presence of clinical signs suggesting sepsis with absence of prior antifungal therapy. Laboratory diagnosis included blood culture, mannan antigen detection of *Candida* species by ELISA and semi-nested PCR. *Candida* species were detected by blood culture in 15.6% of the children and was detected by PCR and antigen detection in 20% for each. Candidemia was more frequently detected among patients with central venous lines (38.5%), children with Diabetes Mellitus (DM) (23.1%) and children with frequent blood transfusions (15.4%). However, these risk factors were not statistically significant. Mortality rate among children with candidemia was 42.9%. The sensitivity, specificity, and accuracy of antigen detection method by ELISA compared to culture, were 71.4, 89.5 and 86.7%, respectively. The sensitivity, specificity and accuracy of semi-nested PCR compared to culture were 85.7, 92.1 and 91.1% respectively. It can be concluded from this study that *Candida* species is a frequent pathogen associated with pediatric sepsis. Blood culture though is a reliable laboratory method for its diagnosis may lack sensitivity and requires prolonged time. Semi-nested PCR for detection of candidemia is sensitive, specific and accurate method. Mannan antigen detection by ELISA is rapid and easy; however, it may lacks specificity; it can be used as a preliminary method for screening. Further studies are recommended to detect the appropriate laboratory algorithm for early diagnosis of candidemia to start antifungal therapy appropriately.

Key words: *Candida*, Mannan antigen, polymerase chain reaction (PCR), blood culture.

INTRODUCTION

Invasive *Candida* infections have emerged as an important pathogen in the last decade especially in immune-compromised patients. There are several factors associated with such increase like the use of broad

spectrum antibiotics, increase use of invasive devices like intravascular catheter and parenteral alimentation (Moran et al., 2009; Watson et al., 2003; Pappas et al., 2003).

Among susceptible patients for infection with *Candida* species are pediatric patients in intensive care units either neonatal (NICU) or pediatric intensive care unit (PICU) (Watson et al., 2003; Pappas et al., 2003). Previous study had demonstrated that infection rate with *Candida* species was increased around 10 folds among neonates in a five years duration (Dutta and Palazzi, 2011). The mortality rates due to invasive candidiasis are high and range from 20 to 30% (Kuzucu et al., 2008; Neu et al., 2009; Singhi and Deep, 2009) that can be increased up to 50% if there is a delay in appropriate laboratory diagnosis (Roilides et al., 2004; Vendettuoli et al., 2008).

Candida species in pediatric patients are usually associated with sepsis (Watson et al., 2003). However, there is a lack of diagnostic clinical signs and symptoms that indicates that the pathogen is *Candida* unless reported by the laboratory results. There is less practice of empirical antifungal treatment in pediatric patients compared to the adults (Zaoutis et al., 2005; Hegazi et al., 2014). Therefore an appropriate rapid laboratory diagnosis of candidemia children has utmost importance for appropriate antifungal therapy.

Blood culture considered as gold standard for candidemia diagnosis, takes several days for detection even with the use of automated blood culture systems. Moreover, phenotypic identification for species levels takes more time either by the use of chromogenic media or manual API identification system (Odds and Bernaerts, 1994; Latouche et al., 1997).

Another laboratory method for diagnosis of invasive candidiasis is serodiagnosis method. Serodiagnosis depends on the detection of cell wall mannan (mannoprotein); components of *Candida* species that is usually a soluble antigen marker detected transiently in blood during the infection (Yeo and Wong, 2002; Sendid et al., 2003).

Molecular method for direct identification of *Candida* species has proven to be an accurate and rapid method for detection of candidal infections (Roilides et al., 2004; Vendettuoli et al., 2008; Imran and Alshammry, 2016).

Different molecular methods for detection of candidia infection, have been used such as real time PCR (Liguori et al., 2007; Li et al., 2003), Multiplex PCR (Sampath et al., 2017) and nested PCR (Ahmad et al., 2002; Çerikçioglu et al., 2010).

PCRs based on amplifications of 18S and 28S rDNA regions have been used to identify different *Candida* species in clinical specimens, including blood. 18SrRNA gene is a highly conserved region in Fungi and is the primary target transcript used for the detection of *Candida* by PCR (Weerasekera et al., 20013; Makene, 2014).

Thus the aim of the present study was to evaluate the detection of *Candida* in blood samples from pediatric patients with sepsis by blood culture method versus semi-nested PCR and mannan antigen detection method by enzyme linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

This cross-sectional study was carried out in Mansoura University Children hospital, Egypt from March 2016 till March 2017. The study was approved by Mansoura Faculty of Medicine ethical committee. Written approval consent was obtained from the parents of the studied children. The inclusion criteria for children were the presence of clinical signs suggesting sepsis with the absence of prior antifungal therapy. Children with underlying malignancy or neutropenia and receiving antifungal empirical therapy were excluded from the study.

Sample collection

Each child participating in the study was subjected to full medical history taking and clinical examination. Candidemia was defined as positive blood culture for *Candida* species either in one or two blood cultures (Neu et al., 2009; Lagrou et al., 2007). Six milliliters blood samples were obtained from children under complete aseptic techniques, divided as follows; two milliliters sample in plain vacutainer for serum separation for ELISA, two milliliters over EDTA for PCR and two milliliters was inoculated into blood culture Bact/Alert system.

Candida identification

Blood cultures were incubated for 7 days at 37°C and cultured on Sabouraud dextrose agar (SDA) with chloramphenicol and blood agar. *Candida* colonies were identified by colony morphology on SDA, germ tube formation and chlamydospore production. Further identification to species level was performed by API *Candida* (bioMérieux, France) (Ahmad et al., 2002).

Mannan antigen detection by ELISA

Mannan antigen was determined using Platelia *Candida* Antigen kits Platelia™ *Candida* (Bio-Rad, France) following the manufacturer's recommendations, is a one-stage immunoenzymatic sandwich microplate assay for quantitative detection of soluble mannan antigen in human blood. The absorbance (optical density) of samples and calibrator were determined with a spectrophotometer set at 450/620 nm wavelength. Each experiment included a calibration curve, which was made with a pool of normal human serum supplemented with known concentrations of mannan ranging from 0.1 to 2 ng/ml.

For mannanemia levels exceeding 2 ng/ml (above the range of the calibration curve) was further diluted and retested to obtain a precise concentration (Sendid et al., 2003).

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Semi-nested PCR for detection of *Candida*

Two sets of primers were used to increase these sensitivity and specificity of the assay. DNA was extracted from ethylenediamine tetraacetic acid (EDTA) blood using QIAamp DNA blood Mini Kit supplied by QIAGEN companies. Then 10 µl of extracted DNA was subjected to the first round of amplification using ready to go PCR beads which contain all the reagents necessary for PCR reaction except primers and the template. In the first round of amplification the Panfungal primers targeting 18S rRNA gene were used

F: 5` AGGGATGTATTTATTAGATAAAAAATCAA 3`.
R : 5 CGCAGTAGTTAGTCTTCAGTAAATC 3`.

All tubes were transferred to the thermal cycler where they were subjected to initial one cycle of denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 20 s, and final one cycle of extension at 72°C for 7 min. Three microliter of the amplified panfungal products were used in semi-nested PCR for further amplification using the *Candida* genus specific primer by the same method of amplification with the exception of annealing temperature, which was 66°C.

***Candida* genus specific primers**

F: 5´ GGGAGGTAGTGACAATAAATAAC 3´.
R: 5´CGTCCCTATTAATCATTACGAT 3´ (Jaeger et al., 2000)

In every PCR run, a negative control (distilled water) and a positive control (Purified *Candida* DNA) were included.

The final PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide. Generated PCR products of 728 and 402 bp for panfungal and *Candida* species; respectively. Negative control was included in each amplification set consisting of sterile distilled water. Positive control was extracted DNA from *Candida* isolated from clinical sample in the laboratory. The process was performed under entirely sterile conditions for PCR to avoid contamination (Van Burik et al., 1998).

Statistical analysis

The data were analyzed using Statistical Package for Social Science software computer program version 17 (SPSS, Inc., Chicago, IL, USA). Quantitative non parametric data were presented in median and interquartile range (IQR). Mann-Whitney U test was used for comparing quantitative no- parametric data. Chi-square "$\chi 2$" or Fischer's exact tests, as indicated, were used to compare the qualitative data. *P* value less than 0.05 was considered statistically significant

The sensitivity, specificity, positive predictive value, accuracy of antigen and PCR to differentiate between positive and negative *Candida* culture were calculated.

The sensitivity and specificity of antigen (quantitative) to differentiate between positive and negative *Candida* culture was examined at different cutoff points using ROC curve analysis to determine the best cutoff point as well as the diagnostic power of each test.

RESULTS

The present study included 90 children affected with sepsis. They were 51.1% males and 48.9% females. The risk factors for the development of sepsis were presence of central venous line in 34.8%, urinary catheter in10.1%, low birth weight preterm newborn 19.1%, DM type 1 24.7%. The outcome of the studied children ranged from death 24.7% to discharge with complete recovery in 74.2%. The majority of the studied children were on ampicillin+sulbactam (unasyn) (75%) and/or Cefotaxime (65.5%) as empirical antibiotics therapy (Table 1).

Candida species were detected by blood culture in 15.6% of the children and was detected by PCR and antigen detection in 20% for each (Table 1).

Species identification by API *Candida* showed eight isolates of *Candida albicans*, three isolates *of Candida tropicalis* and three isolates of *Candida parapsilosis* (Figure 1).

Candidemia was more frequently detected among patients with central venous lines (38.5%), children with DM type 1(23.1%), low birth weight (15.4%) and children with frequent blood transfusions (15.4%). However, these risk factors were not statistically significant. The mortality rate among children with candidemia was 42.9% (Table 2).

Mannan antigen detection by ELISA was associated with a positive blood culture in 10 patients while it was negative in 4 patients with positive culture. Moreover, antigen detection by ELISA was positive in 8 patients with negative culture. The sensitivity, specificity, and accuracy of antigen detection method by ELISA 71.4, 89.5 and 86.7%, respectively (Tables 3 and 4).

In comparison between detection of *Candida* by blood culture and PCR, PCR was positive in association with culture in 12 patients (66.7%) while it was negative in 2 positive cultures and positive in 6 cases with negative blood culture. The sensitivity, specificity and accuracy of PCR were 85.7, 92.1 and 91.1%, respectively (Tables 5 and 6).

The cutoff value of *Candida* antigen by ELISA was 49.5 pg/ml with sensitivity 88.9% and specificity 54.2% (Figure 2).

DISCUSSION

Invasive infection by *Candida* species is common among critically ill children. Blood culture is the gold standard method for diagnosis of sepsis. Nevertheless, the time between sampling and obtaining the results of culture may be as long as 7 to 10 days, which may be too long for the introduction of effective treatment. Moreover, the sensitivity of this method for the isolation of fungi is low (Badiee et al., 2014; Sherman-Weber et al., 2004). *Candida* species was detected by blood culture in 15.6%. The most common species detected by API were *C. albicans* followed by *C. tropicalis* and *C. parapsilosis*. This is similar to results reported previously by Taira et al. (2014).

The non-*Candida albicans* species isolated from the present study were *C. tropicalis* and *C. parapsilosis,* which were recorded previously to be common fungal species among children (Colombo et al., 2013; Santolaya et al., 2014).

The mortality rate among children with candidemia was

Table 1. Demographic, clinical and microbiological results of the studied patients.

Age (Median -IQR)		0.30	0.10-4.00
Sex	Male	46	51.1%
	Female	44	48.9%
Outcome	Death	22	24.7%
	Discharge without improvement	2	2.2%
	Discharge with improvement	66	74.2%
Risk factors	Central line	31	34.8%
	Urinary catheter	9	10.1%
	Frequent blood transfusion	10	11.2%
	Low birth weight	17	19.1%
	DM	22	24.7%
Culture of *Candida*	P	14	15.6%
	N	76	84.4%
Antigen	P	18	20.0%
	N	72	80.0%
PCR	Yes	18	20.0%
	No	72	80.0%
Unasyn	Yes	66	75.0%
	No	22	25.0%
Cefotaxime	Yes	57	65.5%
	No	30	34.5%
Amikacin	Yes	18	20.5%
	No	70	79.5%

Data expressed as median(IQR) or as frequency (Number-percent); IQR: interquartile range; P: positive; N: Negative; DM: Diabetes Mellitus.

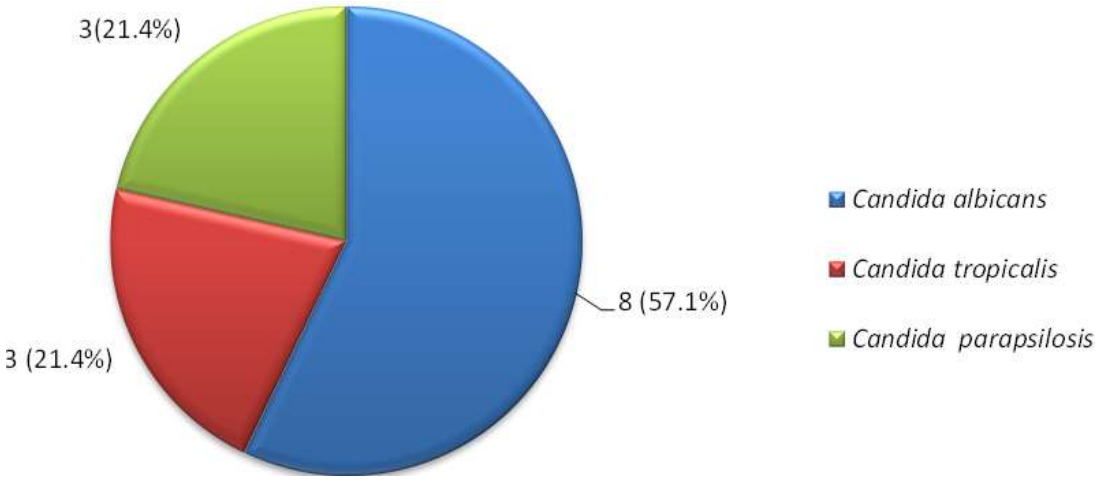


Figure 1. Distribution of isolated *Candida* species by API *Candida.*

42.9%. This rate was similar to study reported previously by Hegazi et al. (2014) and Santolaya et al. (2014).

Table 2. Association between *Candida* culture positive and some clinical risk factors.

Parameter		Culture of *Candida*				P
		P		N		
		No.	%	No.	%	
Risk factors	Central line	5	38.5	26	34.2	
	Urinary catheter	1	7.7	8	10.5	
	Frequent blood transfusion	2	15.4	8	10.5	0.97
	Low birth weight	2	15.4	15	19.7	
	DM type 1	3	23.1	19	25.0	
Outcome	Death	6	42.9	16	21.3	
	Discharge without improvement	0	0.0	1	1.3	0.2
	Discharge with improvement	8	57.1	58	77.3	

Data expressed as median (IQR) or as frequency (number-percent), IQR: Interquartile range; P: Probability; significance <0.05; DM: Diabetes Mellitus.

Table 3. Association between *Candida* culture and antigen detection.

		Antigen					
		P		N		Total	
		No.	%	No.	%	No.	%
***Candida* culture**	P	10	71.4	4	28.6	14	15.6
	N	8	10.5	68	89.5	76	84.4
	Total	18	20.0	72	80.0	90	100.0

Table 4. Sensitivity, specificity, PPV, NPV and accuracy of antigen detection compared to culture.

Mannan antigen detection	True positive	False negative	True negative	False positive	Sensitivity	Specificity	PPV	NPV	Accuracy
	10	4	68	8	71.4%	89.5%	55.6%	94.4%	86.70%

PPV: Positive predictive value; NPV: Negative predictive value.

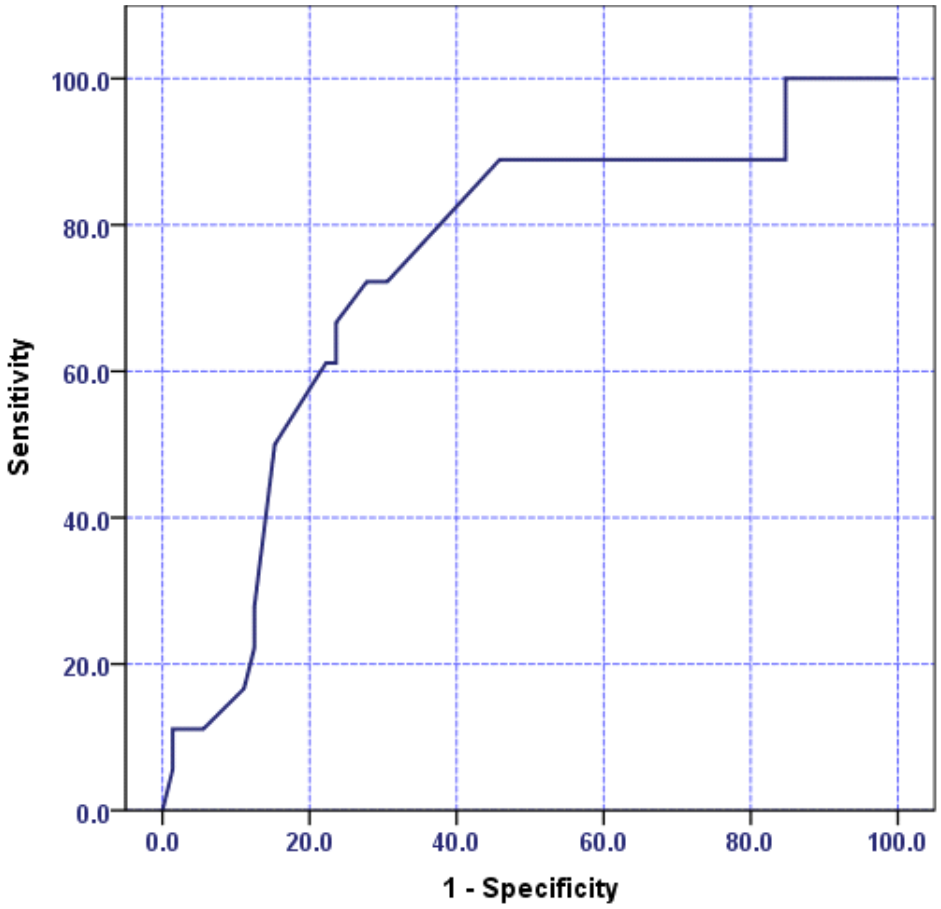
Table 5. Association between *Candida* culture and PCR.

		PCR					
		P		N		Total	
		No.	%	No.	%	No.	%
***Candida* culture**	P	12	66.7	2	2.8	14	15.6
	N	6	33.3	70	97.2	76	84.4
	Total	18	100	72	100	90	100.0

Table 6. Sensitivity, specificity, PPV, NPV and accuracy of PCR compared to culture.

PCR	True positive	False negative	True negative	False positive	Sensitivity	Specificity	PPV	NPV	Accuracy
	12	2	70	6	85.7%	92.1%	66.6%	97.2%	91.11%

PPV: Positive predictive value; NPV: Negative predictive value.



	AUC (95-CI%)	P value	Cutoff value	Sensitivity	Specificity	PPV	NPV	Accuracy
Antigen	74.4 %(61.7%-87.1%)	<0.001*	49.50	88.9%	54.2%	32.65%	95.12	61.11%

Figure 2. ROC curve of mannan antigen versus culture. ROC: Receiver operating characteristic; AUC: Area under the curve; CI: Confidence interval; PPV: Positive predictive value; NPV: Negative predictive value.

Positive blood culture for *Candida* was more frequently detected among patients with central venous lines (38.5%), children with DM type 1 (23.1%) and children with frequent blood transfusions (15.4%). However, these risk factors were not statistically significant. The presence of invasive devices, immunocompromized conditions and frequent blood transfusion are known risk factors for invasive *Candida* infections (Celebi et al., 2007; Baldesi et al., 2017; Chapman et al., 2017).

Modern technologies have been developed for rapid detection and identification within two hours of low concentration of *Candida* species in bloodstream infections approximating 1 CFU/ml such as magnetic resonance-based technology T2*Candida* ® (Lau et al., 2010). Other tests that could eventually be used such as the β-1,3 glucan and mannan tests (Montagna et al., 2009; Nguyen et al., 2012).

The sensitivity, specificity, and accuracy of the antigen detection method by ELISA were 71.4, 89.5 and 86.7% respectively. The sensitivity and of *Candida* antigen detection in the present study was similar to previous study (Alam et al., 2007) and it was less than that reported by Kurita et al. (2009). While the specificity was higher than that previously reported (Badiee et al., 2014). The difference in sensitivity may be attributed to the fact that mannan antigen of *Candida* species is rapidly cleared from the patient's blood (Kurita et al., 2009)

The study tried to detect the best cutoff value for quantitative detection of mannan antigen. This increases the sensitivity of the test with lowering the specificity. The cutoff value of *Candida* antigen by ELISA was 49.5 pg/ml with sensitivity 88.9% and specificity 54.2%. Thus detection of mannan antigen by ELISA can be used as a promising screening test for detection of candidemia (Duettmann et al., 2016). However, it has to be confirmed by more specific laboratory methods before the start of

antifungal therapy.

In a comparison between detection of *Candida* by blood culture and PCR, PCR was positive in association with culture in 12 patients (66.7%) while it was negative in 2 positive cultures. Similarly, Taira et al. (2014) reported that PCR was positive in blood samples with negative culture. This can be attributed to the presence of transient episodes of candidemia that leads to systemic infection in those patients.

In the present study, PCR was positive in 6 cases with negative blood culture. There are some concerns about the comparison of PCR to blood culture as gold standard technique due to lack of blood culture sensitivity to detect all positive cases with candidemia (Avni et al., 2011). It has been estimated that a single blood culture can miss from 25 up to 50% of disseminated candidiasis (Olaechea et al., 2004). Thus the reporting of positive PCR results in negative blood culture samples may not be reported as false positive as these patients may actually have a *Candida* bloodstream infection that was not detected by a single blood culture. Moreover, these cases may represent nonviable organisms, free *Candida* DNA in the blood, or persistence of a low-level infection. Several studies have previously demonstrated that molecular techniques perform better than culture methods (Ahmad et al., 2002; Lau et al., 2008; Avni et al., 2011).

The overall accuracy of semi-nested PCR was 91.1% with good sensitivity 85.7% and specificity 92.1%. Thus, this method can be applied as a specific rapid laboratory diagnosis associated with appropriate clinical evaluation to start antifungal therapy in suspected patients (Tirodker et al., 2003; Wellinghausen et al., 2009; Ruppenthal et al., 2005). Similar conclusion was made by a previous study from Egypt with PCR for 16 srRNA (Hassan et al., 2014).

On the other hand other studies with higher number of patients are required to evaluate its role as a screening method for highly susceptible patients. The advantage of PCR as rapid and specific method for accurate diagnosis of candidemia is extremely important in rapid introduction of antifungal therapy, improvement of candidemia outcomes, reducing the costs of unnecessary antibiotics therapy and reducing the hospital costs (Zilberberg et al., 2010; Kaufman, 2010).

It can be concluded from this study that *Candida* species is a frequent pathogen associated with pediatric sepsis. Blood culture though is a reliable laboratory method for its diagnosis may lack sensitivity and requires prolonged time. Semi-nested PCR for detection of candidemia is sensitive, specific and accurate method. Mannan antigen detection by ELISA is rapid and easy; however, it may lacks specificity can be used as preliminary method for screening. Further studies are recommended to detect the appropriate laboratory algorithm for diagnosis of *Candida* sepsis to start antifungal therapy appropriately and the clinical outcome associated with earlier diagnosis achieved with direct PCR

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Bacteriological quality of drinking water from source to point of use among rural communities of Farta Woreda in North West, Ethiopia

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Access to safe water is a universal need. However, many of the world's population lack access to adequate and safe water. Consumption of contaminated water with viruses, bacteria and parasites causes health risk to the public and the situation is serious in rural areas. So this study is aimed at assessing the bacteriological quality of drinking water at source and point of use among rural Communities of Farta Woreda, North Western, Ethiopia. A descriptive cross sectional study was conducted from February 15th to February 30th 2016 in 41 rural Kebeles of Farta Woreda. A total of 120 water samples were obtained from protected water sources and household water storage containers and tested for *Escherichia coli* by using membrane filter methods. The contaminant risk of water sources and household storage containers were assessed by sanitary inspection checklist of World health Organization. Descriptive statistics (proportion and percentage) were used to count the *E. coli* load and the results were interpreted using World Health Organization (WHO) guidelines for drinking water quality. All household water storage containers and majority 22(91.7%) of the protected wells and 5(83.3%) springs in the study area were not in compliance with WHO recommended values (0 CFU/100 ml of drinking water) for drinking water. Majority 10(41.7%) of protected well, 5(83.3%) protected spring and 42(46.7%) household storage containers had high sanitary risk score for *E. coli*. The water sources and also household water storage containers were heavily contaminated with *E. coli.* Source protection strategies, awareness creation on safe water handling practices as well as monitoring are necessary to enhance good drinking water quality.

Key words: Drinking water, *E-coli*, bacteriological quality, rural community, storage container.

INTRODUCTION

Water in sufficient quantity and good quality is essential for life. However, many of the world's population lack access to adequate and safe water (Tadesse et al., 2010). The use of improved sanitation facilities is low

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especially in Sub-Saharan Africa and Southern Asia. Eight hundred and eighty four million people in the world still do not get their drinking-water from improved sources; Sub-Saharan Africa accounts for over a third of that number. Fresh water has become a scarce commodity due to over exploitation and pollution. Increasing population and its necessities have led to the deterioration of surface and sub surface water (Shyamala et al., 2008).

Regrettably, it is no surprise that much ill health is attributable to a lack of hygiene sanitation and water supply. No access to good quality drinking water leads to a high risk of water-borne or diarrheal-related diseases such as cholera, typhoid fever, hepatitis A, amoebic and bacillary dysentery and other diarrhoeal diseases (WHO/UNICEF,2010). Each year, 4 billion cases of diarrhoea causes 2.2 million deaths, mostly among the under-fives. Eighty-eight percent of cases of diarrhoea worldwide are attributable to unsafe water, inadequate sanitation or insufficient hygiene (Prüss-Üstün et al., 2008) and kills more children than HIV/AIDS, malaria and measles combined (UNICEF/WHO, 2009).

Bacteriological water quality refers to the bacteriological appearances of water. Hence, it is defined in terms of the absence or presence of indicator organisms. The absence of indicator organisms in drinking water indicates its bacteriological quality and do not pose health risk if consumed (WHO, 2011).

The technique that has been recently adopted is analyses for *Escherichia coli* (*E. coli)* as an indicator organism (Cheesbrough, 2006; WHO, 2011); because it provides conclusive evidence of recent faecal contamination (Odonkor and Ampofo, 2013; WHO, 2011), more specific, and used to estimate disease risk (Edberg et al., 2000; WHO, 2012). WHO recommends zero *E. coli* per 100 ml of drinking water (WHO, 2012).

Tadesse et al. (2010) revealed that water collected from good bacteriological quality sources is likely to become contaminated at its point of use. Water supplies and quality in Ethiopia are not different from the general situation of developing countries as a whole. The situation is worse in rural areas where coverage is only 20% when compared with 80% in urban areas which are the least among the continent (Admassu et al., 2004).

One-fifth of all drinking water supplies in Ethiopia are at “high” risk to human health. As a result, 60 to 80% of the population suffers from water-borne diseases (MoH, 2004). The highest (12.9%) prevalence diarrhoea was recorded among children of households that drink water from unprotected wells (CSA and ICF International, 2012).

People living in rural communities are the population sector most affected by hydro-transmissible infectious pathogen agents. Therefore, controlling water quality is one of the essential issues of drinking water management (Sehar et al., 2011; Udousoro and Umoren, 2014).

The provision of safe and adequate water supply for the population has far reaching effects on health, productivity and quality of life, as well as on the socioeconomic development of the nation. The most important aspect to provide safe water supply is therefore determine whether an indicator organism is present. Therefore, this study aimed at assessing the bacteriological quality of drinking water at source and point of use among rural communities of Farta Woreda, North Western, Ethiopia**,** which will help in the intervention actions to be taken by the concerned bodies for further improvements of community health and will provide baseline information for further study.

MATERIALS AND METHODS

Study design and description of study area

A descriptive cross sectional study was conducted from February 15th to February 30th 2016 in Farta Woreda which is found in Amhara regional state of Ethiopia (Figure 1). Woreda consists of 2 urban Kebeles and 41 peasant associations (PAs). There are 10 health centers and 54 health posts providing health service for the Woreda population. Woreda has 88.4, 85.2 and 75.7% health service, latrine and improved water supply coverage respectively. The main water sources are protected springs, unprotected springs protected hand pumps dug wells and unprotected hand dug well for all domestic uses. All protected water sources found in 41 rural Kebeles and water sources in selected 8 Kebeles were the source and study population respectively. Functional protected water sources that provide at least 6 months service to the community and households that used that protected water sources were included in the study

Sampling procedures

From the total 41 rural Kebeles found in the Woreda, 8 Kebeles were selected randomly and included in the study. A representative sample of 30 protected water sources (n=24 from protected hand pump dug wells and n= 6 from protected spring) were obtained from the total 150 functional protected water sources found in 8 Kebeles of the Woreda. In addition a total of 90 households were included for household water handling practice and bacteriological analysis of household storage containers. The sample from the source was taken correspondingly with the household water sample after asking the inhabitants where they fetch water during the time of household water sample collection and for each source three households were included to see the contamination variation from household to household.

Data collection tools and procedures

Data were collected by using rapid water testing kit and sanitary inspection checklist prepared by WHO. Three hundred milliliter of water samples were collected by using sterile plastic bottle after washing out let pipe for protected spring and hand dug well following the procedure of membrane filtration drinking water quality testing by using Oxfam DelAgua field test kit as indicated by the American Public Health Association (APHA) (1998). Similarly water samples were taken from household drinking water storage containers following similar procedures. In addition the contaminant risks of water sources and household drinking water storage containers were assessed by Sanitary Survey using WHO drinking water source observation check list.

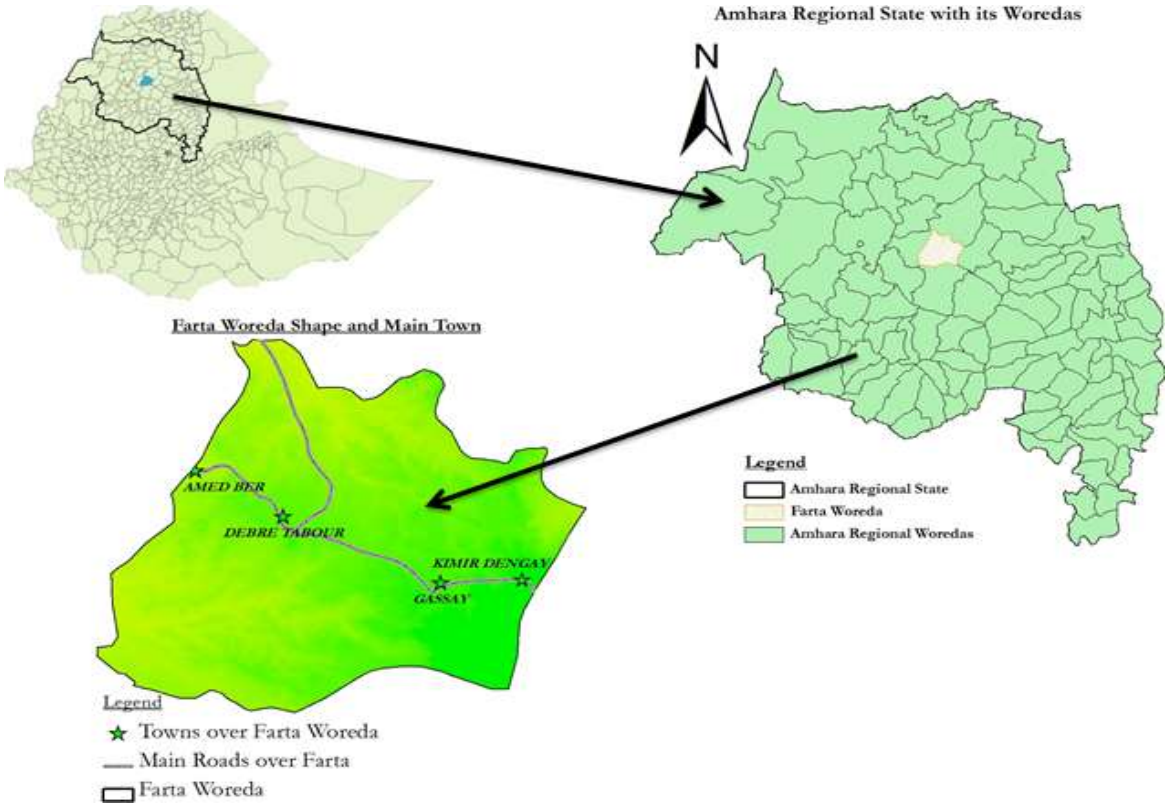

Figure 1. Map of Farta Woreda, Northwest Ethiopia, March, 2016.

Table 1. Bacteriological quality (*E. coli*) of protected well and Springs in Farta Woreda, North West Ethiopia.

Type of water sources	*E. coli* level /100 ml sample of water					Total
	0	1-10	10-100	100-1000	≥1000	
Protected spring (n=6)	1 (16.7%)	0	5(83.3%)	0	0	6
Protected well (n=24)	2 (8.3%)	5 (20.85%)	10 (41.7%)	4 (16.7%)	3 (12.5%)	24
Total	3 (10%)	5 (16.7%)	15 (50%)	4 (13.3%)	3 (10%)	30
WHO risk category*	In conformity with WHO guidelines	Low risk	Intermediate risk	High risk	Very High risk	

*WHO (2011). Guideline for drinking water quality, Vol. I.

Data quality management and analysis

The data quality was assured by closed follow up and supervision of the laboratory technicians by both principal and co-investigators. The water samples were immediately transported to the Amhara regional laboratory unit for water quality analysis. During transportation, the samples were stored below 4°C using cold closet or ice box. Descriptive statistics, which included mean, proportions and percentages, were used. The bacteriological counts recorded were compared and interpreted with the WHO guidelines for drinking water.

RESULTS

Bacteriological analysis of protected source water samples

Of the total 30 water samples collected from protected water sources 27 (90%) were above the standard limit of WHO (0 CFU/100 ml of drinking water) and 3 (10%) within the acceptable range of WHO. Of the total 24 water samples collected from protected wells 3 (12.5%), 4 (16.7%), 10 (41.7%) and 5 (20.85%) had *E. coli* concentrations ranging from ≥1000, 100-1000, 10-100 and 1-10, respectively. Similarly from the total 6 water samples obtained from protected spring 5(83.3%) had *E. coli* concentrations ranging from 10 to 100 *E. coli*/100 ml of water (Table 1).

Bacteriological analysis of household container water samples

Of the total 90 household water containers all had *E. coli* Eleven (12.2%), 8 ((8.9%), 61 (67.8%) and 10 (11.1%)

Table 2. Bacteriological quality (*E-coli*) of household water storage containers in Farta Woreda, North West Ethiopia.

Type of water sources	*E. coli* level /100 ml sample of water					Total
	0	1-10	10-100	100-1000	≥1000	
Household container water samples (n=90)	0	10 (11.1%)	61 (67.8%)	8 (8.9%)	11 (12.2%)	**90**
Risk category*	In conformity with WHO guidelines	Low risk	Intermediate risk	High risk	Very high risk	

*WHO (2011). Guideline for drinking water quality, Vol. I.

Table 3. Levels of risk of contamination of 6 protected springs and 24 protected wells and 90 household storage containers in Farta Woreda, North West Ethiopia.

Sanitary inspection score	WHO risk category*	*E. coli* (CFU/100 ml of water)					Total
		0	1-10	10-100	100-1000	≥1000	
Protected well (n=24)							
0-2	Low risk	2 (8.3%)	0	0	0	0	2 (8.3%)
3-5	Medium risk	0	1 (4.2%)	2 (8.3%)	1 (4.2%)	0	4 (16.7%)
6-8	High risk	0	2 (8.3%)	6 (25%)	2 (8.3%)	0	10 (41.7%)
9-11	Very High risk	0	2 (8.3%)	2 (8.3%)	1 (4.2%)	3 (12.5%)	8 (33.3%)
Total		**2 (8.3%)**	**5 (20.8%)**	**10 (41.7%**	**4 (16.7%)**	**3 (12.5%)**	**24**
Protected spring (n=6)							
0-2	Low risk	1 (16.7%)	0	0	0	0	1 (16.7%)
3-5	Medium risk	0	0	0	0	0	0
6-8	High risk	0	0	5 (83.3%)	0	0	5 (83.3%)
9-11	Very High risk	0	0	0	0	0	0
Total		**1**	**0**	**5**	**0**	**0**	**6**
Household (n=90)							
0-2	Low risk	0	8 (8.8%)	11 (12.3%)	0	0	19 (21.1%)
3-5	Medium risk	0	1 (1.1%)	23 (25.6%)	2 (2.2%)	3 (3.3%)	29 (32.2%)
6-8	High risk	0	1 (1.1%)	27 (30%)	6 (6.7%)	8 (8.8%)	42 (46.7%)
9-12	Very High risk	0	0	0	0	0	0
Total		**0**	**10**	**61**	**8**	**11**	**90**

*Source: Howard AG (2002). Water Quality Surveillance: A practical guide, Loughborough University, UK and WHO (2011). Guideline for drinking water quality, Vol. I.

had *E. coli* concentration of ranging from ≥1000, 100-1000, 10-100 and 1-10, respectively (Table 2).

Level of risk of contamination of water sources and household drinking water storage containers

In case of risk classification, 8 (33.3%),10 (41.7%), 4 (16.7%) and 2 (8.3%) of protected well water samples had very high, high, medium and low sanitary risk score for *E. coli* respectively. Similarly the majority 5(83.3%) of water samples from protected springs had high sanitary risk score for *E-coli*. Using *E. coli* count as a microbiological indicator to determine the overall risk to health status, 42 (46.7%), 29 (32.2%) and 19 (21.1%) household water samples had high, medium and low sanitary risk score for *E. coli*, respectively. The bacteriological analysis results of both the protected sources and household storage containers clearly indicated the increment of *E. coli* per 100 ml of water with increasing sanitary risk score from low to high (Table 3).

DISCUSSION

The World Health Organization recommends that water directly intended for human consumption be free from *E. coli* contamination, since the presence of *E. coli* indicates a potential health risk for consumers (WHO, 2011). However, the current study showed that majority 22 (91.7%) of examined samples from wells and 5 (83.3%) spring had *E. coli* concentration above the WHO acceptable range (*E. coli* counts must not be detected in any 100 ml of drinking water samples). for drinking water.

The same study in rural communities of Dire Dawa administrative council showed that all and majority (83.3%) of protected spring and well were positive for total coloforms (TC), respectively (Desalegn et al., 2013) .Another study in North Gonder zone showed that 71.43% of the samples obtained from protected springs had all kind of indicator bacteria. Fifty percent of the positive samples had fecal coli forms, of these 35.7% had *E. coli* (Abera et al., 2011). Edessa et al. (2017) also found that 92.6% of well water samples are contaminated with *fecal coliforms*. The contamination of these water sources might be due to poor source protections. This suggested poor protection and sanitation practice in the water sources and household water handling practices.

Despite the standard limit established by WHO (2011) water intended for human consumption should contain no microbiological agents that are pathogenic to humans, the bacteriological analysis of water at household storage containers in the current study revealed that all (100%) of samples were contaminated with *E. coli*. This is supported by a study conducted in Kolladiba town of Ethiopia which showed that all the water samples from household (HHs) storage containers were found to be positive for total coliforms, while 32.5% were contaminated with fecal coliforms (Sharma et al., 2013). Another study in Shashemane Rural District of Ethiopia showed that 33.3% of sampled water were contaminated with *E. coli* (Edessa et al., 2017). A study in Bahirdar city of Ethiopia also indicated that, analysis of household water samples revealed that 19(54.2%) and 12(34.2%) had total coliform count from 10-100 and 1.01-9.99 CFU/100 ml and no household water sample had total coliform count from 0.01 to 1.01 CFU/100 ml of water (Milkiyas et al., 2011). Similarly a study conducted in Bona district of southern Ethiopia, Jimma zone of south west Ethiopia and Adama Town of oromiya regional state showed that majority of water samples taken from household storage containers were not compliance with WHO guideline value of 0 CFU/100 ml (Abebe and Dejene, 2015; Mohammed et al., 2015; Temsgen and Hameed, 2015). The poor water quality observed in storage containers might be due to the poor handling practice of the inhabitants in collection and storage. The behavioral and hygienic practices of the community might also be contributing to this high load of indicator organisms.

The sanitary inspection result of all water sources and household storage containers this study had sanitary risk score ranging from low to high and majority 15(50%) and 42(46.7%) of protected sources and household storage containers had high sanitary risk score for *E. coli* contamination. This finding is in agreement with a study conducted in rural communities of Ethiopia (Amenu et al., 2014; Tsega et al., 2013). They reported that All dug wells and springs were at high risk category for total coliforms. Another study by Abebe and Dejene (2015) revealed that all of the protected spring examined had risks ranging from low to high .This study demonstrated that adequate protection of water sources could improve their bacteriological quality by effectively preventing faecal coli form from entering water system prior to their delivery point. Source protection almost invariably is the best method of ensuring safe drinking water. However, failure to provide adequate protection, poor site selection, and unhygienic practices of the consumers and deterioration of construction materials may contribute the contamination of water sources. It implies that water with high sanitary risk had high chance of contamination with fecal coli forms.

Limitations of the study

This research measures only microbial water quality by using *E. coli* as an indicator for fecal pollution. As a limitation, the physico-chemical analysis was not done due to logistics constraints. However, it was believe that the information obtained about fecal contamination of the water sources at Farta Woreda is the first in its kind and revealed the hygienic condition of water sources which are used by the community.

Conclusion

Bacteriological quality of most water samples analyzed in the current study did not meet the standards set for drinking water by the WHO. Similarly none of the water samples taken from household drinking water storage containers were in compliance with the WHO guideline value 0 CFU/100 ml. In addition majority of water points and household water storage containers were found to have high sanitary risk score for contamination of *E. coli*. Source protection strategies as well as monitoring are recommend for this community. Moreover, further action in the improvement of water supply schemes in the area and awareness creation on safe water handling practices are necessary.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of the fermentation on the microbial population occurring during the processing of zoom-koom, a traditional beverage in Burkina Faso

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Zoom-koom is a traditional fermented beverage from Burkina Faso based on cereals such as millet or sorghum. Samples were collected from two local production sites of microenterprises (Zogona and Dassasgho). Microorganisms dynamic during the production of zoom-koom were enumerated using pour plate methods. The titratable acidity, pH and temperature of fermentation were determined using respectively titrimetric and electrochemical methods. The results showed a decrease in pH and an increase in acidity during soaking and fermentation step. While the enterobacteria and yeasts counts decreased ($p<0.05$), lactic acid bacteria (LAB) counts increased ($p<0.05$). On average, the pH decreased from 5.7 to 4.1; the lactic acid concentration ranged from 0.45 to 0.71 (lactic acid g/100 g) and the LAB ranged from 2.2×10^8 to 5.6×10^8 CFU/g for the millet dough. For the red sorghum dough the pH decreased from 6.2 to 4.2; the lactic acid concentration increased from 0.15 to 0.49 (lactic acid g/100 g) and LAB ranged from 8.9×10^6 to 5×10^{10} CFU/g. The ambient temperature and nature of the grains had an impact on the fermentation process. Unfermented red sorghum zoom-koom had the lowest load of yeast and enterobacteria than unfermented millet zoom-koom. Short rods in pair or short chains (3 or 4 rods) bacteria are the main microorganisms responsible of the fermentation process.

Key words: Sorghum, millet, fermentation, zoom-koom, lactic acid bacteria (LAB).

INTRODUCTION

Spontaneous or natural fermentation has been used in Africa for centuries to preserve and improve the nutritional status of foods. Fermented sorghum or millet-based foods, alcoholic and non-alcoholic drinks or beverages are prepared in many African countries for human consumption (Odunfa et al., 1996; Usha et al., 1996; Muyanja et al., 2002). The desired properties of these beverages are their nutritional value, taste, mouth file and

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fruity aroma. Generally, drinks are not taken as food but in Africa, cereals-based beverages are considered as foods because of their nutritional value and their contribution to the diet of people (Sawadogo-Lingani et al., 2008). These beverages are highly appreciated by consumers, and play an important role in the culture of African people. Often attached to the traditions of hospitality and conviviality, these beverages are part of the etiquette of most families and serve to seal the relationships between individuals (Aka, 2008). Among these beverages, we have tchapalo or dolo and zoom-koom in Burkina Faso, pito in Ghana, doro in Zimbabwe, bouza in Egypt, kunun-zaki in Nigeria and mougoudji in Mali (Olasupo et al., 1997; Djé et al., 2008; Sawadogo-Lingani, 2010).

The zoom-koom is a non-alcoholic beverage, prepared from millet and rarely from sorghum, and much appreciated by consumers in Burkina Faso. It is produced from shelled cereal or whole grains. The grain of sorghum is distinguished from the grain of millet by its richness in polyphenols (tannins). Cereals aleuronic cells are rich in mineral salts, B-complex vitamins and lipids; they contain some hydrolyzing enzymes (Kiemtoré, 2005; FAO, 2012), but the husking and the blasting eliminate a good part. The rate of dietary fiber is variable (2 to more than 30%) and it depends on the size of the grain (Kiemtore, 2005; FAO, 2012). Fibers play an important physiological role in allowing the normal progression of the alimentary bowl in the digestive tract and promoting certain metabolisms (cholesterol, triglycerides). The husking removes a good part of the bran, therefore fibers. The advantage of consuming a zoom-koom based on cereal not cracked is its richness in nutrients and dietary fibers. To get this drink, the grains of millet or sorghum are soaked overnight and soaked grains are washed and mixed with ginger and mint. The blend is grinded into a dough, diluted with water, and then filtered using a fabric to obtain zoom-koom, in which sugar and tamarind juice are added to give a sweet and sour taste (Soma, 2014).

However, according to Barro et al. (2007), zoom-koom is the street food which contains more count of thermotolerants and coliforms bacteria. To improve the microbiological quality of this beverage, a fermentation step of the dough is necessary. In fact, Soma (2014) had shown throughout a control fermentation of millet zoom-koom with *Lactobacillus fermentum* strain used as a starter, that the lactic fermentation allowed the reduction of enterobacteria counts and kept safe the final product. More ever, many works showed that the organic acids produced during the fermentation of pito in Ghana, tchapalo in Ivory Coast and ben-saalga in Burkina Faso, helped to obtain a better microbial stability of the product (Tou et al., 2006; Dje et al., 2008). Several investigations have shown the involvement of LAB in African traditional fermented cereal-based foods and beverages, including *Lactobacillus, Leuconostoc, Lactococcus, Pediococcus* and *Weissella* species (Olasupo et al., 1997; Hayford et al., 1999; Lei and Jakobsen, 2004). The microbiota of many African traditional fermented cereal products have been investigated, e.g. maize based products like kenkey (Halm et al., 1993; Olasupo et al., 1997; Hayford et al., 1999), mawè (Hounhouigan et al., 1993a,b, 1994) and ogi (Johansson et al., 1995; Olasupo et al., 1997), sorghum based product like kisra (Hamad et al., 1992) and dolo (Sawadogo-Lingani et al., 2007), millet based products like kunu-zaki (Olasupo et al., 1997) and ben-saalga (Tou et al., 2006). However, only a few studies have been done to date on zoom-koom (Barro et al., 2007; Soma, 2014) and its fermentation process and no information is available on the kinds of micro-organisms involved in the spontaneous fermentation of the dough.

This study is to evaluate the effect of spontaneous fermentation on microbial population during the production of a zoom-koom based on fermented and unfermented whole millet (*Pennisetum glaucum*) and red sorghum (*Sorghum bicolor* L. Moench) dough.

MATERIALS AND METHODS

Monitoring of the zoom-koom processing and sampling

The processes of zoom-koom based on fermented and unfermented dough of whole millet (*P. glaucum*) or red sorghum (*S. bicolor* L. Moench) were followed in two production sites at Ouagadougou (Burkina Faso): one site is located in the district of Zogona and the other one is located in the district of Dassasgho. The diagrams of production process were established and the different steps of sampling were identified. The main steps and components were illustrated in diagram of the processes of both fermented and unfermented fresh zoom-koom by local producers in Zogona and Dassasgho at household level (Figure 1).

From this diagram, the different steps of sampling were indicated such as: the soaking of millet or red sorghum grains (start and end), the fermentation of millet or red sorghum dough (start and end) and their corresponding zoom-koom samples (without sugar and with sugar), the unfermented dough of millet or red sorghum and their corresponding zoom-koom samples (without sugar and with sugar after acidifying with tamarind juice). As shown in Figure 1, the production process of zoom-koom includes the following main steps:

(1) Soaking: After washing, the millet or sorghum grains were weighed and soaked in a quantity of water equal to twice of the mass of millet or sorghum grains (2:1, w/w). The soaking time was set at 16 h (soak time observed in micro-workshops).
(2) Grinding: The flavored and aromatization ingredients were added to the soaked grains (millet or sorghum) at the rate of 3 g/100 g for the mint and 6 g/100 g for the ginger, before the wet grinding.
(3) Fermentation: The dough is left to stand for fermentation for 10 h (average time observed in the field). The fermentation was realized at ambient temperature (33 to 42°C).
(4) Filtration: A quantity of water equal to 3 times the mass of the wet dough was added to the dough. The suspended is poured onto a muslin (mesh ≤ 0.5 mm) to get a zoom-koom without sugar.
(5) Mixture: After filtration, a sugar solution was added to filtrate to give the fresh zoom-koom with sugar (Figure 2). For the case of the unfermented fresh zoom-koom, a tamarind juice was added to filtrate in order to give the zoom-koom, a sour taste. The tamarind juice is not added to fermented zoom-koom because this product is

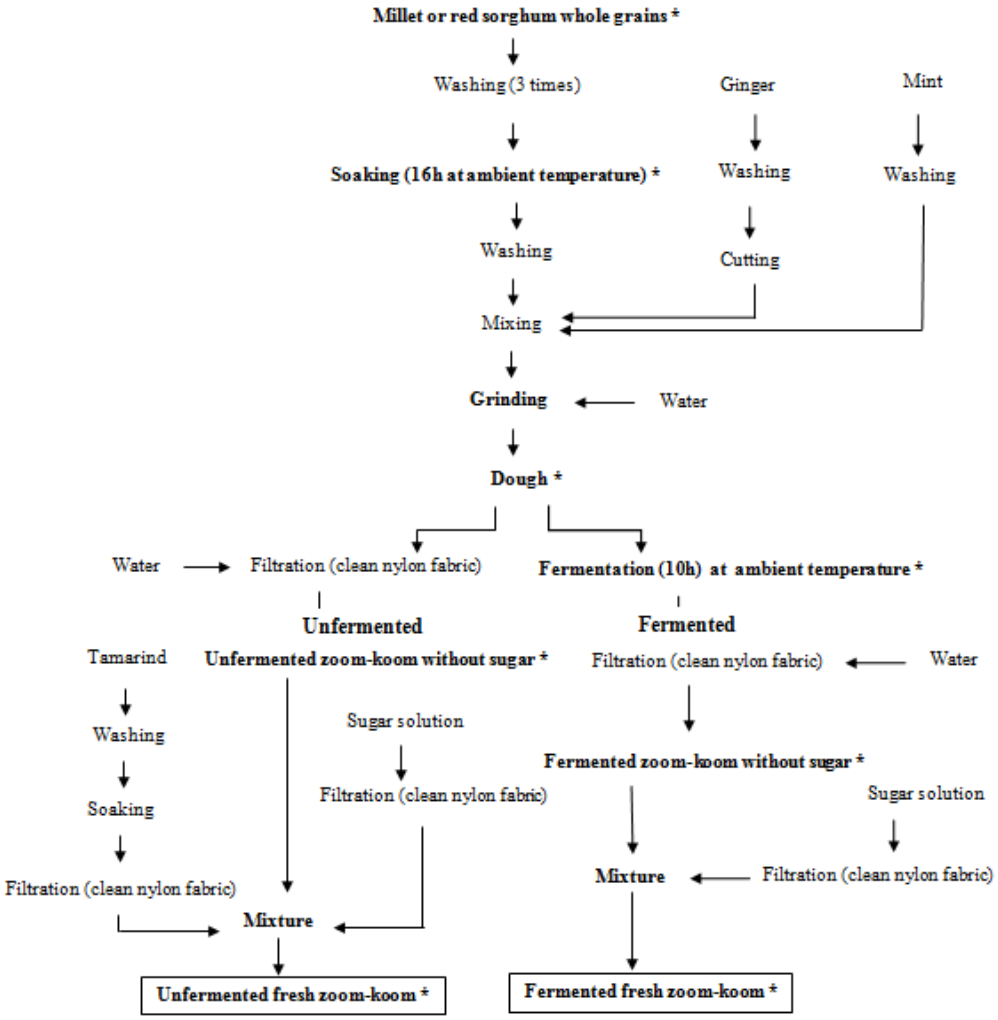

Figure 1. Flow diagram of fresh *zoom-koom* to the producers at Zogona and Dassasgho sites in Ouagadougou (Burkina Faso). *Steps of sampling.

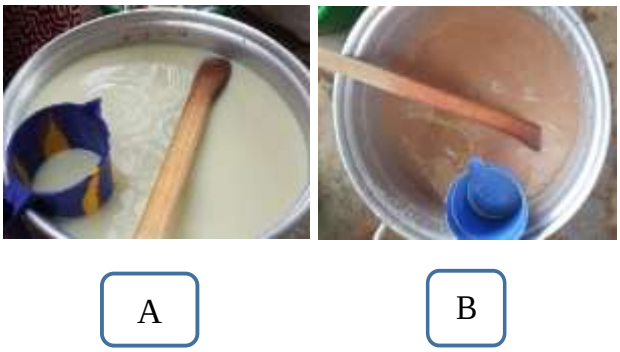

Figure 2. *Zoom-koom* of millet (A) and *Zoom-koom* of red sorghum (B).

already acid. To make the sugar solution, a quantity of sugar is weighed and dissolved in a quantity of water equal to twice the mass of sugar (2:1, w/w).

Prior to sampling, all materials used for sampling (glass bottle) were sterilized at 121°C for 15 min. For each sampling, 200 g or 200 ml of sample were taken in sterile screw-cap bottles at the different

steps of zoom-koom processes at both production sites. The samples were collected during three successive productions and transported to the laboratory in an icebox containing the ice. All the samples were preserved at 4°C before analysis which was carried out in triplicate, within the 24 h of the sampling. The temperature of the place from where the samples were collected was around 40°C for the production realized in Zogona site. (Months of April and May) and around 33°C for the production realized in Dassasgho site (Months of July and August).

Enumeration of microorganisms

For all samples, 10 g were homogenized in 90 ml sterile diluent (0.1% peptone, 0.8% NaCl, pH 7.0 ± 0.2) in a stomacher bag and homogenized in a stomacher (stomacher 400 lab blender, England) for 30 s at normal speed. For the solid raw material, the product was soaked for 30 min in the diluent at the laboratory temperature (25°C) before homogenization in the stomacher for 2 min at normal speed. From appropriate ten-fold dilutions, total mesophilic cells count were enumerated by pour plate on Plate Count Agar (Liofilchem, Spain) incubated at 30°C for 72 h (ISO 4833, 2003). Yeasts were enumerated by pour plate on Dextrose Chloramphenicol Agar (Liofilchem, Spain), pH 6.6 ± 0.2, and incubated at 30°C for 3 to 5 days according to ISO 7954 (1988). LAB were enumerated on modified Man, Rogosa and Sharpe (mMRS: MRS-IM agar + maltose) agar (Liofilchem, Spain), incubated anaerobically in an anaerobic jar with anaerocult A at 37°C, for 72 to 96 h according to ISO 15214 (1998). Enterobacteria were enumerated on Violet Red Bile Glucose (VRBG) agar (Liofilchem, Spain), incubated at 37°C for 24 h according to ISO 7402 (1993). Dishes containing 15 to 150 colonies were retained for the counting. The results were given as cfu/g of sample.

Isolation and preliminary characterization of LAB isolates

For LAB, the colonies from the highest dilution on mMRS were picked and further subcultured by streaking on mMRS agar anaerobically and growing in broth media (MRS broth) until pure cultures were obtained. A total of 366 isolates of presumed LAB were isolated. The isolates were first characterized based on colony and cell morphology using microscope (Olympus optical, BX 40F-3, Japan). Gram reaction was carried out by the KOH (3%) method (Gregersen, 1978), catalase production was determined by adding to a colony on a glass slide a drop of H_2O_2 solution (30%). Oxidase reaction was carried out by using oxidase disc. LAB isolates were subcultured and stored at -80°C in MRS broth with 30% (v/v) glycerol (87% v/v) for their identification.

Physico-chemical analyses

The pH of the samples was measured with an electronic pHmeter (Model HI 8520; Hanna Instrument, Singapore). For solid samples, 10 g of product were mixed with 20 ml of distilled water prior to pH measurement. For liquid samples, the pH was measured directly (Sawadogo-Lingani et al., 2007). The temperature at the beginning and at the end of fermentation was measured with a thermometer (Rweger V104, Swiss). For titratable acidity determination, 5 g or 5 ml of sample suspended in ethanol (90%) was centrifuged for 5 min at 3500 g. Of the supernatant, 10 ml was transferred to a 50-ml measuring flask and filled up to 50 ml with distilled water. After mixing, 10 ml of the diluted sample was titrated with NaOH 0.1 N using 1% phenolphthalein as indicator (Sawadogo-Lingani et al., 2007). The titratable acidity (as g lactic acid per 100 ml or g of sample) was calculated according to Amoa-Awua et al. (1996).

Statistical analyses of the data

All the data were submitted to analysis of variance (ANOVA) and a principal component analysis (PCA) with the statistical software XLSTAT-Pro 7.5.2 and the means were compared using the test of Student Newman-keuls to the probability level $p<0.05$. The curves were obtained using Microsoft Excel 2013.

RESULTS

Microbial populations during the processing of zoom-koom from fermented and unfermented millet or red sorghum dough

Zogona production sites

Table 1 presents the evolution of microbial populations associated to the processing of zoom-koom produced from fermented and unfermented millet or red sorghum dough from the raw grains to the final products (zoom-koom) ready to drink. The counts of enterobacteria, yeasts, LAB and total mesophilic cells were respectively 2.7×10^3, 1.5×10^4, 1.4×10^3, and 2.3×10^5 CFU/g in the millet grains and 2.9×10^4, 6.3×10^5, 5.9×10^6 and 1.7×10^7 CFU/g in the red sorghum grains. During the soaking steps, a significant increase of enterobacteria, yeasts and LAB counts was observed ($p<0.05$). However, during the fermentation of the millet dough, a significant decreasing of the enterobacteria and yeasts counts was observed ($p<0.05$), respectively from 8.7×10^7 to 3.2×10^2 CFU/g and 4.4×10^6 to 8.4×10^3 CFU/g. For the fermentation of the red sorghum dough, the enterobacteria counts were decreased significantly ($p<0.05$) from 4.7×10^6 to 4.8×10^4 CFU/g, while the yeasts counts followed a non-significant increase ($p<0.05$) from 5.5×10^4 to 7.6×10^4 CFU/g. The LAB counts were increased (from 2.2×10^8 to 5.6×10^8 CFU/g for millet dough and from 8.9×10^6 to 5.0×10^{10} CFU/g for the red sorghum dough) and remain the main flora during the fermentation step of the millet and red sorghum dough ($r=0.9$). No significant difference ($p<0.05$) was observed between unfermented zoom-koom without sugar and unfermented zoom-koom with sugar and tamarind juice, except for the enterobacteria counts from unfermented red sorghum zoom-koom.

Dassasgho production site

Table 2 presents the evolution of microbial populations associated to the processing of zoom-koom produced from fermented and unfermented millet or red sorghum dough, from the raw grains to the final products (zoom-koom) ready to drink. The counts of enterobacteria, yeasts, LAB and total mesophilic cells were, respectively 8.4×10^4, 1.5×10^4, 8.2×10^4 and 5.1×10^6 CFU/g in the millet grains, and 2.9×10^4, 1.8×10^3, 1.6×10^4 and 5.0×10^5 CFU/g in the red sorghum grains, respectively. During the soaking steps, a significant increase of enterobacteria,

Table 1. Microbial populations during the production of millet or sorghum *zoom-koom* at Zogona sites.

Sample	Products		Microorganisms count (cfu/g)							
			Total mesophilic cells		Enterobacteria		Yeasts		Lactic acid bacteria	
	Steps of sampling		Millet	Sorghum	Millet	Sorghum	Millet	Sorghum	Millet	Sorghum
Fermented samples	Soaked Grains	Raw grains	$(2.3 \pm 0.1)10^{5c}$	$(1.7 \pm 1.5)10^{7bc}$	$(2.7 \pm 1.0)10^{3b}$	$(2.9 \pm 0.6)10^{4b}$	$(1.5 \pm 1.1)10^{4b}$	$(6.3 \pm 0.1)10^{5a}$	$(1.4 \pm 0.1)10^{3b}$	$(5.9 \pm 5.9)10^{6bc}$
		Start of soaking	$(8.2 \pm 1.8)10^{5c}$	$(4.7 \pm 3.5)10^{5c}$	$(1.9 \pm 1.7)10^{4b}$	$(1.6 \pm 0.9)10^{4b}$	$(3.3 \pm 0.3)10^{3b}$	$(5.4 \pm 0.4)10^{2b}$	$(2.0 \pm 2.0)10^{5b}$	$(2.3 \pm 1.2)10^{3c}$
		End of soaking	$(1.5 \pm 1.5)10^{10\,a}$	$(4.3 \pm 4.3)10^{9ab}$	$(2.0 \pm 1.4)10^{8a}$	$(3.4 \pm 3.4)10^{7a}$	$(6.7 \pm 5.3)10^{6a}$	$(2.0 \pm 1.810^{5ab}$	$(3.7 \pm 0.5)10^{8a}$	$(7.5 \pm 7.4)10^{7ab}$
	Dough	Start of fermentation	$(4.4 \pm 2.1)10^{8ab}$	$(1.8 \pm 1.4)10^{7bc}$	$(8.7 \pm 6.1)10^{7a}$	$(4.7 \pm 2.6)10^{6a}$	$(4.4 \pm 3.3)10^{6a}$	$(5.5 \pm 1.4)10^{4ab}$	$(2.2 \pm 1.2)10^{8a}$	$(8.9 \pm 7.4)10^{6ab}$
		End of fermentation	$(9.7 \pm 0.03)10^{8ab}$	$(6.0 \pm 0.5)10^{10a}$	$(3.2 \pm 3.1)10^{2b}$	$(4.8 \pm 2.5)10^{4b}$	$(8.4 \pm 1.3)10^{3b}$	$(7.6 \pm 0.7)10^{4ab}$	$(5.6 \pm 0.9)10^{8a}$	$(5.0 \pm 0.9)10^{10a}$
	Zoom-koom	Without sugar	$(1.2 \pm 0.1)10^{8b}$	$(5.3 \pm 0.9)10^{9ab}$	$(8.6 \pm 6.8)10^{2b}$	$(4.1 \pm 2.4)10^{3b}$	$(1.7 \pm 0.4)10^{3b}$	$(4.6 \pm 0.9)10^{3b}$	$(8.4 \pm 0.1)10^{7a}$	$(2.2 \pm 0.4)10^{8ab}$
		With sugar	$(2.0 \pm 0.3)10^{8ab}$	$(1.6 \pm 0.03)10^{8ab}$	$(8.8 \pm 7.4)10^{2b}$	$(4.4 \pm 2.9)10^{3b}$	$(2.2 \pm 0.4)10^{3b}$	$(4.4 \pm 0.7)10^{3b}$	$(2.6 \pm 0.4)10^{8a}$	$(1.5 \pm 0.02)10^{8ab}$
Unfermented samples	Dough	Dough	$(4.4 \pm 2.1)10^{8a}$	$(1.8 \pm 1.4)10^{7a}$	$(8.7 \pm 6.1)10^{7a}$	$(4.7 \pm 2.6)10^{6a}$	$(4.4 \pm 3.3)10^{6a}$	$(5.5 \pm 1.4)10^{4a}$	$(2.2 \pm 1.2)10^{8a}$	$(8.9 \pm 7.4)10^{6a}$
	Zoom-koom	Without sugar	$(1.7 \pm 0.1)10^{8a}$	$(1.1 \pm 0.7)10^{7a}$	$(2.8 \pm 0.9)10^{7a}$	$(2.3 \pm 0.04)10^{6a}$	$(1.2 \pm 0.5)10^{6a}$	$(4.6 \pm 0.8)10^{4a}$	$(1.1 \pm 0.1)10^{8a}$	$(5.1 \pm 2.8)10^{6a}$
		With sugar and Tamarind	$(4.4 \pm 4.3)10^{7a}$	$(2.4 \pm 0.9)10^{6a}$	$(6.5 \pm 6.2)10^{6a}$	$(2.2 \pm 0.2)10^{4b}$	$(4.0 \pm 3.6)10^{5a}$	$(1.5 \pm 1.1)10^{5a}$	$(2.0 \pm 2.0)10^{7a}$	$(7.6 \pm 4.2)10^{5a}$

Each column values having a common letter are not significantly different according to the Student Newman Keuls test at the 5% threshold.

Table 2. Microbial population during the production of millet or sorghum *zoom-koom* at Dassasgho production site.

Sample	Products and Steps of sampling		Microorganisms count (cfu/g)							
			Total mesophilic cells		Enterobacteria		Yeasts		Lactic acid bacteria	
			Millet	Sorghum	Millet	Sorghum	Millet	Sorghum	Millet	Sorghum
Fermented samples	Soaked Grains	Raw grains	$(5.1 \pm 3.9)10^{6b}$	$(5.0 \pm 0.5)10^{5d}$	$(8.4 \pm 7.5)10^{4a}$	$(1.2 \pm 0.1)10^{3\,e}$	$(1.5 \pm 1.1)10^{4a}$	$(1.8 \pm 0.2)10^{3b}$	$(8.2 \pm 5.8)10^{4b}$	$(1.6 \pm 0.1)10^{4d}$
		Start of soaking	$(4.0 \pm 2.1)10^{6b}$	$(1.3 \pm 0.1)10^{5e}$	$(1.7 \pm 1.6)10^{5a}$	$(4.7 \pm 0.2)10^{2f}$	$(3.3 \pm 0.3)10^{3a}$	$(1.3 \pm 0.7)10^{3b}$	$(1.0 \pm 0.9)10^{5b}$	$(1.5 \pm 0.5)10^{3e}$
		End of soaking	$(1.9 \pm 0.5)10^{8a}$	$(2.2 \pm 0.5)10^{7c}$	$(5.3 \pm 4.6)10^{7a}$	$(1.0 \pm 0.1)10^{7a}$	$(1.6 \pm 1.2)10^{5a}$	$(1.1 \pm 1.0)10^{6a}$	$(1.6 \pm 0.6)10^{8a}$	$(5.0 \pm 0.5)10^{6c}$
	Dough	Start of fermentation	$(7.8 \pm 3.2)10^{7a}$	$(1.3 \pm 0.6)10^{8b}$	$(9.1 \pm 2.8)10^{6a}$	$(2.7 \pm 0.1)10^{6c}$	$(1.8 \pm 0.5)10^{5a}$	$(4.4 \pm 2.3)10^{5a}$	$(7.1 \pm 4.9)10^{7a}$	$(3.4 \pm 0.2)10^{7b}$
		End of fermentation	$(8.8 \pm 2.1)10^{8a}$	$(3.2 \pm 0.7)10^{8a}$	$(1.8 \pm 0.7)10^{6a}$	$(8.5 \pm 0.4)10^{6b}$	$(1.8 \pm 1.0)10^{5a}$	$(2.1 \pm 0.9)10^{5a}$	$(7.1 \pm 1.7)10^{8a}$	$(2.1 \pm 0.3)10^{8a}$
	Zoom-koom	Without sugar	$(6.0 \pm 3.6)10^{8a}$	$(5.9 \pm 0.7)10^{7bc}$	$(1.4 \pm 1.3)10^{6a}$	$(1.2 \pm 0.1)10^{6d}$	$(1.7 \pm 1.5)10^{5a}$	$(2.7 \pm 0.6)10^{5a}$	$(5.0 \pm 3.0)10^{8a}$	$(3.2 \pm 1.5)10^{7b}$
		With sugar	$(1.8 \pm 0.1)10^{8a}$	$(3.5 \pm 1.0)10^{7c}$	$(9.2 \pm 8.7)10^{5a}$	$(1.3 \pm 0.1)10^{6d}$	$(2.5 \pm 2.2)10^{5a}$	$(1.5 \pm 0.3)10^{5a}$	$(8.0 \pm 6.0)10^{7a}$	$(2.6 \pm 0.5)10^{7b}$
Unfermented samples	Dough	Dough	$(7.8 \pm 3.2)10^{7a}$	$(1.3 \pm 0.6)10^{8a}$	$(9.1 \pm 2.8)10^{6a}$	$(2.7 \pm 0.1)10^{6a}$	$(1.8 \pm 0.5)10^{5a}$	$(4.4 \pm 2.3)10^{5a}$	$(7.1 \pm 4.9)10^{7a}$	$(3.4 \pm 0.2)10^{7a}$
	Zoom-koom	Without sugar	$(3.4 \pm 2.4)10^{7a}$	$(9.4 \pm 2.6)10^{6b}$	$(2.4 \pm 0.1)10^{6a}$	$(1.9 \pm 0.5)10^{6a}$	$(5.1 \pm 1.1)10^{4a}$	$(2.1 \pm 0.9)10^{5a}$	$(2.4 \pm 1.9)10^{7a}$	$(5.1 \pm 0.9)10^{6b}$
		With sugar and Tamarind	$(4.9 \pm 2.7)10^{6b}$	$(2.3 \pm 0.6)10^{6c}$	$(2.2 \pm 1.2)10^{5b}$	$(2.6 \pm 2.2)10^{5a}$	$(5.3 \pm 1.9)10^{3b}$	$(1.1 \pm 0.3)10^{4b}$	$(2.6 \pm 2.1)10^{6a}$	$(7.8 \pm 0.4)10^{5c}$

Each column values having a common letter are not significantly different according to the Student Newman Keuls test at the 5% threshold.

Table 3. Physico-chemical parameters of samples during the processing of millet or sorghum zoom-koom at Zogona production site.

Sample	Products and steps of sampling		pH		Titratable acidity (Lactic acid g/100 g)		Temperature (°C)	
			Millet	Sorghum	Millet	Sorghum	Millet	Sorghum
Fermented samples	Soaked Grains	Raw Grains	6.2 ± 0.0^{a}	6.4 ± 0.3^{a}	0.20 ± 0.15^{a}	0.06 ± 0.01^{d}	nd	nd
		Start of soaking	6.2 ± 0.1^{a}	6.2 ± 0.2^{a}	0.10 ± 0.06^{a}	0.08 ± 0.01^{d}	nd	nd
		End of soaking	4.7 ± 0.4^{b}	5.1 ± 0.1^{b}	0.22 ± 0.01^{a}	0.13 ± 0.02^{cd}	nd	nd
	Dough	Start of fermentation	5.7 ± 0.0^{a}	6.2 ± 0.1^{a}	0.45 ± 0.15^{a}	0.15 ± 0.01^{cd}	26.5 ± 1.5^{a}	26.0 ± 0.0^{a}
		End of fermentation	4.1 ± 0.0^{c}	4.2 ± 0.0^{c}	0.71 ± 0.28^{a}	0.49 ± 0.05^{a}	29.5 ± 1.5^{b}	26.5 ± 0.5^{a}
	Zoom-koom	Without sugar	3.9 ± 0.1^{c}	4.2 ± 0.0^{c}	0.25 ± 0.15^{a}	0.25 ± 0.03^{bc}	nd	nd
		With sugar	3.8 ± 0.0^{c}	4.2 ± 0.0^{c}	0.39 ± 0.25^{a}	0.33 ± 0.07^{b}	nd	nd
Unfermented samples	Dough	Dough	5.7 ± 0.0^{a}	6.2 ± 0.1^{a}	0.45 ± 0.15^{a}	0.15 ± 0.01^{a}	nd	nd
	Zoom-koom	Without sugar	5.5 ± 0.3^{a}	6.3 ± 0.0^{a}	0.19 ± 0.12^{a}	0.12 ± 0.04^{a}	nd	nd
		With sugar and Tamarind	3.9 ± 0.1^{b}	4.5 ± 1.0^{a}	0.24 ± 0.11^{a}	0.26 ± 0.09^{a}	nd	nd

Each column values having a common letter are not significantly different according to the Student Newman Keuls test at the 5% threshold. nd: Not determined.

yeasts and LAB counts was observed ($p<0.05$). The results from Table 2 also show a non-significant decrease ($p<0.05$) of the enterobacteria counts during the fermentation steps of the millet dough (from 9.1×10^{6} to 1.8×10^{6} CFU/g). A significant increase ($p<0.05$) of enterobacteria counts during the fermentation of red sorghum dough (from 2.7×10^{6} to 8.5×10^{6} CFU/g). The yeast counts followed a non-significant decrease ($p<0.05$) during the fermentation of red sorghum dough (from 4.4×10^{5} to 2.1×10^{5} CFU/g) and a stability for the fermented dough of millet (1.8×10^{5} to 1.8×10^{5} CFU/g). The LAB which remain the main flora ($r=0.8$) faced a significant raise during the fermentation (from 7.1×10^{7} to 7.1×10^{8} CFU/g and from 3.4×10^{7} to 2.1×10^{8} CFU/g, respectively for millet and red sorghum dough). A comparison between fermented and unfermented millet and red sorghum zoom-koom, showed that the unfermented zoom-koom of millet and red sorghum contain almost the same counts of enterobacteria and yeasts. But, the fermented millet dough contains less enterobacteria than the fermented dough of red sorghum. A significant difference between the unfermented zoom-koom without sugar and the unfermented zoom-koom with sugar and tamarind juice was observed ($p<0.05$), except for the enterobacteria counts from unfermented red sorghum zoom-koom and LAB counts from unfermented millet zoom-koom.

Evaluation of physico-chemical parameters during the processing of zoom-koom from fermented and unfermented millet or red sorghum dough

Zogona production site

Table 3 presents the evaluation of pH, titratable acidity and the temperature associated to the processing of zoom-koom produced from fermented and unfermented millet or red sorghum dough. The pH and titratable acidity of the grains were respectively 6.2 and 0.20 g/100 g (as lactic acid) for the millet grain and also respectively 6.4 and 0.06 g/100 g (lactic acid) for the red sorghum grain. During the soaking step of the grains, the pH of the soaking water decreased significantly ($p<0.05$) from 6.2 to 4.7 and from 6.2 to 5.1, respectively for millet and sorghum grain. Therefore, during the fermentation of the dough, a significant decrease ($p<0.05$) in pH from 5.7 to 4.1 and from 6.2 to 4.2, respectively for fermented millet dough and fermented red sorghum dough. The titratable acidity calculated as lactic acid content raised significantly ($p<0.05$) from 0.45 to 0.71 g/100 g and from 0.15 to 0.49 g/100 g, respectively for fermented millet dough and fermented red sorghum dough. The pH of the unfermented zoom-koom had shown a non-significant decrease except for the millet zoom-koom. The titratable acidity has also shown a non-

Table 4. Physico-chemical parameters of samples during the processing of millet or sorghum zoom-koom at Dassasgho production site.

Sample	Products and steps of sampling		pH		Titratable acidity (Lactic acid g/100g)		Temperature (°C)	
			Millet	Sorghum	Millet	Sorghum	Millet	Sorghum
Fermented samples	Grain	Grain	6.2 ± 0.0^{a}	6.2 ± 0.0^{a}	0.10 ± 0.03^{b}	0.07 ± 0.01^{c}	nd	nd
		Start of soaking	6.3 ± 0.0^{a}	6.1 ± 0.0^{a}	0.10 ± 0.03^{b}	0.04 ± 0.01^{c}	nd	nd
		End of soaking	5.4 ± 0.4^{a}	4.5 ± 0.3^{b}	0.15 ± 0.05^{b}	0.10 ± 0.01^{c}	nd	nd
	Dough	Start of fermentation	5.7 ± 0.5^{a}	5.8 ± 0.1^{a}	0.37 ± 0.06^{b}	0.13 ± 0.01^{bc}	21.0 ± 1.0^{a}	21.5 ± 0.5^{a}
		End of fermentation	4.2 ± 0.0^{b}	4.6 ± 0.1^{b}	0.90 ± 0.14^{a}	0.45 ± 0.05^{a}	21.5 ± 0.5^{a}	22.0 ± 0.0^{a}
	Zoom-koom	Without sugar	4.2 ± 0.1^{b}	4.9 ± 0.1^{b}	0.36 ± 0.04^{b}	0.25 ± 0.05^{b}	nd	nd
		With sugar	4.1 ± 0.1^{b}	4.9 ± 0.0^{b}	0.34 ± 0.08^{b}	0.15 ± 0.05^{bc}	nd	nd
Unfermented samples	Dough	Dough	5.7 ± 0.5^{a}	5.8 ± 0.1^{a}	0.37 ± 0.06^{a}	0.13 ± 0.01^{a}	nd	nd
	Zoom-koom	Without sugar	5.2 ± 0.8^{a}	6.0 ± 0.1^{a}	0.17 ± 0.04^{a}	0.07 ± 0.01^{a}	nd	nd
		With sugar and Tamarind	3.5 ± 0.1 ^{a}	3.8 ± 0.0 ^{b}	0.40 ± 0.04 ^{a}	0.15 ± 0.05 ^{a}	nd	nd

Each column values having a common letter are not significantly different according to the Student Newman Keuls test at the 5% threshold. nd: Not determined.

significant slight increase after tamarind juice adding. The pH of zoom-koom based on fermented millet or red sorghum dough was almost similar to those of unfermented zoom-koom mixed with tamarind juice. During the fermentation, the temperature of the dough showed a significant variation ($p<0.05$) for fermented millet dough (from 26.5 to 29.5°C) and a slight non-significant variation ($p<0.05$) for the fermented red sorghum dough (from 26 to 26.5°C). It is noticed that fermentation using red sorghum dough is slower than fermentation using millet dough. This led to less important reduction of pH than for millet dough.

Dassasgho production site

Table 4 presents the values of pH, titratable acidity and the temperature associated to the processing of zoom-koom produced from fermented and unfermented millet or red sorghum dough. The pH and the titratable acidity of the grains were respectively 6.2 and 0.10 lactic acid g/100 g for the millet grain and also respectively 6.2 and 0.07 lactic acid g/100 g for the red sorghum grain. During the soaking steps of the grains, the pH was decreased non-significantly ($p<0.05$) from 6.3 to 5.4 and significantly from 6.1 to 4.5, respectively for millet and sorghum grain. During the fermentation, the pH of the fermented millet dough showed a significant decrease ($p<0.05$) from 5.7 to 4.2, but the pH of red sorghum dough showed a significant decrease (from 5.8 to 4.6), but less important than that of millet dough. The titratable acidity calculated as lactic acid content increased significantly ($p<0.05$) from 0.37 to 0.90 g/100 g and from 0.13 to 0.45 g/100 g, respectively for fermented millet and red sorghum dough. The pH of the unfermented zoom-koom had shown a non-significant decrease excepted for the red sorghum zoom-koom. The lactic acidity had also shown a non-significant increase after tamarind juice adding. The pH of zoom-koom based on fermented millet or red sorghum dough was slightly similar to those of unfermented zoom-koom mixed with tamarind juice. The fermentation temperature showed a non-significant variation ($p<0.05$) for fermented millet dough (from 21.0 to 21.5°C) and for red sorghum dough (from 21.5 to 22°C). It is noticed that fermentation using red sorghum dough is slower than fermentation using millet dough. This lead to less important reduction of pH than for millet dough.

Main presumptive LAB isolates collected from the zoom-koom production at the two productions sites

A total of 350 presumptive isolates of LAB (Gram +, catalase -, oxidase -, rods or cocci) have been collected from the samples taking at zoom-koom production process (Tables 5 and 6). The production made in the district of Zogona allowed

Table 5. Distribution of LAB isolates from the raw grains to the final product throughout the processing of millet and red sorghum zoom-koom at Zogona production site.

Sample	Zogona								Total of isolates collected by Samples
	Millet				Sorghum				
	Cocci in pair and tetrad	Cocci in pair and short chains	Long rods in pair and short chains	Short rods in pair and short chains	Cooci in pair and tetrad	Cocci in pair and short chains	Long rods in pair and short chains	Short rods in pair and short chains	
Raw Grains	-	-	-	2	6	-	-	-	8
Soaked Grains, Start of soaking	-	6	-	-	2	-	-	-	8
Soaked Grains, End of soaking	1	3	1	4	1	-	-	-	10
Soaking Water, start of soaking	-	7	-	-	-	-	-	-	7
Soaking Water, end of soaking	-	2	1	6	-	-	-	-	9
Dough, start of fermentation	-	-	1	11	-	-	-	9	21
Dough, end of fermentation	-	-	1	12	-	-	1	10	24
Fermented zoom-koom without sugar	1	-	-	11	-	1	2	12	27
Fermented zoom-koom with sugar	-	-	1	21	-	-	-	18	40
Unfermented zoom-koom without sugar	-	2	-	11	2	1	-	11	27
Unfermented zoom-koom with sugar and tamarind	1	3	2	6	-	2	-	16	30
Total	3	23	7	80	11	4	3	76	207

-: Not found.

to collect 207 presumptive LAB isolates. From the district of Dassasgho, a total of 143 presumptive LAB isolates were collected. The isolates were cocci in pair and tetrad, cocci in pair and short chains, long rods in pair and short chains, short rods in pair and short chains. The results shown that presumptive LAB associated to the production of the millet and red sorghum zoom-koom were dominated by short rods: 70.79 and 80.85%, respectively for millet zoom-koom and red sorghum zoom-koom in Zogona production site (Table 5); 91.56 and 86.66%, respectively for millet zoom-koom and red sorghum zoom-koom, in the district of Dassasgho (Table 5). Further works will be focused on the characterization and the identification of the isolates and will give more informations on the microbial species involved.

DISCUSSION

The microorganisms (yeasts, moulds, entero-bacteria, and LAB) present in the raw grains of millet and red sorghum represent the endogenous flora of these grains. During the soaking steps, the decrease in pH and the increase in titratable acidity can be attributed to the activities of LAB, which population increased during this step. It means that a natural lactic fermentation occurred during the soaking step of the millet and sorghum grains. This has been reported by previous investigations (Sawadogo-Lingani et al., 2010). The increase of enterobacteria and yeasts counts during the soaking could be due to the availability of nutrients and the increase in moisture content. Moreover, some studies conducted on the sorghum have shown that the soaking is a very critical step in the malting of sorghum (Tawaba et al., 2013). In fact, the growth of the micro-organisms present in the grains during the soaking step, may be due to the favorable moisture content (35-40% or even more) (Ogbonna et al., 2004) and therefore the activity of the water, thus creating conditions favorable to the activation of the spores and the development of the bacteria, yeasts and molds (Tawaba et al., 2013).

The decrease in enterobacteria and yeasts counts during the fermentation of millet and red

Table 6. Distribution of LAB isolates from the raw grains to the final product throughout the processing of millet and red sorghum zoom-koom at Dasssasgho production site.

Sample	Dassasgho								Total of isolates collected by samples
	Millet				Sorghum				
	Cocci in pair and tetrad	Cocci in pair and short chains	Long rods in pair and short chains	Short rods in pair and short chains	Cooci in pair and tetrad	Cocci in pair and short chains	Long rods in pair and short chains	Short rods in pair and short chains	
Raw grains	-	-	-	-	-	-	-	-	-
Soaked grains, start of soaking	2	-	-	-	1	-	-	2	5
Soaked grains, end of soaking	-	-	2	3	-	1	-	1	7
Soaking Water, start of soaking	-	-	-	5	-	-	-	3	8
Soaking Water, end of soaking	1	-	-	12	-	-	-	-	13
Dough, start of fermentation	-	-	-	9	1	-	1	6	17
Dough, end of fermentation	1	1	-	12	-	-	-	10	24
Fermented zoom-koom without sugar	-	-	-	4	-	-	-	8	12
Fermented zoom-koom with sugar	-	-	-	8	-	-	1	9	18
Unfermented zoom-koom without sugar	-	-	-	11	-	-	1	8	20
Unfermented zoom-koom with sugar and tamarind	-	-	-	12	-	-	2	10	24
Total	4	1	2	76	2	1	5	52	143

sorghum dough is probably due to the effect of the acidification of the dough (decrease in pH and the increase in titratable acidity) resulting to the concomitant growth of LAB during this step. These results corroborate with those of Vieira-Dalodé et al. (2008) in the production of *gowè*, a sour beverage from Benin, who found a significant increase of LAB count after 4 h of fermentation and during the fermentation step as well as a significantly lower count of yeasts. Our results are also in line with those of Soma (2014) in the production of zoom-koom, Sawadogo-Lingani et al. (2007) in the production of dolo and pito, Lei and Jakobsen (2004) in the production of koko and Muyanja et al. (2002) in the production of bushera, who found a LAB counts of 10^8 and 10^9 cfu/g during the fermentation process. Sawadogo-Lingani et al. (2010) in the traditional malting of sorghum had found a LAB counts which increased from 10^5 to 10^{10} cfu/g during the steeping of the sorghum grains.

In fact, LAB are well known to be producers of natural antimicrobial substances like organic acids (lactic, acetic, formic, phenyllactic caproic), carbon dioxide, hydrogen peroxide, ethanol and bacteriocins (Messens and De Vuyst, 2002). The production of organic acid during the fermentation process induce an important decrease in pH, which in association with the formation of antibacterial substances determine the microbial stability as well as the level of the growth of pathogenic bacteria and other undesirable micro-organisms. Previous works showed that the organic acids produced during the fermentation of pito in Ghana, tchapalo in Ivory Coast and ben-saalga in Burkina Faso, helped to obtain a better microbial stability of the products (Tou et al., 2006; Dje et al., 2008), which can be also applied for zoom-koom. LAB are able to synthesize some active bacteriocins not only against other lactic bacteria, but also against other gram positive bacteria and moulds as well as against gram negative bacteria, among which enterobacteria and pathogenic germs (Raimbiault, 1995) are important. It can be noted that the dilution (addition of water) reduced the microbial counts. The growth of yeasts during the fermentation of red sorghum dough may be partially attributed to the presence of lactic acid. Our results are similar to those obtained by Mohammed et al. (1991), who found a high number of yeasts during the

fermentation of sorghum. These results can explain the increase in yeasts counts during the fermentation of sorghum dough at Zogona's site and its decrease during the fermentation at Dassasgho's site. The difference in the number of LAB between millet and sorghum dough during the fermentation in Zogona production site could be explained by the fact that at the start of sorghum fermentation the number of LAB was low, resulting in less competition. When this number rises it could have more competition and fewer sugars available.

The stationary state of enterobacteria counts and the increase in yeasts counts during the fermentation process at Dassagho site may be due in part to the low acidification of the dough and in other hand to the low temperature of fermentation (21 to 22°C) because of the climatic factors in that periods (July and August with a room temperature around 33°C (period of the raining season in Burkina Faso). If the initial acidification is not fast enough, some pathogenic or undesirable germs can partially develop, maintain themselves and deviate or evolve later the product, during the conservation period. For this reason, it is recommended to assure that the initial phase of acidification is sufficiently fast for the good microbiological quality of product (Raimbiault, 1995). Some studies showed that, for an efficacy stable action, the acidification may be fast, in less than 24 h, and the pH may drop below 4.0 (Raimbiault, 1995). According to Muyanja et al. (2002), a pH of 3.5 to 4.0 has been reported to inhibit Enterobacteriaceae and other Gram-negative bacteria. The results found in the production of Dassasgho corroborate with those of Brisabois et al. (1997) on milk pathogens which found that a small variation in pH can reduce (pH 4.55) or promote (pH 4.95) the development of pathogens (Brisabois et al., 1997). Indeed, studies on milk pathogens carried out in France and in Europe have concluded that most pathogenic bacteria develop at pH intervals of between 4.5 and 9.6 (Brisabois et al., 1997). These results corroborate with those found during the fermentation realized in Dassasgho production site, where the pH for the fermentation of red sorghum dough was decreased from 5.8 to 4.6 resulting in a small reduction in enterobacteria counts.

The slowness of the fermentation of red sorghum dough compared to the millet dough could be due to the structural composition of the two grains. The red sorghum is rich in tannins. This compound impact the enzymatic digestibility, thereby, the availability of sugars which are the basic substrate for lactic fermentation. Studies also showed that those phenolic compounds in one hand are capable of protecting the grains against fungus attack, insects and birds, which is an obvious agronomic advantage, reducing the enzymatic digestion as well as the proteins, starch and others polysaccharides (Tawaba et al., 2013). This slowness of the fermentation causes a less important drop of the pH of zoom-koom and also a less important production of lactic acid compared to the fermented zoom-koom realized with millet dough. The enterobacteria counts are lower in the sorghum zoom-koom for some case like Zogona's productions. This result could be explained by the low acidity. The most of the isolates involved in the fermentation process of sorghum and millet grains for the production of zoom-koom, are Gram positive, catalase negative and oxydase negative. Rods in pair and short chains could belong to the genus of *Lactobacillus*. These partial results corroborate with previous studies (Sawadogo-Lingani et al., 2007; Lei and Jakobsen et al., 2004) where Lactobacillus species had been identified as dominant LAB involved in the fermentation of sorghum (dolo, pito) or millet based products (koko).

Conclusion

The present study highlighted the importance of fermentation in food processing by improving the microbiological quality of final products. During the processes of millet and red sorghum zoom-koom, the fermentation of the dough before the filtration steps was able to reduce the number of enterobactieria and yeasts. Fermentations taking place under low temperatures need more fermentation time for a better acidification of the dough. Fermented zoom-koom is better than unfermented zoom-koom, in terms of microbiological quality for the case of millet. In the case of sorghum zoom-koom, the unfermented zoom-koom is better than fermented zoom-koom. Fermented millet zoom-koom is better than fermented sorghum zoom-koom, but the unfermented sorghum zoom-koom is better than unfermented millet zoom-koom in terms of microbiological quality. LAB isolates of the genus of *Lactobacillus* seem to be the dominant bacteria involved in the fermentation of zoom-koom. The identification of the isolates and the determination of their technological properties are essential for the optimization of fermentation processes and the improvement of the quality of the final products by the use of selected LAB starter cultures.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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