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Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Review

Insights of maize lethal necrotic disease: A major constraint to maize production in East Africa

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Maize Lethal Necrotic Disease (MLND) is a new disease in East Africa, first reported in Kenya in 2011 and then spread to Tanzania, Uganda and Rwanda. The disease is caused by *Maize Chlorotic Mottle Virus* (MCMV) in combination with viruses of genus *Potyvirus,* mostly *Sugarcane Mosaic Virus* (SCMV). The co-infection is the one that results in intensive to complete yield loss. Diagnosis of MLND based on symptoms is reported ineffective because symptoms like stunting and chlorosis resembles nutrient deficiencies or maize mosaic. Detection and characterization of MLND causing viruses have been done by techniques such as enzyme-linked immuno-sorbent assay (ELISA), polymerase chain reaction (PCR) and next generation sequencing. Relatively little work has been done to characterize MLND causing viruses in Tanzania prior to those techniques. The disease can be managed through the use of certified seeds, sanitation, quarantine, crop rotation, the use of resistant/tolerant maize varieties and other cultural practices. The use of resistant maize varieties is considered the most reliable, eco-friendly, effective and economical way of managing MLND.

Key words: Enzyme-linked immuno-sorbent assay (ELISA), etiology, *Maize Chlorotic Mottle Virus*, Maize lethal necrotic disease, nucleic acid based methods, resistant maize varieties, *Sugarcane Mosaic Virus.*

INTRODUCTION

Maize (*Zea mays*) is important staple crop in east Africa (FAOSTAT, 2013) and is one of the most widely cultivated gramineous plants in the regions (Acland, 1977) due to its ability to grow in diverse climates (Agbonifo and Olufolaji, 2012). In 2011, a disease with virus like symptoms (chlorotic mottle on maize leaves, mild to severe mottling and necrosis) were reported in east Africa causing dramatic maize damage in farmers fields (Wangai et al., 2012a,b). The disease was identified as Maize Lethal Necrotic Disease (MLND) (Wangai et al., 2012b; Adams et al., 2013), a new disease in Africa and perhaps the worst enemy of the maize crops in recent times. This review discusses MLND in east Africa, including its importance, diagnostics, etiology, managements and therefore highlights the future research needs.

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MAIZE LETHAL NECROTIC DISEASE

Causative agents/pathogens

MLND is caused by *Maize Chlorotic Mottle Virus* (MCMV) as a single virus infection or in combination with other Potyviridae family like *Sugarcane Mosaic Virus* (SCMV), *Wheat Streak Mosaic Virus* (WSMV) or *Maize Dwarf Mosaic Virus* (MDMV) (Bockelman et al., 1982). The double infection (co-infection) which is more severe than single infection (Niblett and Claflin, 1978; Scheets, 1998) occurs mostly with two viruses; MCMV and SCMV and this gives rise to what is known as MLND, also referred to as Corn Lethal Necrosis (CLN) (Uyemoto et al., 1980; Uyemoto et al., 1981).

History and geographical distribution of MLND

In September 2011, the first outbreak of MLND was reported in east Africa along rift valley regions of Kenya (Wangai et al., 2012a, b). Regions that were reported to have the disease includes; Bomet, Naivasha, Narok, Chepalungu, Sotik, Transmara, Bureti, Nakuru, Konoin, South Narok, Mathira East, Imenti South Districts and Nyeri (Wangai et al., 2012c). In August 2012, this disease was also reported in Tanzania around border regions especially Northern zone and along Lake Zone (Makumbi and Wangai, 2013). Northern zone includes Arusha (Karatu, Mlangarini, Longijave and Ngaramtoni), Kilimanjaro (Hai district in Nshara and lower Moshi) and Manyara in Kiru, Babati, Mbulu and Simanjiro. Lake Zone includes Mwanza, Mara and Shinyanga. In Uganda, the disease was first reported in October 2012 in Busia then in border district of Tororo, Mbale and Kapchorwa (ASARECA, 2013). MLND was first reported in February 2013 in Gisesero site, Musanze District in Northern Province of Rwanda and it then spread to western Province (Adams et al., 2014; ASARECA, 2013).This disease is not reported yet in Burundi (ASARECA, 2013).

This disease is new in east Africa but not new in the other parts of the world as it was identified as corn lethal necrosis in 1976 in Kansas (Niblett and Claflin, 1978; Uyemoto, 1983), Peru (Castillo, 1977; Uyemoto, 1983), Hawaii (Kaua'i) in the early 1990s (Nelson et al., 2011), Nebraska in 1976 (Uyemoto, 1983), Argentina (Gordon et al., 1984), Texas and Brazil (Uyemoto, 1983).The possibility of spreading to other areas cannot be ruled out and hence need to quantify its distribution in a wider context.

The extent of yield loss due to the impact of the disease

MLND is a big threat to maize production in East Africa as it can cause intensive to complete yield loss (Wangai et al., 2012b). Maize is susceptible to this disease at all stages of development specifically from seedling stage to near maturity (CGIAR Research Program MAIZE, 2012). The loss is due to infected maize plants with small ears, distorted and set little or no grains. On the other hand maize production costs are increasing as farmers use herbicides and insecticides to control weeds and insect vectors transmitting the disease. Furthermore, seed production costs also increases as extra cost of seed treatment is incurred by the seed companies. Therefore, proper solution must be found to properly manage the MLN disease to reduce the losses and maximize production.

Diagnosis of the disease

The best method of controlling plant diseases is proper identification of the causative agents (Webster et al., 2004; Adams et al., 2013) and this is supported by the best diagnostic tools. Several methods have been used to diagnose plant viral diseases. These methods include; serological methods, nucleic acids based methods (Singh and Singh, 1995; Naidu et al., 2003; Webster et al., 2004; Punja et al., 2007; Trigiano et al., 2008), electron microscopy (EM) (Singh and Singh, 1995), physical properties of a virus (that is, thermal inactivation point, dilution end point, and longevity *in vivo*) (Trigiano et al., 2008), transmission tests, and symptomatology (Naidu et al., 2003). In this review, only three methods viz; symptomatology, serological and nucleic acids based methods mostly used in the diagnosis of plant virus diseases specifically MLND are discussed.

Symptomatology: Diagnosis based on symptoms

Symptoms are one of the indications of plants being affected either by biotic (pests and pathogens) or abiotic (environmental conditions) factors in fields (Agrios, 2005). They are important in disease management as some of the management practices such as rouging are based on the observed symptoms.

Symptoms of MLND

Symptoms of MLND includes; elongated yellow streaks parallel to leaf veins, streaks may coalesce to create chlorotic mottling, chlorotic mottling may be followed by leaf necrosis (Nelson et al., 2011; Makone et al., 2014) which may lead to "dead heart" symptom and plant death (Wangai et al., 2012a), premature aging of the plants (Gordon et al., 1984), failure to tassel and sterility in male plants, malformed or no ears (Uyemoto et al., 1981; Gordon et al., 1984), failure of cobs to put on grains and rotting of cobs (Wangai et al., 2012a).

Diagnosis of MLND causative agents based on observation of symptoms has been reported be less accurate because some of the symptoms like stunting and chlorosis may not be virus infection but nutrient deficiencies or maize mosaic (Nelson et al., 2011). Additionally, factors like unfavorable environmental conditions, damage by pests, air pollution, herbicides applications, and infection by non-viral pathogen can also induce virus like symptoms (Naidu et al., 2003). Furthermore, symptoms may be very slight and inconclusive, infected plants may be symptomless (Lima et al., 2012) or different viruses may cause similar symptoms in a plant (Webster et al., 2004). Therefore, to be certain and to avoid misdiagnosis, other confirmatory tests must be done to ensure accurate diagnosis of virus infection (Bock, 1982).

Serological methods

Detection and diagnosis of plant viruses have included serological tests since the 1960s (Martin et al., 2000). These tests are believed to be the best in identification of large number of field samples (Wu et al., 2013). They are reported as one of the most specific and easiest methods for rapid and precise identification (Naidu et al., 2001; Astier et al., 2007). Such tests include enzyme-linked immuno-sorbent assay (ELISA) which includes (triple antibody sandwich ELISA (TAS-ELISA), double antibody sandwich ELISA (DAS-ELISA) and direct antigen coating-ELISA (DAC-ELISA) (Kumar et al., 2004), dot-immuno-binding assay (DIBA), and immuno-capture reverse transcription-polymerase chain reaction (IC-RT-PCR) by using the MAb 4B8 that is developed for sensitive, specific, and rapid detection of MCMV in fields (Wu et al., 2013). Other serological tests include; tissue blot immunoassays, immuno-electron microscopy (trapping and decoration), western blots, double immune diffusion and lateral flow rapid tests (Lima et al., 2012). These serology tests are based on antigen-antibody reaction (Lima et al., 2012).

Among serological methods, ELISA has been extensively used in many studies to identify viral diseases of plants (Punja et al., 2007). The reason being relatively high sensitivity and specificity (highly strain specific) (Lima et al., 2012), low cost and simple for routine diagnosis (Webster et al., 2004; Kimar et al., 2004). This test is based on the basic principle in which the virus antigens are recognized by their specific antibodies (IgG) in association with colorimetric properties (Lima et al., 2012). ELISA method have been used to identify WSMV in wheat (Montana et al., 1996; Ilbagi et al., 2005), MCMV in maize (Jensen et al., 1991; Xie et al., 2011; Adams et al., 2013; Lukanda et al., 2014), SCMV in maize (Louie, 1980; Adams et al., 2013; Lukanda et al., 2014) and MDMV in maize (McDaniel and Gordon, 1985; Giolitti et al., 2005). DAS-ELISA has been used to identify MLND causing viruses in Kenya but gave negative results (Adams et al., 2013) probably due to low sensitivity and poor specificity for unusual or variant isolates (Adams et al., 2013). Similar study was done to identify MCMV and SCMV by ELISA (DAS-ELISA and Indirect ELISA) with polyclonal antibodies produced against the East African strains of MCMV and SCMV and it was successful. (Mahuku et al., 2015a, b).

In spite of serological methods such as ELISA being less accurate in identifying unusual or variant isolates because of being too specific to a particular species or even strain of a virus as reported by Adams et al. (2013), still it can be used in identification because it is the easiest method associated with low cost. Furthermore, it is rapid and can be used in the identification of large number of samples and that is why it is intensively used in quarantine/movement of seeds and plants across countries to identify diseases of quarantine importance including MLND (Mezzalama et al., 2015). However, there must be proper selection of good reagents and ensuring the level of antibodies' sensitivity and specificity toward the pathogen under study, proper handling, storage of reagents and incubation time and temperature must be done carefully as these factors have been reported by Hewings and D'Arcy (1984) to affect ELISA results.

Nucleic acid based methods

Nucleic acid based methods have been used in identification and characterization of many viral diseases of plants (Henson and French, 1993; Hadidi et al., 1995; Lopez et al., 2003). Polymerase chain reaction (PCR) and next generation sequencing (NGS) are among nucleic acid based methods used in the diagnosis of many plant virus diseases including MLND (Zhang et al., 2011; Wangai et al., 2012b; Adams et al., 2013, Lukanda et al., 2014; Mahuku et al., 2015a, b).

Polymerase chain reaction (PCR)

PCR is a molecular technology that facilitates the amplification of rare copies of specific nucleic acid sequences to produce a quantity of amplified product that can be analyzed (Coleman and Tsongalis, 2006). This method is used in many applications (Doughari et al., 2009) including diagnostics of plant virus diseases (Henson and French, 1993; Hadidi et al. 1995; Lopez et al., 2003) because of its speed, specificity, sensitivity, and versatility (Naidu et al., 2003). Apart from detection of viruses, PCR products (amplicons) can be sequenced to provide further data on strain types (Webster et al., 2004). There are several PCR variants including basic PCR, reverse-transcription-PCR (RT-PCR) common for

RNA viruses, real-time PCR (Lopez et al., 2003; Kumar et al., 2004; Rao et al., 2006; Punja et al., 2007; Hardingham et al., 2012), Multiplex PCR, Nested PCR (Lopez et al., 2003; Webster et al., 2004; Rao et al., 2006; Punja et al., 2007; Hardingham et al., 2012), immunocapture PCR (IC–PCR), competitive fluorescence PCR (CF–PCR) and fluorescence RT–PCR using TaqmanÔ technology (Webster et al., 2004). These PCR variants are designed to increase sensitivity, alter specificity or allow automation of detection (Webster et al., 2004).

PCR has been used in diagnosis of many viral diseases of plants including detection of MCMV by real-time PCR in maize seeds (Zhang et al., 2011) and in maize leaves (Adams et al., 2014). Real-time PCR is considered as the best confirmatory test and for routine diagnosis and it is species specific (Adams et al., 2013). Additionally, RT-PCR has been used to detect/verify MCMV and SCMV in maize (Wangai et al., 2012b; Mahuku et al., 2015a), MCMV in sugarcane (Wang et al., 2014) and in maize (Xie et al., 2011), SCMV, *Sorghum Mosaic Virus* (SrMV), *Sugarcane Streak Mosaic Virus* (SCSMV) and *Sugarcane Yellow Leaf Virus* (SCYLV) in sugarcane (Xie et al.,2009), and *SCMV* in maize and sorghum (Rafael et al., 2014).

PCR results can be affected by a number of factors including improper handling and storage of reagents, PCR contaminants, quality of enzyme (that is, Taq polymerase), type of primers and annealing temperature and the presence of inhibitors that can affect amplification of the target DNA which may be the result of improper purification of DNA/RNA (Viljoen et al., 2005). These inhibitors may lead into false negative results and contaminated amplicons may lead to false positive results. Therefore, considerable care is required throughout the process. It is essential to include proper positive and negative control reactions to guard against systematic contamination of PCR reagents and to ensure that the desired amplicon is produced in positive reaction (Coleman and Tsongalis, 2006). Moreover, Rao et al. (2006) reported on non-uniform distribution of most viruses in plant and even less in the plot, orchard or nursery, therefore studies on sampling methodologies and sample processing is urgently needed in to avoid false negative results.

Nevertheless, PCR is considered as the best confirmatory and reliable method for routine diagnosis. However, the need of expertise and high costs of reagents hinders it to be used extensively in detection and identification of viral diseases of plants such as MLND especially in low income-developing countries including east Africa, thus affecting proper diagnosis of viral diseases of plants in regions.

Sequencing

Sequencing is a very reliable technique for virus identification and has led to development of strain specific probes and primers from extensive sequence data available from many viral isolates (Punja et al., 2007). Next-generation sequencing (NGS) is one of modern techniques that have been used in the diagnosis of new unidentified viral plant diseases. This technique involves generation of sequences in non-specific fashion and identification is based on similarity searching against GenBank (Adams et al., 2013). It has been used in several studies to identify and characterize plant viruses including MLND (Adams et al., 2013, 2014; Mahuku et al., 2015a, b). Among those studies includes characterization of MCMV and SCMV in Kenya whereby MCMV showed a similarity of more than 96% to the Yunnan strain from China but different from US strains while SCMV was found most similar to a strain from China (Adams et al., 2013). Other similar study, complete nucleotide sequence of MCMV isolates in Nebraska was done, whereby sequences of MCMV-NE (Nebraska isolates) and MCMV-KA (Kansas isolates) were closely related sharing 99.5% nucleotide sequence identity suggesting that the two virus isolates share a very recent common ancestor (Stenger and French, 2008). However, in spite of NGS being the most modern and effective method for detection of novel unidentified viral plant diseases, it is not used extensively because of high associated cost. This has severely affected proper diagnosis of plant diseases (including MLND) in the region's leading to very low level of molecular diagnosis. Therefore, there is a need of capacity building and enhancing developing countries in plant disease diagnostics.

Because of low level of molecular diagnosis of plant diseases in east Africa (specifically Tanzania), virus strains causing MLND are not well known. Therefore, there is a need of using modern techniques to identify and characterize viruses causing MLND across regions of east Africa and hence set strategic plans to manage the disease and thereby secure food and alleviate poverty.

Etiology of pathogens causing MLND

Sufficient knowledge of causative agents of a disease, their origin, their disseminations and survival properties usually results in adequate control of the disease.

Taxonomy of the pathogens

Maize Chlorotic Mottle Virus (MCMV): MCMV is the only species in the genus *Machlomovirus* family Tombusvirideae (Stenger and French, 2008; King et al., 2011), closely related to members of the genus *Carmovirus*. It is an isometric single component particle containing 4.4 kb single stranded positive sense genomic RNA (ssRNA) (Goldberg and Brakke, 1987; Lommel et

al., 1991) and has a smooth spherical or hexagonal shape with a capsid protein of 25 kDa (Lommel et al., 1991).

Sugarcane Mosaic Virus (SCMV): SCMV is one of the major viruses in the genus *Potyvirus,* family Potyviridae. The virus is not enveloped having filamentous flexuous particles (700-760 nm long and 13-14 nm in diameter) of single stranded positive sense RNA (Teakle et al., 1989).

Wheat Streak Mosaic Virus (WSMV): WSMV is one of viruses in genus *Tritimovirus*, family Potyvirideae (Kumar et al., 2004). It is single stranded positive sense RNA (ssRNA) approximately 9.4 to 9.6 kb sizes with a 3'-poly A terminus. It has a filamentous particle of 15 nm diameter and 690 to 700 nm long (Kumar et al., 2004; Wegulo et al., 2008).

Maize Dwarf Mosaic Virus (MDMV): MDMV belongs to genus *Potyvirus,* family Potyviridae (Giolitti et al., 2005). The virus is a single stranded positive sense RNA (ssRNA) with a flexuous filaments viral particle of 750 nm long and 13 nm wide (Williams and Alexander, 1965; Bancroft et al., 1966; Autrey, 1983).

Life cycle of the pathogens

Survival between cropping seasons: MLND causing viruses can survive in infected maize residuals and contaminate soil, alternative hosts like sorghum, (Toler, 1985), millet, (Bockelman et al., 1982; ASARECA, 2013), Johnson grasses (Knoke et al., 1974; Toler, 1985; ASARECA, 2013) and other grasses in the family Poaceae (Scheets, 2004) can also harbor MLND viruses and act as source of inoculums in the next seasons of maize production.

Transmission

MCMV is transmitted by vectors mainly beetles (Nault et al., 1978; Gordon et al., 1984; Jensen et al., 1991) rootworms (Nault et al., 1978; Uyemoto, 1983; Jiang et al., 1992) thrips (Jiang et al., 1992) and stem borers. SCMV is transmitted by several species of aphids in non-persistent manner (Brandes, 1920; Pemberton and Charpentier, 1969; Zhang et al., 2008). WSMV is transmitted by mites in persistent manner (Kumar et al., 2004; Wegulo et al., 2008). MCMV is transmitted by aphids in non-persistent manner (Knoke et al., 1974; McDaniel and Gordon, 1985; Toler, 1985; Simcox et al., 1995). Additionally, infected soil (Nelson et al., 2011) and seeds have been reported as a reservoir and a means of viruses' transmission (Jensen et al., 1991; Delgadillo Sánchez et al., 1994). Human activities such as using utensils in infected field without thorough washing can transmit the disease causing viruses from infected to uninfected fields.

Initial infection on maize plants

Generally, plant cells have a robust cell wall and viruses cannot penetrate them unaided. Therefore, they penetrate through wounds created by the feeding mode of insect vectors (Ellis et al., 2008) or mechanical injury by human activities. The feeding insect deposits/injects MLND causing viruses rapidly when feeding on a non-infected plant. Such a relationship is termed "non-persistent" and this is common transmission for Potyvirus by aphids (Zhang et al., 2008; Trigiano et al., 2008). Beetles spread a layer of pre-digestive materials known as regargitant on the leaves as they feed, when viruliferous beetles spread this layer they also deposit virus particles in the wound at the feeding site (Trigiano et al., 2008). Once inside the cell, the viral protein coat is removed and nucleic acid enters the nuclear membrane and alters the maize DNA machinery so as to produce many of its copies. Since MLND causing viruses are RNA viruses, they first change their RNA to complementary DNA (cDNA) to mimic its host maize DNA. When more copies of viral particles have been synthesized, their movement between cells is through plasmadermata and the whole maize plant through phloem (Ellis et al., 2008). This results in disease manifestation and secondary cycles to alternative hosts (sorghum, millet, sugarcane and Johnson grasses etc.) and therefore continue repeated cycles during seasons and off seasons by the aid of vectors.

Disease management

Disease management is the selection and use of appropriate techniques to suppress disease to a tolerable level (Fry, 2012). The goal of plant disease management is to reduce the economic and aesthetic damage caused by plant diseases (Maloy, 2005). Proper disease management is achieved when the causation and the effect that the disease could cause are known. Disease management in this context is described based on basic principles of disease control by Whetzel (1929) with modifications as explained by Maloy (2005) and other studies(http://www.apsnet.org/edcenter/advanced/topics/EpidemiologyTemporal/Pages/ManagementStrategies.aspx)

Reduction of initial inoculums

Pathogen exclusion/strict quarantine: Pathogen exclusion is the prevention of disease establishment in areas where it does not occur. This is a major objective of

plant quarantine procedures throughout the world. Maize seeds are inspected before entering and going out countries and within country regions to prevent transmission of the disease especially by seed transmission. Plant quarantine is a national service and is organized within the framework of Food and Agriculture Organization (FAO) (Kumar et al., 2004). It is considered as one of the best procedures of controlling movement of MCMV, rather than attempting to control the endemic SCMV (Adams et al., 2014). This is because MCMV is new in East Africa, reported in Kenya in 2011 (Wangai et al., 2012a, b) but SCMV is not and was reported in East Africa in 1973 (Louie, 1980). Enforcement of this practice will have significant effects in limiting the introduction of MLND into other areas and prevent their spreading and hence reducing threats of food security.

Pathogen eradication: This method reduces pathogen from infected areas before it becomes well established (Maloy, 2005). Pathogen eradication includes sanitation which involves cleaning of tools such as tractor and clothing used in infected fields, removal of infected maize plant debris that will act as source of inoculums in the next season, rouging of diseased maize plants (Mawishe and Chacha, 2013), eliminating weeds and other alternative hosts (insect vectors) which serve as reservoir for viruses (Webster et al., 2004; Maloy, 2005; Trigiano et al., 2008). Crop rotation can be done by planting a non-host crop, this can reduce (but not eliminate) density of the viruses and manage MLND (Uyemoto, 1983). Non-host crops include Irish potatoes, sweet potatoes, cassava, beans, bulb onions, spring onions, vegetables and garlic (Wangai et al., 2012a). Additionally, the use of techniques that disfavor vectors/movement for example, reflective mulches for aphids and sticky cards for other insect vectors that feed on maize can be used to reduce vectors for transmission and thereby reducing density of inoculums.

Reducing the rate of infection

Avoidance: This method aims at avoiding contact between host (maize) and pathogen (viruses) by planting maize in field with no history of the disease, provide adequate plant spacing and avoid crowding, avoiding injury to the maize plants because viruses penetrates the plants through wounds and avoiding the use of recycled maize seeds by using certified seeds (Trigiano et al., 2008; Wangai et al., 2012a), planting maize on the onset of the main rainy season and not during the short rain season so as to create a break in maize planting seasons (Wangai et al., 2012a). This will reduce the population of vectors and hence low rate of infection and disease severance.

Plant protection: This method involves protection of the host (maize) from invading pathogens (viruses). It is achieved by spraying chemicals and modification of plant nutrient (the use of manure and fertilizers) and environment. MLND viruses cannot be controlled by the use of chemicals, but chemicals can be used to kill vectors that transmit/spread those viruses. Several insecticides, formulated either as granules or spray applications can be used to manage vectors (e. g. aphids, rootworms, stem borers, mites, thrips) that transmit MLND. Such insecticides include Imidacloprid, Thiamethoxam, Deltamethrin, Abamectin, Permethrin, Endosalphan and Dimethoate (TPRI, 2011). For effective control of vectors, appropriate insecticides must be sprayed once every 1 to 2 weeks and there should be rotation of multiple chemicals every month to avoid immunity development of the target vector (Mezzalama et al., 2015). The use of chemicals has been reported insufficient in the management of plant virus diseases (Satapathy, 1998; Perring et al., 1999). Other protection techniques include the use of manure, basal and top dressing fertilizers to strengthen the resistance of plants to disease and pests (Wangai et al., 2012a).

Resistant or tolerant varieties: This is the most reliable, effective, environmental friendly and economical way of controlling plant diseases (Kumar et al., 2004). This is because it is durable, reduces crop losses due to disease and no or little use of chemicals (pesticides) that could affect human and the environment. Many Efforts are being done to produce resistant varieties of maize in eastern Africa (ASARECA, 2014). For example, strong collaboration between CIMMYT and National maize programs has been established to effectively tackle the MLN challenge in eastern Africa (CGIAR Research Program MAIZE, 2012; IRIN, 2013). This resulted in establishment of a centralized MLN screening facility for eastern Africa at the KALRO Livestock Research Farm in Naivasha (CGIAR Research Program MAIZE, 2012; IRIN, 2013). Additionally, Ngotho (2013), reported on the funding from the Bill and Melinda Gates Foundation and Syngenta Foundation for Sustainable Agriculture that will be used to develop fast tracking maize varieties that are tolerant to the disease and drought by scientists and researchers within Pan-Africa and the eleven ASARECA countries, Kenya, Uganda, Tanzania, Rwanda, Burundi, Ethiopia, Sudan, Eritrea, DRC Congo, Madagascar and South Sudan.

If proper management of this disease is not taken seriously, the disease will spread throughout Africa where maize is produced as there are reports of MLND in Democratic Republic of Congo (Luanda et al., 2014) South Sudan (FAO REOA, 2013; ASARECA, 2013), Ethiopia (Mahuku et al., 2015b) and Somalia. This may result in serious economic impacts, food insecurity as well as affecting livelihoods and well-being of Africa.

FUTURE RESEARCH NEEDS

In order to manage MLND effectively in east Africa, the

following questions needs to be answered: How do the virus strains causing MLND present in regions of east Africa differ in the rate of infection? What insect vectors are responsible for transmission of MLND causing viruses in EA? What is the relationship between MLND causing viruses and their insect vectors? How can these insect vectors be managed? How much seeds can contribute to transmission of the viruses causing MLND? What genes are responsible for host resistance? How can these genes be incorporated into seed stocks by breeders? What is the prevalence/incidence of MLND in each region of EA? And what is the contribution of climate change to the spread of MLND? Therefore, there is a need to conduct studies to address these questions to properly manage MLND.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Status of inducible clindamycin resistance among macrolide resistant *Staphylococcus aureus*

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Clindamycin has long been an option for treating both methicillin sensitive *Staphylococcus aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) infections. So, it is utmost important to perform the susceptibility test for erythromycin and clindamycin. And, there is concern on use of this antibiotic in the presence of erythromycin resistance because of the possibility of induction of cross-resistance among members of macrolide, lincosamide and streptogramin B (MLS_B) group. During August 2011 to May 2012, a total of 207 isolates of *S. aureus* were isolated and among which 29.47% (61) isolates were confirmed as MRSA by cefoxitin (30 µg) disc. All the isolates were further processed for MLSB resistance test by double disc diffusion test of erythromycin (2 µg) and clindamycin (15 µg) at a distance of 15 and 22 mm between them. This study result show 12.56% (26) and 14.49% (30) of inducible macrolide-lincosamide-streptogramin B phenotype ($iMLS_B$) resistance type at 22 and 15 mm disc distance, respectively, showing 15 mm disc distance is potential than 22 mm and 17.39% (36) of $cMLS_B$ resistance type. Similarly, both iMLSB and cMLSB are greater in MRSA than MSSA and constitutes 18.05 (11) and 36.06% (22), respectively. Thus, this study concludes that D-test should be used as a mandatory method and is more potential in 15 mm disc apart.

Key words: *Staphylococcus aureus,* methicillin resistant *S. aureus* (MRSA), methicillin sensitive *S. aureus* (MSSA), inducible macrolide-lincosamide-streptogramin B phenotype ($iMLS_B$), cMLSB, D-test.

INTRODUCTION

Staphylococcus aureus acquisting *mecA* gene which encodes PBR-2a with low affinity for β-lactams, is methicillin resistant *S. aureus* (MRSA) (Brumfitt and Hamilton, 1989), which is the major cause of nosocomial and community acquired infection (Frank et al., 1999). Changing pattern in antimicrobial resistance and increasing incidence of MRSA infection have led to treating such infection with MLS antibiotics (Jadhav et al., 2011). However, their wide use resulted in increasing number of Staphylococci strains resistant to MLS_B

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antibiotics (Saiman et al., 2003). Macrolide, lincosamide and Streptogramin B (MLS_B) antibiotics are chemically distinct but have a similar mode of action (Gadepalli et al., 2006; Leclercq and Courvalin, 1991). The MLS family of antibiotics has three different mechanisms of resistance such as; target site modification, enzymatic antibiotic inactivation and macrolide efflux pumps (Jadhav et al., 2011).

As the methicilin-resistant *S. aureus*are emerge, the clindamycin has become an excellent drug for some staphylococcal infections, particularly skin and soft tissue infections and as an alternative in penicillin-allergic patients (Drinkovic et al., 2001). Clindamycin, is among the limited choice of antimicrobials effective against MRSA, has good oral bioavailability making it a good option for outpatient therapy and changeover after intravenous antibiotics (Jadhav et al., 2011; Leclercq, 2002). There is concern about use of this antibiotic in the presence of Erythromycin resistance because of the possibility of induction of cross-resistance among members of the macrolide, lincosamide, strepto-gramin B (MLS_B) group (Hussain et al., 2000). Clindamycin has long been an option for treating both MSSA and MRSA infections. So, it is utmost important to perform the susceptibility test for erythromycin and clindamycin as *S. aureus* possesses two types (constitutive and inducible) of clindamycin resistance pattern. This resistance mechanism can be constitutive where rRNA methylase is always produced ($cMLS_B$) or can be inducible where methylase is produced only in the presence of an inducing agent ($iMLS_B$). MRSA has adapted to survive treatment with beta-lactam antibiotic such as penicillins, cephalosporins including methicillin, dicloxacillin, nafcillin and oxacillin. MRSA is especially troublesome in hospital-associated (nosocomial) infection (Boucher and Corey, 2008; Creechs et al., 2005; Eveillard et al., 2004).

It is very important that microbiologists keep a close eye on the developing patterns of drug resistance to be able to guide therapy effectively. Inducible resistance to clindamycin could limit the effectiveness of this drug. Demonstration of $iMLS_B$ phenotype in isolates that are susceptible to clindamycin and resistant to erythromycin is possible by using double disk diffusion agar inhibitory assay or simply D-test (Jadhav et al., 2011; Gadepalli et al., 2006; Steward et al., 2005; Reddy and Reddy, 2012). In this study, we have attempted to characterize MLSBi resistance in both hospital and community associated *S. aureus* isolates, including MRSA and MSSA, at KIST medical college and hospital.

MATERIALS AND METHODS

The present study was conducted from August 2011 to May 2012. During the study, a total of 4230 clinical samples were processed and among which 207 isolates of *S. aureus* were isolated. Clinical samples include pus, blood, wound swab, body exudates, tips and urine.

S. aureus isolates were identified using the standard conventional methods (Frank et al., 1999; Saiman et al., 2003; Fiebelkorn et al., 2003). Antimicrobial susceptibility testing were done by Kirby Bauer's disc diffusion method on Muller-Hinton agar plates using Penicillin (10 U), Ampicillin (10 µg), Cloxacillin (5 µg), Erythromycin (15 µg), Clindamycin (2 µg), Cotrimoxazole (1.25/23.75 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Cefotaxime (30 µg), Chloramphenicol (30 µg) as first line antibiotics and Amikacin (30 µg), Gentamicin (10 µg), Ceftazidime (30 µg), Amoxycillin/Clavulanic acid (20/10 µg) and Vancomycin (30 µg) as second line anitibiotics. *S. aureus* (ATCC 25923) was used as quality control for disc diffusion test as recommended by CLSI (2011).

The organisms which showed resistant to Ampicillin, Penicillin and Cloxacillin were subjected to test with Cefoxitin (30 µg) to confirm MRSA. The isolates with resistant to at least two classes of first line antibiotics were regarded as MDR (Sahm et al., 2001; Simner et al., 2011). MRSA isolates were preserved in nutrient agar containing 20% glycerol at -7°C until further investigation.

Isolates were plated on a Muller Hinton Agar plate at a Mac Farland concentration of 0.5 to eventually cover the agar surface.Clindamycin and Erythromycin disks, containing 2 and 15µg each respectively were placed in the center of the plate separated by a distance of 15 and 22 mm from the centre of discs. Plates were incubated at 37°C for 24 h. Inducible resistance to Clindamycin was defined as blunting of the clear circular area of no growth around the Clindamycin disc on the side adjacent to the Erythromycin disc and was designated D-test positive. Absence of a blunted zone of inhibition was designated D-test negative. Three different phenotypes were interpreted as follows (Deotale et al., 2010; Kloos and Banerman, 1999). *S. aureus* ATCC 29213 (D-test negative) and *S. aureus* ATCC 25923 (D-test positive) were used as quality control.

RESULTS

Among 207 isolates of *S. aureus* isolated from different clinical specimens, 29.47% (61) were confirmed as MRSA distributing higher percentage in IPD than OPD (35.71 vs. 23.85%), in age group 51-60 years (42.11%), in female (29.91 vs. 28.89%) and in nephrology ward (31.44%). All MRSA were highly resistant to penicillin (100%), ampicillin (98.36%), ceftazidime (88.53%) and erythromycin (88.53%) while all MRSA were sensitive to vancomycin showing all MRSA isolates were MDR MRSA.

The overall prevalence of $iMLS_B$ resistant phenotype was found to be 14.49% (Table 1) among *S. aureus,* however, 4 more isolates of *S. aureus* were found to be $iMLS_B$ when placed in 15 mm distance than 22 mm distance and higher in MRSA (18.03%) than MSSA (13.01%) (Table 2). Among 30 isolates of $iMLS_B$, *S. aureus* were found to be the highest in female (18.80%), age group 31-40 years and OPD patients (14.68%).

DISCUSSION

An important distinctive feature of *S. aureus* strains is the susceptibility to methicillin; hence, strains are categorised as MSSA or MRSA which was first reported in 1960s in the hospital setting. Most MRSA strains are multidrug-resistant, being commonly resistant to macrolides,

Table 1. Distribution of MLS_B resistance in *S. aureus.*

	Inducible clindamycin test	
	15 mm; n(%)	**22 mm; n(%)**
$cMLS_B$	36 (17.39)	36 (17.39)
Er/Cl* sensitive	103 (49.76)	103 (49.76)
MS_B resistance	38 (18.36)	42 (20.29)
$iMLS_B$	30 (14.49)	26 (12.56)
Total	207 (100.00)	207 (100.00)

Table 2. Distribution of MLS_B resistance among MSSA and MRSA isolates.

Resistant and sensitive phenotypes	Ery	Cld	D-test	MRSA	MSSA
				No. (%)	**No. (%)**
Inducible MLS_B ($iMLS_B$)	R	S	D+	11 (18.03)	19 (13.01)
Constitutive MLS_B ($cMLS_B$)	R	R		22 (36.06)	14 (9.59)
MS_B resistant	R	S	D-	21 (34.43)	17 (11.64)
Ery/Cl * sensitive	S	S		7 (11.48)	96 (65.76)
Total				61 (100.00)	146 (100.000)

aminoglycosides and fluoroquinolones (Pantosti et al., 2007). The emergence of resistance to multiple antibiotics among staphylococci has left very few therapeutic options for clinicians. A therapeutic decision is not possible without the relevant clinical and microbiological data (Frank et al., 2002; Levin et al., 2005). Newer antibacterial agents as tigecycline, dalbavancin, oritavancin and ceftobiprole are now available for staphylococcal infections; however, it is possible that these antibiotics will also gain resistance towards the pathogens in due course of time. So, a wise decision would be to conserve those antibiotics which are still highly effective against staphylococci; clindamycin is one of such drugs due to its pharmacokinetic properties.

Though detection of *mecA* gene is considered as the gold standard for revealing methicillin resistant gene (Arbique et al., 2001; Fatholahzadeh et al., 2008), however in the present study, phenotypic method (test with cefoxitin disc; 30 µg/ml) as described by CLSI (2011) was employed. The results of the study revealed that MRSA were detected in 29.47% which is in accordance with the findings disseminated by other studies (Fatholahzadeh et al., 2008; Mdani et al., 2001; Vaez et al., 2011) and various regions in Nepal as well (Kumari et al., 2008; Sanjana et al., 2010; Shrestha et al., 2009). Some of the previous studies found the percentage of MRSA in different area ranging 15.4-44.90% (Kumari et al., 2008; Sanjana et al., 2010; Shrestha et al., 2009; Subedi and Brahmadathan, 2005). A study at a tertiary care hospital of Nepal has reported 42.42% MRSA in 2008 (Mishra, 2008). All these studies have depicted the alarming condition due to MRSA isolates which is still in increasing trend. The prevalence is still higher in the well developed countries where it ranged from 50-60% by mutated strains of *S. aureus* (Vazquez, 2006). But in the developing countries like Nepal, the higher prevalence of MRSA may have contended the fact that the inappropriate use of antibiotics for community as well as hospital acquired infections has resulted in the increment of the pressure to select MRSA and other resistant bacteria (Kumari et al., 2008; Sanjana et al., 2010; Subedi and Brahmadathan, 2005).

Increasing frequency of MRSA infections and changing patterns in antimicrobial resistance have led to renewed interest in the use of macrolide lincosamidestreptogramin B (MLS_B) antibiotics to treat such infections. However, their widespread use has led to an increase in the number of *Staphylococcus* strains resistant to MLS_B antibiotics (Saiman et al., 2003) and as MRSA infections have become increasingly common in the community setting, the development of empirical antimicrobial therapeutic strategies for staphylococcal infections has become more problematic. The increasing frequency of MRSA with *in vitro* inducible clindamycin resistance raises a concern of clindamycin treatment failures and this is where the D test becomes significant (Frank et al., 2002; Levin et al., 2005).

In this study, 14.49% of *S. aureus* isolates were inducible macrolide-lincosamide-streptogramin B phenotype ($iMLS_B$) and 17.39% were of $cMLS_B$. The results are in accordance with a previous study in Nepal in which 18.2% of $iMLS_B$ were reported (Shrestha et al., 2009). This study also correlates with the study done earlier which reported 34% of $iMLS_B$, 19% $cMLS_B$ and 30% of MS phenotypes (Mohanasoundaram, 2011). This study showed that the S-phenotype is mostly associated

with MSSA than MRSA which is supported by a previous study (Reddy and Reddy, 2012). Similarly, this study showed $cMLS_B$ phenotype is higher among MRSA (36.06%) which is lower than the report of 44.2% $cMLS_B$ among MRSA from Turkish hospital (Yilmaz et al., 2007). The D-test results of staphylococci isolates showed four phenotypes; including D-positive, D-negative, MS (R) and S phenotype. Most of the MRSA showed MS-phenotype followed by D-negative while the most of the MSSA showed S-phenotype followed by $iMLS_B$ phenotype in this study, which is supported by other studies (Jadhav et al., 2011; Yilmaz et al., 2007; Chelae et al., 2009). MRSA exhibit $iMLS_B$ predominantely than MSSA, the result being in accordance with a few studies reported before (Jadhav et al., 2011; Gadepalli et al., 2006; Yilmaz et al., 2007; Chelae et al., 2009; Rahabar and Hajia, 2007).

CLSI has recommended using D-test in which 15 µg Ery and 2 µg Cld should be placed 15-26 mm apart from edge-edge (Clinical and Laboratory Standards Institute, 2011). This study evaluated the efficacy of two inter disc distances for $iMLS_B$ phenotype detection, by placing at 15 and 22 mm from edge to edge of Ery and Cld discs. Four phenotypes failed to be detected as $iMLS_B$ at 22 mm distance than at 15 mm distance in this study which is supported by the study done in India reporting 7 more isolates were detected as $iMLS_B$ strains at 15 mm distance previously reported as D-test negative at 22 mm distance concluding low interdisc distance induces production of methylase by inducible agents (Ajantha et al., 2008).

Due to the restricted range of antibiotics available for the treatment of methicillin-resistant staphylococcal infections and the known limitations of vancomycin, clindamycin should be considered for the management of serious soft tissue infections. In addition, such testing can provide information about resistant to MLS phenotype group of antibiotics and can be useful for surveillance studies related to MLS resistance in staphylococci. If D-test is not performed, nearly half of the erythromycin resistant and clindamycin sensitive *S. aureus* isolates might have been missed and resulting in therapeutic failure with clindamycin. So before declaring the clindamycin sensitivity among the clinical isolates of *S. aureus*, it is necessary to check for inducible resistance (Jadhav et al., 2011; Gadepalli et al., 2006; Reddy and Reddy, 2012; Fiebelkorn et al., 2003; Shrestha et al., 2009; Mohanasoundaram, 2011; Chelae et al., 2009; Rahabar and Hajia, 2007; Ajantha et al., 2008; Delialioglu et al., 2005; Mshana et al., 2009; Rodrigues et al., 2007; Zorgani et al., 2010). Negative D-test among the erythromycin resistant isolates confirm the sensitivity to clindamycin and possible to choose clindamycin as drug of choice in the treatment of staphylococcal infections (Leclercq, 2002). By consistently performing the D-test, the diagnostic laboratory can properly guide the clinician and clindamycin could be a valuable weapon against the staphylococci. It would be better to implement the D-test for iMLSb detection on a routine basis in the hospital laboratory.

Conclusion

In this study, the prevalence of inducible clindamycin resistance was high among macrolide resistant *S. aureus* isolates. Since the results of this study represent the scenario of a single hospital and might not be representative of the rest of the country, it is recommended that D-test for iMLSb detection should be carried out in the hospital laboratory on a routine basis throughout the country. The use of highly advanced molecular methods for such results would be more promising in such studies.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Study on rapid detection of seven common foodborne pathogens by gene chip

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To develop a rapid, effective, specific, and sensitive method to detect foodborne pathogens, 13 sets of primers were designed to amplify the conservative and specific genes of *rfbE, fliC, invA, hilA, ipaH, femA, nuc, hlyA, prfA, tuf, speB, tlh* and *tdh*, respectively. Establishment of foodborne pathogens detection chips was conducted by spotting the target genes on the chips by Nano-PlotterTM NP 1.2 printing system. The DNA of 7 standard pathogenic strains and 147 strains extracts from food samples was amplified and labeled for hybridization. The results demonstrated that *enterhemorrhagic Escherichia coli O157:H7, Salmonella enteritidis, Shigella flexner, Staphylococcus aureus, Listeria monocytogenes, β-hemolytic streptococcus,* and *Vibrio parahaemolyticus* could correctly be identified by the designed gene chip at an optimal temperature of 58°C and were proved as a potential method with good stability and sensitivity (5 pg/µl of template DNA).

Key words: Gene chip, food-borne pathogen, virulence gene, detection.

INTRODUCTION

It is well known that food is the basis of human beings. In recent years, hundreds of outbreaks of foodborne infection cases occur in the world (Keener et al., 2014; Scallan et al., 2015; Centers for Disease Control and Prevention, 2013) and the species of bacteria causing foodborne infections have continuously become more diversified (Van Doren et al., 2013; Crim et al., 2015; Korsak et al., 2015), resulting in serious harmfulness to human's health. Generally, the food-borne pathogens are some micro-organism from food-processing and transporting, which usually are the main murderers making human's diseases. Therefore, analyzing pathogenic bacteria in food is a standard practice to ensure safety and quality of the food. Presently, there are

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many methods, such as culture-medium enriching, culturing, isolating, and biochemical identification available to detect foodborne pathogens (Feng et al., 2013; Wang et al., 2014, 2012). However, these methods are as long as 3-to-7-day duration, laborious, and fewer species detected. Compared with the above methods, though polymerase chain reaction (PCR) is rather rapid, its disadvantages are that only one strain and gene can be detected at a batch sample, which greatly lowers the working effectiveness and prevents the feasibility of detecting high-flux samples. Further studies are needed to develop rapid and objective methods for foodborne pathogen detection. In this study, the multi-gene methods have been successfully applied for the simultaneous detection of common foodborne pathogens in real food samples. The design of primers, the amplification of PCR, and the formation of gene chip matrix of probes are based on the steady virulence gene or non-virulence gene in the *enterohemorrhagic Escherichia coli (EHEC) O157:H7, Salmonella enteritis, Shigella, Staphylococcus aureus, Listeria monocytogenes, β-hemolytic streptococcus*, and *Vibrio parahaemolyticus.* Our methods are highly specific, sensitive, time-saving, and effective in the simultaneous detection of foodborne pathogens. The established methods have shown satisfactory results applied to detect the 7 standard strains of foodborne pathogens and 147 isolation strains from food samples.

MATERIALS AND METHODS

Standard strains and food isolation strains

Seven standard strains

EHEC O157:H7 (CCTCCAB200051), *S. enteritis* (CCTCCAB94018), *S. aureus* (CCTCCAB94006), and *L. monocytogenes* (CCTCCAB97021) were purchased from Chinese Presentative Culture Preservation Center (Beijing, China). *Shigella* (51571-10) and *β-hemolytic streptococcus* (32210-18) were from Chinese Medicine Bacteria Center of Microscobial Preservation Committee (Beijing, China). *V. parahaemolyticus* (VPL4-90) was provided by Guangzhou Microbiological Institute, Chinese Academy of Sciences (Guangzhou, China).

Seven food isolation strains

Each isolation strain is composed of 21 strains, including 5 negative and 16 positive ones, and all were granted by the Food Safety Laboratory of Technology Center, Zhuhai Entry-Exit Inspection, and Quarantine Bureau (Zhuhai, China).

Reagents

TaKaRa *Ex* Taq (5 U/μl), DL2000 Ladder Marker, λDNA, dATP, dGTP, dCTP, dTTP, and TaKaRa Spaced Cover Glass, Code No.: TX702, TX703, Lot: TAGA0508, GB010920 were from Baosheng Biological Engineering Co., Ltd. (Dalian, China). TIANamp Bacteria DNA Kit (Lot: DP302) was provided by Tiangen Biotech (Beijing) Co., Ltd. Omega Bio-tek E.Z.N.A^{TM} Gel Extraction Kit, Lot: D050520 was purchased from Qikete Company (Guangzhou, China) and stored at room temperature. Baio® amido-slide, 2×spotting buffer solution, pre-hybridization and hybridization buffer solution were from Shanghai Baiao High-tech Co. Ltd. (Shanghai, China). Cy3-dCTP: 25 nmol, PA53021, Lot: 334872 was purchased from Amersham Biosciences (UK). Cleaning solution 1: 0.1% SDS 2×SSC; cleaning solution 2: 0.1% SDS 0.2×SSC; cleaning solution 3: 0.2×SSC.

Design of the primers

The good conservative domains were picked by DNAMAN software from the conservative and specific genes of seven types of foodborne bacteria. The various primers (Shanghai Yingjun Co. Ltd., China) were also designed by Array Designer 2.0 software (Primer Biosoft International, CA).

Design of aligned genes (positive references)

The aligned gene was picked up from one specific DNA gene existing in the lambda bacteriophage, a positive reference, used as quality control (QC) in the experimental program. The results will not be reliable unless the aligned gene was found. The designs of prime and the composition of aligned genes are identical to the aforementioned workflow.

Extraction and preparation of the target and the aligned genes in samples

The extraction and preparation of DNA sample template was processed based on the User's Manual from TIANamp Bacteria DNA Kit. The amplification was processed by using PCR gradient instrument and then the production was recovered by the gel-cut after electrophoresis (the annealing temperature of the primers shown in Table 1. The cut gel was amplified as a template after ten-fold dilution and the purification was processed with isopropyl alcohol precipitation method. The amplification gene was sequenced by Shanghai Yingjun Company and Shanghai Biological Engineering Company (Shanghai, China), from which the obtained data was analyzed by Blast search and DNAMAN software.

Preparation of the gene chip of foodborne pathogens

The concentrations of target gene and aligned gene were diluted to 250 to 300 ng/μl prior to spotting on the amido slide by spotting instrument. Three sets of comparative experiments were designed as follows: positive group (aligned gene), negative group (3×SSC), and blank group (Milli-Q water), among which each sample had ten same positions and each slide repeated two arrays. Prior to being fixed at 65°C for an hour in an oven, the prepared samples should first be dried overnight; next hydrated and crosslinked by UV; finally, centrifugated after washing by 0.2% SDS solution and stored at room temperature.

Preparation of the probes and detection of concentrations

Probes were prepared by enzymatic reaction notation and the notated dCTP was taken to detect the target gene and the aligned gene. In total 50 μl solution, where DNA template shared 1 μl, 10×*Ex* Taq Buffer 5 μl, dATP, dGTP, dTTP Mixture (2.5 mmol/L, respectively) 4 μl, 10 mmol/L dCTP 0.5 μl, 1 mmol/L Cy3-dCTP 0.5 μl, each primer 1 μl, and *Ex* Taq (5 U/μl) 0.5 μl. The optimized

Table 1. Related information on the primers of target gene.

Target gene	Primer sequence(5'->3')	Fragment sizes of the target gene (bp)	Tm (°C)
O157:H7 rfbE gene	AAGATTGCGCTGAAGCCTTTG CATTGGCATCGTGTGGACAG	497	52
O157:H7 fliC gene	ATTCAGCAGGTAATATCAC TATCATCCACATAAGACTTC	390	52
Salmonella enteritis invA gene	TCCATTACCTACCTATCTG GGCATCAATACTCATCTG	382	50
Salmonella enteritis hilA gene	TAATCCTGTTCCTGTATCG GAAGTATCGCCAATGTATG	371	50
Shigella ipaH gene	AATTCTGGAGGACATTGC TCATACTTCTGCTCTTCTG	303	50
Staphylococcus aureus femA gene	AGCACATAACAAGCGAGATAAC CCAGCATCTTCAGCATCTTC	300	50
Staphylococcus aureus nuc gene	TGGCGTAAATAGAAGTGGTT GCTTGTGCTTCACTTTTTCT	438	50
Listeria monocytogenes hlyA gene	AACCTACAAGACCTTCCAG CGTATCCTCCAGAGTGATC	498	50
Listeria monocytogenes prfA gene	ATACACGATAACTTTCTCTTGC GAACAGGCTACCGCATAC	336	50
β-hemolytic streptococcus tuf gene	TTCCAGTTATCCAAGGTTC CGGTAGTTGTTGAAGAATG	484	50
β-hemolytic streptococcus speB gene	TAGACAATACAACTGGAACAAC GTCAAGACGGAAGAAGCC	400	50
Vibrio parahaemolyticus tlh gene	TACGCTTGAGTTTGGTTTG GGTGAGTTGCTGTTGTTG	476	50
Vibrio parahaemolyticus tdh gene	CCATCTGTCCCTTTTCCTGC CCACTACCACTCTCATATGC	426	50
λbacteriophage DNA gene as aligned gene	AAAGCGACGCAATGAGGCACT GTTCCACGACCGCAACTGC	500	54

concentration of probes was 3000 pg/μl (Takara Biotechnology (Dalian, China) Co. Ltd., 2014-2015).

Hybridization, scanning and data analysis for gene chip

Gene chip was hybridized with the introduced probes at 58°C for 10n to 18 h after pre-hybridization at 50°C for an hour. After hybridization, the chip was in turn washed by cleaning solution 1, 2, 3, and Milli-Q water, prior to being centrifuged and dried, respectively. The gene chip signal was obtained by a 532-nm excitation light source with 100% laser power, 600 Photo Multiplier Tube (PMT) Gain, and 10-μm resolution. The final information was taken using GenePix Pro Ver. 4.1 software, both mean values of ten-point signals from same target gene and ten-sample signal-to-noise ratio (SNR) were used to characterize the signal value of hybridization for each target gene.

Optimized temperature of hybridization of gene chip

Different tagged probes and prepared chip were hybridized at 52, 56, 58, and 60°C, respectively. The optimized temperature was obtained by analyzing specificity of hybridization of gene chip at different temperature.

Testing of specificity and sensitivity

The testing of specificity has been done based on seven probes of standard foodborned pathogens and detection chip hybridized at an optimized temperature, respectively. The results have shown that there are not distinct interferences in difficult matrix samples. Additionally, DNA template purified by *Shigella* was diluted to 3000, 300, 30, 10, 5, and 1 pg/μl and then hybridized with relevant detection chip before getting data and verifying sensitivity of chip by fluorescence scanner.

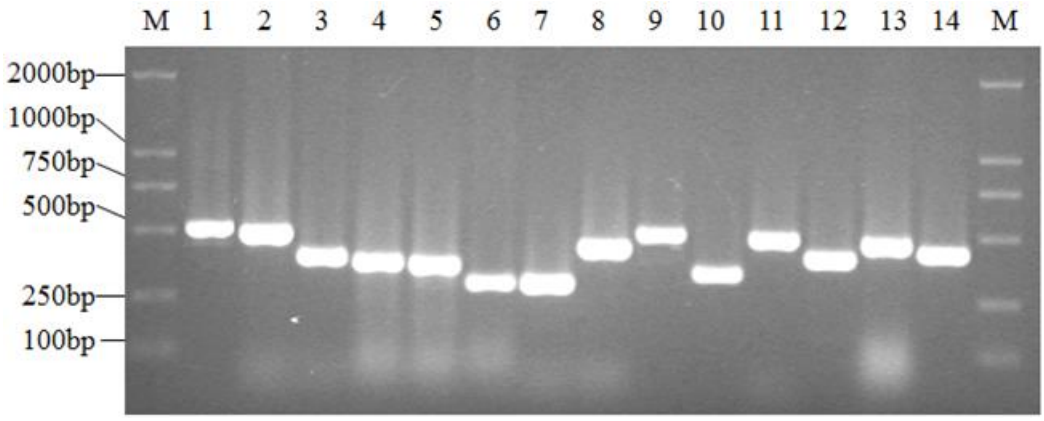

Figure 1. PCR amplification electrophoresis of target genes and aligned genes of seven foodborne pathogens. M: DL2000 Ladder Marker; 1: TλDNA; 2: TrfbE; 3: TfliC; 4: TinvA; 5: ThilA; 6: TipaH; 7: TfemA; 8: Tnuc; 9: ThlyA; 10: TprfA; 11:Ttuf; 12: TspeB; 13: Ttlh; 14: Ttdh.

Testing of isolated food strains

The data was obtained by scanning after the isolated food strains were amplified to notate by relevant primers and hybridized with the detection chips, successively.

RESULTS

Design of primers, sequence comparison of fragment and analysis of homology

Fourteen pairs of primers were made among which *rfbE, tdh,* and *λDNA* were candidates and the remaining was designed by array designer (design software of gene-chip primer). As shown in Table 1, the target gene fragments with other genus or species strains have more than 88% sequence homologies except *tdh* gene. It approved that the designed target gene fragments have shown good conservation and are irrelative with homology with nucleoside sequence of other genus and species. Moreover, Less than 43% ratio of homologies was produced between 13 sets of target genes fragments and aligned genes, greatly preventing gene-gene from being hybridized, which theoretically guaranteed the good specificity of detecting chip. Moreover, double gene combined detection supplied to each foodborne pathogen will greatly lower the probability of false positive and improve the specificity in the difficult matrix samples.

Testing of amplification effect and specificity of target gene and aligned gene

In this research, thirteen target genes and one aligned gene were successfully amplified from seven types of foodborne pathogens and *λbacteriophage* DNA gene, respectively. According to the data obtained by nucleic acid protein spectrophotometer, the concentrations and the purities (A260:A280) of genes were 449.3 to 1917.0 ng/μL and 1.70 to 1.87, respectively. Figure 1 shows that the strips of targeted genes and aligned genes are not distinct interferences and tailing found, meaning that the methods may be reasonable for the successful application of the detection.

Preparation of probes and detection of concentration

Probes were prepared by using enzymatic reaction notation. The experimental data shows that the values of A260:A280 were 1.70 to 1.96 and the concentrations were 307.2 to 846.3 ng/μl, consistent with the experimental requirements (Figure 2).

Experimental results of target gene at different temperature

The gene chip was hybridized at 52, 56, 58 and 60°C, respectively. The experimental data showed that better results can be obtained at 56, and 58°C, where each target gene had a strong signal of hybridization with relevant one on the detection chip and visible interference signals were ‘hidden’. Taking into account the signal intensity and stability, 58°C was employed as the optimized temperature.

Experimental results of hybridization of seven pathogens

The tagging probes can well hybridize with the detection chips (Figure 3a to g). The results showed that more than both 500 strong signals and 4.0 SNR were obtained and invisible interference signal appeared. These proved that the prepared chips had good specificity and did not hybridize with irrelevant genes.

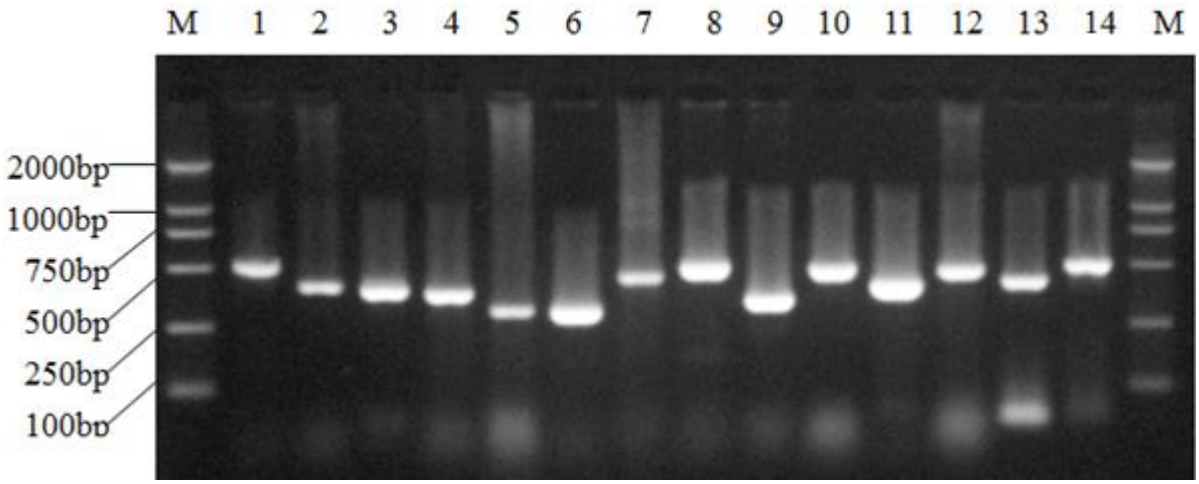

Figure 2. PCR amplification electrophoresis of the probes. M: DL2000 Ladder Marker; 1: PbrfbE; 2: PbfliC; 3: PbinvA; 4: PbhilA; 5: PbipaH; 6: PbfemA; 7: Pbnuc; 8: PbhlyA; 9: PbprfA; 10: Pbtuf; 11: PbspeB; 12: Pbtlh; 13: Pbtdh; 14: PbλDNA.

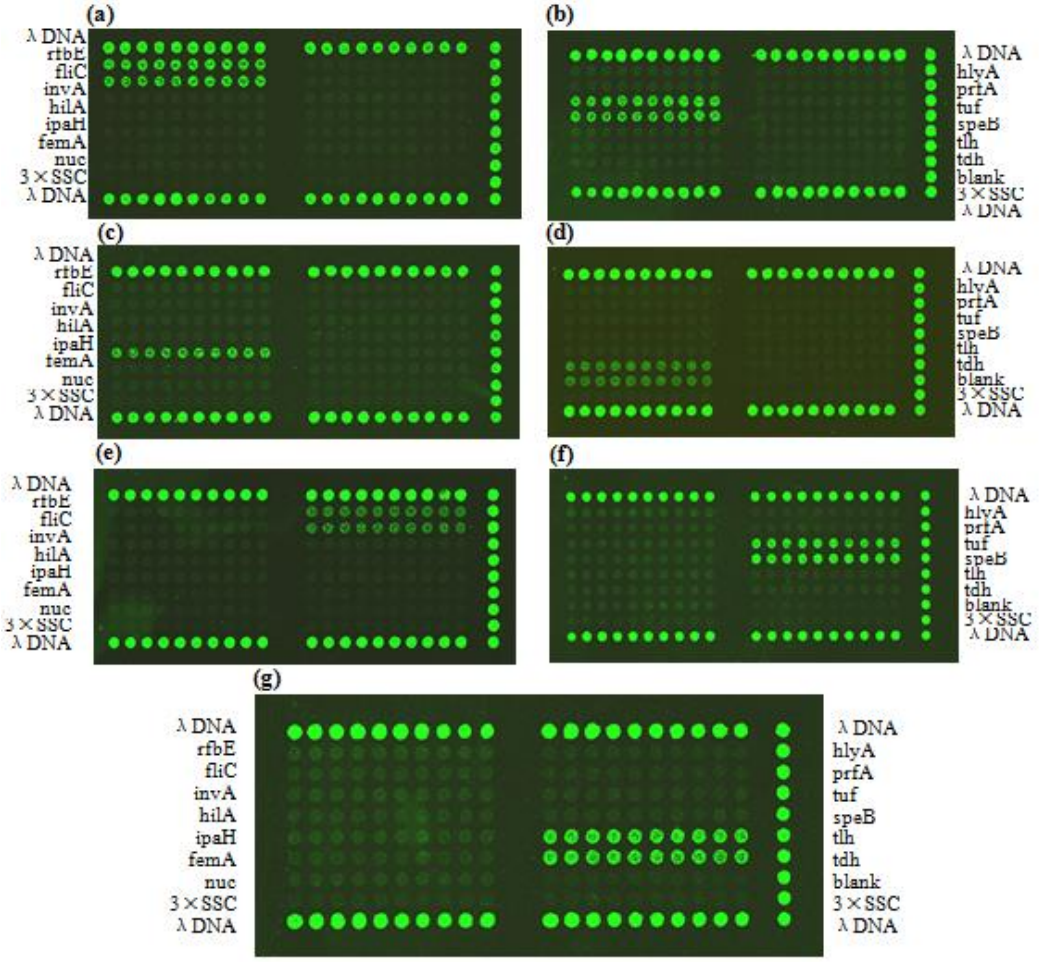

Figure 3. Fluorescence images of hybridization of seven food-borne pathogens taken by nucleic acid protein spectrophotometer: **(a)** *O157:H7* hybridization; **(b)** *Salmonella enteritis* hybridization; **(c)** *Shigella* hybridization; **(d)** *Staphylococcus aureus* hybridization; **(e)** *Listeria monocytogene* hybridization; **(f)** *β-hemolytic streptococcus* hybridization; **(g)** *Vibrio parahaemolyticus* hybridization.

Sensitivity of gene-chip detection for *Shigella*

When the concentrations of template DNA varied from 5 to 3000 pg/µl, SNR was 1.5 above and all signal intensities of hybridization were more than 150, with distinct hybridization spots. Notably, when the concentration was down to 1 pg/µl, SNR was 0.40 and the signal intensities of hybridization were less than 100, without visible signals that appeared. These indicated that 5 pg/µl was the sensitivity of detection in this study.

Specificity, repeatability and sensitivity of the isolation strains for food samples

Using the detecting chips of gene, the detecting results of 147 food isolation strains of seven foodborne pathogens have shown that the probes from 16 positive strains and the targeted genes on the chips had strong hybridization signal, meaning good repeatability; at the same time, there are no distinct interferences between positive strains and targeted genes of other strains, also five negative strains did not show any hybridization signal, bringing high specificity.

In difficult matrix samples, the detecting results of 147 food isolation strains displayed that the limit of detection can be 5 pg/µl.

Analytical applications

Each probe appeared strong signal of hybridization with relevant target gene but did not display similar phenomenon with irrelevant ones. High sensitivity of detection and good specificity had proved that our methods can well be applied to rapid and simultaneous detection of foodborne pathogens in real food samples.

DISCUSSION

Design of the target gene chip

The selection of the target genes will greatly affect the detection chip, so the target genes must be designed in accordance with conservative and specificity in its genus or species. Using the homology analysis of the detection genes, it can be clearly know whether target gene may be detected. Girke et al. (2000) have reported that the hybridization of crossing can be processed in the case of more than 70 to 80% homologies of gene sequences, whereas the hybridization will least or even do not appear under 75% homologies of sequences (Schena, 2003). In this study, the homology between 13 target genes and 1 aligned gene is less than 43%, indicating a good specificity. The homologies of the fragments are more than 88% in the remaining target genes and different genus or species except 70% homology in *tdh*, displaying a satisfactory conservative. The remaining bacteria were simultaneously detected by bi-genes except *Shigella*, thus greatly lowering the ratio of false positive and improving the specificity of detection.

UV-crosslinked and hydration time

To obtain a uniform distribution after inoculation, DNA must be re-hydrated and dried quickly. It is worthy of mentioning that excessively low water temperature and insufficient time will greatly suffer irregular sampling sites which affect the following hybridization and data analysis. By contrast, the sampling sites may be rapidly enlarged under excessively high water temperature and time and thus lead to the pollution of blending of the sampling sites. Generally, the optimized temperature and time is 70 to 80°C and 10 s, respectively. In addition, the chip should be crosslinked from a 10-cm vertical distance by a 15-mJ UV irradiation, which will successfully form a crosslink bond between a fraction of thymine residues in DNA and amino groups on the surface of slide, thus greatly improving the fixed effect. Noticeably, DNA will severely be destroyed for excessive crosslinking time, 20 min were taken as the optimization time.

Treatment parameters of images and choice of signal output mode

The images were scanned to produce the exactly aligned sites of gridding by GenePix Pro Ver.4.1 chip data analysis software and then automatically analyze signal median value, mean value, SNR and standard deviation in each sampling site. Among the mentioned data, the median value is widely used (He and Zhou, 2008; Sarder et al., 2008; Wentzell and Karakach, 2005) owing to its less sensitivity with fragments and dust pollution (Wentzell and Karakach, 2005). In this work, compared with mean value, one is that the median value can better embody hybridization signal in the case of pollution or high background signal; the other is that SNR is relative to signal and background and may well evaluate reliability of the obtained data, bigger SNR value with stronger sampling site signal and weaker background signal. Based on the discussion, median value and SNR were used to characterize the hybridization results.

Detection standard of positive signal

So far, there has still not been a standard model for gene chip adjustment. Al-Khaldi et al. (2004) have reported that the adjustment standard is to contrast the fluorescence signals in the sampling sites and the probe sites. Murray et al. (2001) have suggested that 55% pixels in the

sampling sites are above 1.5-fold local background and the intensities of signals are more than the standard deviation of 2-fold mean value of background signal (Murray et al., 2001). Until now, it is an accepted-widely method to find a proper threshold such as background mean value plus 2-fold variance or negative contrast mean signal plus 2-fold variance, in which the value of less than threshold should directly be ignored and the remaining should be kept for further data analysis. It was considered that the reliable adjustment standard of positive signals is based on more than 1.5 SNR, visible hybridization sampling sites in the scanned images and more than 150 median value of fluorescence intensity in the 10 repeated sampling sites of target genes.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Characteristics of *Streptococcus* and *Staphylococcus* strains isolated from acute cellulitis of dental origin in Ouagadougou, Burkina Faso

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Patients afflicted by acute cellulitis of dental origin are usually in need of urgent treatment. The most frequently isolated bacterial strains associated with this condition are Streptococcal and Staphylococcal species, which are also most commonly implicated with cellulitis in general. The aim of this study was to determine the antibiotic resistance profiles of *Streptococcus* and *Staphylococcus* isolated from patients with acute cellulitis of dental origin in a developing country such as Burkina Faso. Samples (exudates) taken from 52 patients (25 male [48.1%], 27 female [51.9%]) suffering from acute cellulitis were analyzed using conventional microbiology methods. Patients who were 19-40 years of age were the most commonly afflicted by acute cellulitis (representing 59.6% of the subjects in this study). Of the 52 samples taken, 25 (48.1%) were positive and 27 (51.9%) negative for *Staphylococcus* and/or *Streptococcus*. Seventeen *Staphylococcus* (32.7% of the samples) and 8 *Streptococcus* (15.4% of the samples) strains were isolated and characterized using antibiotic susceptibility profiling methods. All the *Streptococcus* strains were found to be resistant to trimethoprim/sulfamethoxazole, chloramphenicol, oxacillin, cefixim, cefuroxim, cefotaxim and ceftriaxon. The *Staphylococcus* strains were mostly resistant to cefixim (88.2%), piperacillin (70.6%), penicillin G (94.1%) and amoxicillin (76.5%). All strains were resistant to metronidazole. Given the high resistance of isolates to antibiotics, it may be necessary to assay bacterial antibiotic susceptibility patterns prior to prescribing these medications.

Key words: Acute cellulitis, tooth, *Streptococcus*, *Staphylococcus*, antibiotics, resistance, Ouagadougou, Burkina Faso.

INTRODUCTION

Cervicofacial cellulitis is an inflammation of the fat cell tissues that entails an interesting head and neck anatomy which is often associated with microbial infections (Lakouichmi et al., 2014). Emergency diagnosis and therapy are generally necessary because the pathology's manifestation is usually not limited to a single area, and it tends to spread through tissue spaces to vital organs (Odzili et al., 2014). Furthermore, cervicofacial cellulitis is frequently associated with high mortality rates in sub-Saharan Africa (Odzili et al., 2014). Yet, despite its considerable morbidity and mortality, there have been few investigations of the etiology of this disease in Africa.

The most common form of cellulitis is a mixed infection (aerobic, facultative anaerobic and obligate anaerobes) which is of dental origin. Most treatments aim to eradicate the etiological agents of the disease. In most of these infections, the bacteria are part of the oropharyngeal flora, with the predominant genera being Gram-positive cocci such as *Streptococcus*, *Staphylococcus* and *Peptostreptococcus*, as well as Gram-negative bacilli (Oberoi et al., 2015).

Staphylococcus and *Streptococcus* are involved in several human infectious diseases, and they play an important role in the severity of the infections that they cause (Petti et al., 2014). The existence of multi-drug resistant (MDR) strains and the appearance of new resistance represent major challenges in the treatment of microbial infections and they have major implications regarding the choice of treatment (Kityamuwesi et al., 2015). Guidance for therapeutic decisions regarding the choice of antibiotic depends on the frequency of the bacteria isolated, and their sensitivity to different classes of antibiotics (Boisramé-Gastrin et al., 2011). There is ample evidence that antibiotic misuse is the most important risk factor for the development of bacterial resistance. Furthermore, an increase in the relative frequency of bacteria producing extended spectrum β-lactamases (ESBL) has been reported both in hospitals and in the wider community. While exhibiting large geographical disparities, the spread of resistance is currently a worldwide public health problem (Laxminarayan and Heymann, 2012).

The acquisition of data on bacterial resistance to antibiotics is necessary in order to achieve better therapeutic management of infections, and to develop an antimicrobial resistance control strategies (Oberoi et al., 2015). This study aimed to determine the prevalence and antibiotic susceptibility of *Streptococcus* and *Staphylococcus* involved in acute cellulitis of dental origin in Burkina Faso.

MATERIALS AND METHODS

Study design and location

This was a prospective study conducted in Ouagadougou (Burkina Faso) (Figure 1) between June and October of 2014. Exudate samples were collected at the Municipal Center for Bucco-dental Health from patients suffering from acute cellulitis, and these were analyzed at the Laboratory of Molecular Biology, Epidemiology and Surveillance of Food-borne Bacteria and Viruses ("LaBESTA") at the University of Ouaga I Professeur Joseph KI-ZERBO School of Doctoral Science and Technology ("EDST") Centre for Research in Biological Sciences, Food and Nutrition ("CRSBAN").

Clinical data

All patients gave informed consent to provide samples, for the epidemiological investigations, and to participate in the study. Data were collected using a standard form containing information regarding the patients' identity, medical history and dietary habits. Oral hygiene was assessed using the Björby and Löe's (1967) retention index, with a scale of 0-3 (Table 1). Upon clinical examination, written and image-based records of teeth affected by bacterial infection were compiled (for example, using panoramic or periapical radiography). Personal income levels were assessed by grouping patients into three occupational categories: low-income participants (for example, farmers, students, pupils and homemakers), high-income patients (for example, commercial and private sector employees) and moderate incomes (for example, public sector employees, informal sector workers, retirees and others similarly not in the work force). The type of food consumed was noted across four of the main food groups: meat products, seafood products, dairy products, sugar-based products and fruits and vegetables.

Samples and processing

Fifty-two exudate samples were collected from patients presenting with acute cellulitis on an everyday basis over the study period (for 5 months). Patients with prior incidences of immunosuppressive diseases (for example, patients with HIV, cancer, diabetes, patients receiving corticosteroid therapy, etc.) were not excluded. Only participants with non-fistulized skin or oral mucosa cellulitis were included in the study (Figure 2). All other cases were excluded. Sampling was performed according to the method described by Rôcas and Siqueira (2013). Patients were asked to rinse their mouth for one minute with chlorhexidine (using a 0.12% solution). The inflated mucosa was then sanitized with 2% chlorhexidine solution prior to collection of up to 2 mL of exudate by piercing the infected area with a sterile needle (Figure 3). The exudates were then immediately transferred into a sterile tube containing thioglycollate resazurin broth (Liofilchem, Italy) (Figure 4**)**. Tubes were conditioned in a cooler at 4°C and transported to the laboratory for microbiological analysis within two hours.

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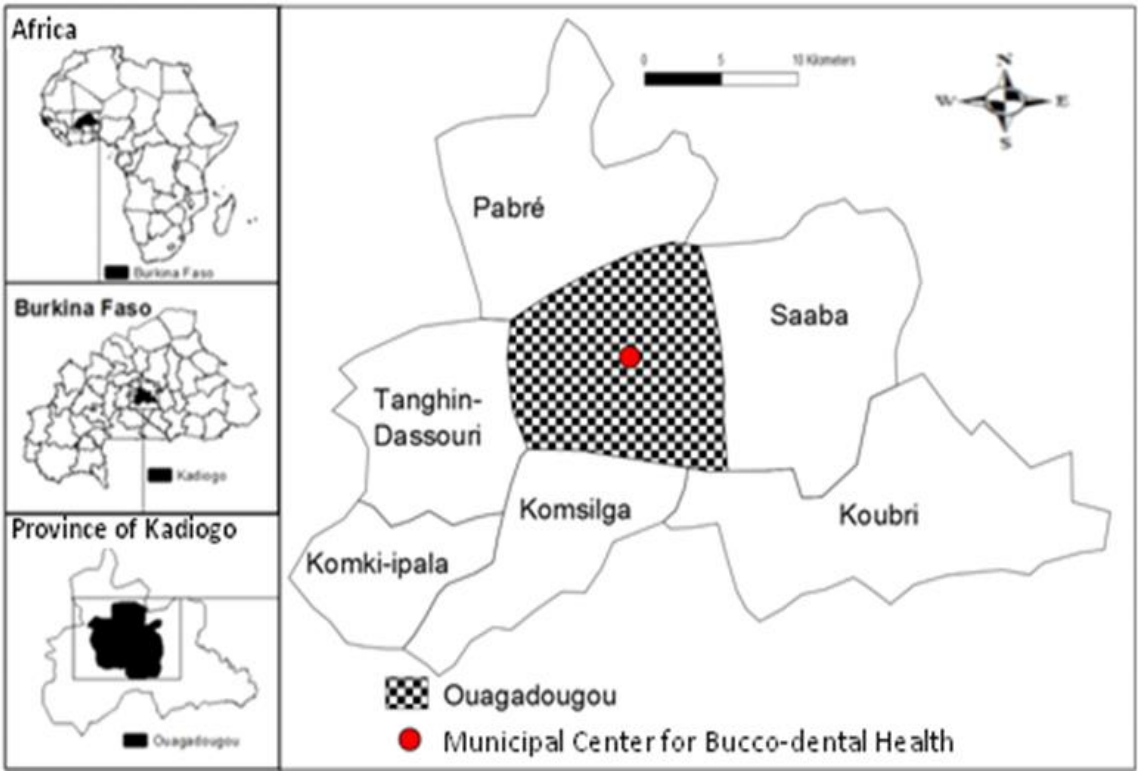

Figure 1. Map of Kadiogo province with the study sites.

Table 1. Oral hygiene index.

0	1	2	3
Absence of tartar, tooth decay or fillings	Tooth decay or fillings close to the gum	Tooth decay, tartar, or filling in contact with the marginal gingiva, a degree of subgingival calculus	Tooth decay, tartar, or filling in the marginal gingiva, abundant subgingival calculus

0 = Score of zero, 1 = score of one, 2 = score of two, 3 = score of three.

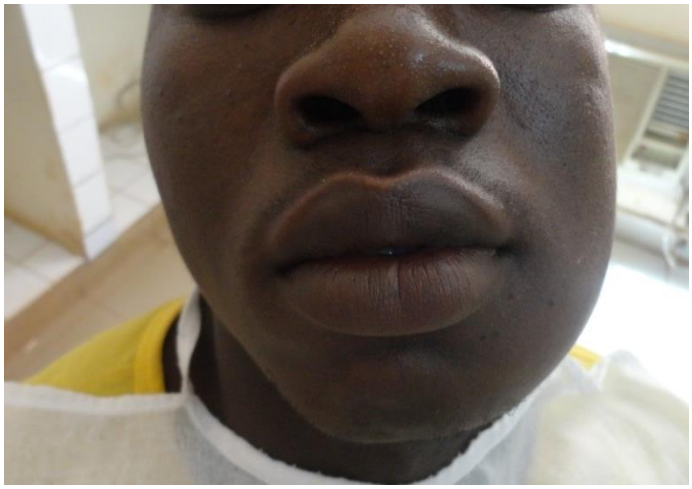

Figure 2. Cellulitis of dental origin.

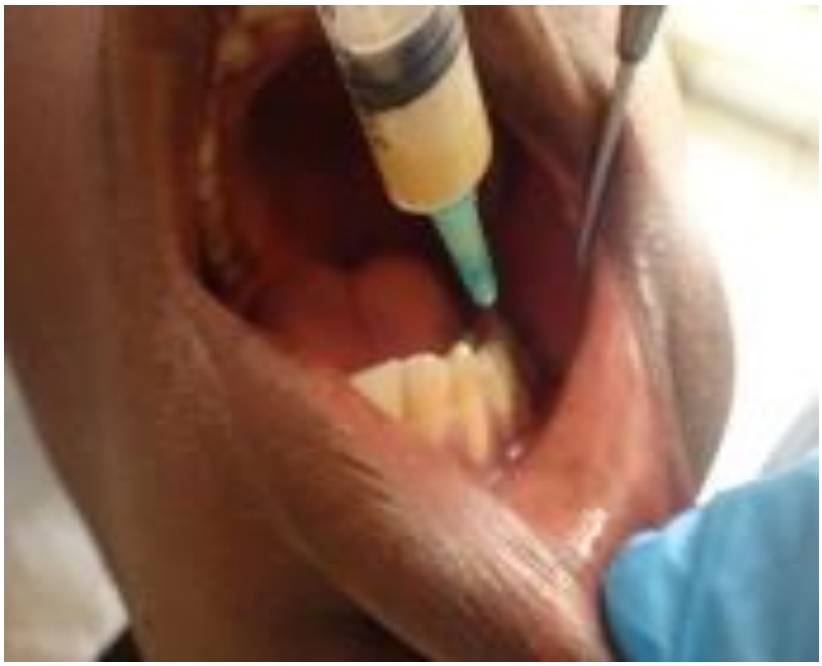

Figure 3. Sampling of exudate.

Figure 4. Specimen storage.

Isolation and identification of *Streptococcus*

Ten microlitres aliquots of anaerobically transported broth (thioglycollate resazurin) (Liofilchem, Italy) were streaked onto plates containing Columbia agar (Liofilchem, Italy) supplemented with hemoglobin (Liofilchem, Italy) and anaerobically incubated at 37°C for 48-72 h (Ellner et al., 1966). Colonies suspected to be *Streptococcus* (with small, white to grayish appearance) were then subcultured on Mueller-Hinton agar (Liofilchem, Italy) prior to biochemical confirmation of their identity using the API 20 Strep kit (bioMérieux, France). Interpretation of the results was done using APIWEB V7.0 software (bioMérieux, France).

Isolation and identification of *Staphylococcus*

Ten microlitres aliquots of anaerobically transported broth (thioglycollate resazurin) (Liofilchem, Italy) were streaked onto plates containing mannitol salt agar (Liofilchem, Italy) and anaerobically incubated at 37°C for 48-72 h (Chapman, 1945). Colonies suspected to be *Staphylococcus* (with a lush, pigmented appearance and surrounded by a yellow halo) were then subcultured on Mueller-Hinton agar (Liofilchem, Italy) and

characterized using the API Staph kit (bioMérieux, France). Interpretation of the results was done using APIWEB V4.1 software (bioMérieux, France).

Antibiotic susceptibility testing

Antimicrobial susceptibility test was carried out using the agar disc diffusion method (Bauer et al., 1966); for *Staphylococcus* strains, Müller-Hinton agar (Liofilchem, Italy) was used; while for *Streptococcus,* Müller-Hinton agar (Liofilchem, Italy) supplemented with 5% defibrinated horse blood was used. The Müller-Hinton agar (Liofilchem, Italy) was inoculated with a 0.5 McFarland standard inoculum in each case. After depositing the antibiotics, plates were incubated anaerobically at 37°C for 24 h. The following 21 antibiotics were used: oxacillin (5 µg), amoxicillin (25 µg), amoxicillin-clavulanic acid (20+10 µg), cefotaxim (30 µg), cefuroxim (30 µg), cefixim (5 µg), ceftriaxon (30 µg), erythromycin (15 µg), trimethoprim/sulfametoxazole (1.25/23.75 µg), chloramphenicol (30 µg), gentamicin (30 µg), tobramycin (10 µg), netilmicin (30 µg), piperacillin (100 µg), piperacillin/tazobactam (100+10 µg), metronidazole (5 µg), penicillin G (10 IU), lincomycin (15 µg), spiramycin (100 µg), clindamycin (10 µg) and ciprofloxacin (5 µg) (Liofilchem, Italy). Inhibition zones were measured and bacterial strains classified as either 'resistant', 'intermediate sensitive', or 'sensitive' according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST, 2014) guidelines, and those of the French Microbiology Society's Antibiogram Committee (CASFM, 2012).

Statistical analysis

Statistical data analysis was performed using Epi-Info Version 7. The Chi-square test was used to determine the difference between two statistical variables. Differences were considered significant at $p<0.05$.

RESULTS

Patient characteristics

Patients in the study population were mostly (59.6%) in 19-40 years age bracket, with 51.9% being female and 48.1% male (Table 2). The first mandibular molar (50% of cases), second mandibular molar (9.7% of cases), and the first maxillary molar (7.8% of cases) were the most commonly affected by bacterial infection. Anamnesis revealed prior surgery (in 5.8% of cases) and hypertension (in 1.9% of cases); no other medical history was observed. Fish and meat items were the food products that were consumed the most, at 46.2 and 30.8% of total, respectively (p=0.0001) (Table 3). Low-income participants represented the group most afflicted by this type of bacterial disease (57.7% of the study population; p=0.0009). The proportion of high-income patients with an oral infection was 19.2%, and those with moderate incomes: 23.1% of the study population. In terms of oral hygiene, 50 patients (96.2%; p=0.0001) were given a retention index score of 3; only 2 patients (3.8 %) had a score of 1.

Table 2. Age and sex distribution of cellulitis cases.

Age group (year)	Sex N (%)		Total N (%)
	Male	Female	
0-6	1 (4)	0 (0)	1 (1.9)
7-12	3 (12)	2 (7.4)	5 (9.6)
13-18	2 (8)	6 (22.2)	8 (15.4)
19-40	14 (56)	17 (63)	31 (59.6)
41-60	3 (12)	1 (3.7)	4 (7.7)
> 60	2 (8)	1 (3.7)	3 (5.8)
Total N (%)	25 (48.1)	27 (51.9)	52 (100)

Bacterial etiologies

Of the 52 samples collected, 25 (48.1%) tested positive and 27 (51.9%) tested negative for *Staphylococcus* and *Streptococcus* species (p>0.05). Seventeen were *Staphylococcus* (32.7% of the samples) and 8 were *Streptococcus* (15.4% of the samples) strains were isolated (p>0.05). Eight *Staphylococcus* species were isolated: *Staphylococcus xylosus* (n=6; 11.5%), *Staphylococcus hominis* (n=3; 5.8%), *Staphylococcus lentus* (n=2; 3.8%), *Staphylococcus warneri* (n=2; 3.8%), *Staphylococcus saprophiticus* (n=1; 1.9%), *Staphylococcus cohnii* ssp*. cohnii* (n=1; 1.9%), *Staphylococcus haemolyticus* (n=1; 1.9%) and *Staphylococcus aureus* (n=1; 1.9%). Five *Streptococcus* species and 2 subspecies of *Streptococcus dysgalactiae* were found: *S. mitis* (n=2; 3.8%), *Streptococcus uberis* (n=1; 1.9%), *Streptococcus dysgalactiae* ssp. *dysgalactiae* (n=1; 1.9%), *Streptococcus pneumoniae* (n=2; 3.8%), *Streptococcus dysgalactiae* ssp. *equisimilis* (n=1; 1.9%) and *Streptococcus agalactiae* (n=1; 1.9%). A single case (representing 4% of the study population) of co-infection by *Streptococcus dysgalactiae* ssp. *dysgalactiae* and *Staphylococcus xylosus* was identified.

Antimicrobial susceptibility test

All *Streptococcus* strains were fully resistant to trimethoprim-sulfamethoxazole, chloramphenicol, oxacillin, cefixim, cefuroxim, cefotaxim and ceftriaxon. The *Streptococcus* strains also exhibited the following degrees of resistance to amoxicillin-clavulanic acid (83.3%), piperacillin-tazobactam (83.3%), ciprofloxacin (83.3%), penicillin G (83.3%) and amoxicillin (83.3%) (Figure 5A). The *Staphylococcus* species were largely resistant to cefixim (88.2%), piperacillin (70.6%), penicillin G (94.1%) and amoxicillin (76.5%) (Figure 5B). All the

Table 3. Dietary habits of the patients.

Age group (year)	Meat products	Dairy products	Fish products	Sweet products	Fruits and vegetables
0-6	1 (1.9)	0 (0)	0 (0)	0 (0)	0 (0)
7-12	1 (1.9)	1 (1.9)	2 (3.9)	5 (9.6)	1 (1.9)
13-18	3 (5.8)	1 (1.9)	4 (7.7)	1 (1.9)	0 (0)
19-40	10 (19.2)	2 (3.9)	14 (26.9)	5 (9.6)	0 (0)
41-60	0 (0)	1 (1.9)	3 (5.8)	0 (0)	2 (3.9)
> 60	1 (1.9)	0 (0)	1 (1.9)	0 (0)	1 (1.9)
Total N (%)	16 (30.8)	5 (9.6)	24 (46.2)	7 (13.5)	4 (7.7)

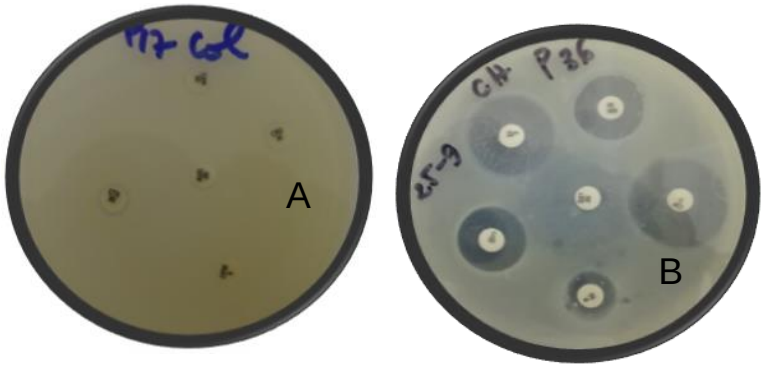

Figure 5. A: Antibiotic susceptibility of *Streptococcus* and B: susceptibility of *Staphylococcus.*

isolates were resistant to metronidazole (100%) (Table 4).

DISCUSSION

This study showed that cervicofacial cellulitis of dental origin afflicts individuals of all ages; and those in the 19-40 years-old age group in particular, as they represented 59.6% of the total patient sample (Table 2). Similar prevalence was reported by others for this same age group (46.8% of the total) (Njifou et al., 2014).

In this study, 27 women (51.9%) and 25 men (48.1%) were afflicted by cellulitis of dental origin ($p>0.05$); which is similar to the result obtained by Miloundja et al. (2011), who found that 30 women (56%) and 25 men (43%) in their patient sample were afflicted. In a similar study, carried out in Morocco, a higher prevalence of dental cellulitis was reported in men (57%) when compared with the women (43%) (Rouadi et al., 2013).

The most frequently represented age group in this study was the one that also consumed the largest percentage of meat and fish products. An earlier study conducted in Ouagadougou, Burkina Faso by Barro et al. (2005) reported that these foods products were more likely to be contaminated with *Staphylococcus* and *Streptococcus*. This could hence well explain the bacterial etiology of cellulitis of dental origin that can be greatly exacerbated by poor oral hygiene and by pre-existing carious lesions that can serve as receptacles. The 19-40 years group was comprised mainly of students and low to mid-level employees. They may pay less attention to their diet, have an affinity for fast food, and tend not to heed oral hygiene recommendations. These factors may underlie the high percentage of cellulitis in this age group.

Several authors have established that *Staphylococcus* is carried as a commensal microorganism on the skin and nasal passages of humans and animals (Hanning et al., 2012). Humans can become contaminated by these pathogens through direct contact with animals, while animal feces can also contaminate dam water intended for human consumption (Mehanned et al., 2014). There are additional studies that suggest that the risk of environmental contamination and infection in dental healthcare settings may be quite considerable (Petti et al., 2014); *S. aureus*, and its carriers, are sources of healthcare-associated infections, and these can hence occur in dental healthcare settings. Dental therapy may promote the dissemination of airborne human bacteria in

Table 4. Antibiotic susceptibility of *Streptococcus* and *Staphylococcus* strains.

Antibiotics	Susceptibility of bacteria isolated N (%)					
	Streptococcus			*Staphylococcus*		
	R	I	S	R	I	S
AUG	7 (87.5)	0(0)	1 (12.5)	8 (47.1)	0 (0)	9 (52.9)
CRO	8 (100)	0 (0)	0 (0)	8 (47.1)	4 (23.5)	5 (29.4)
CFM	8 (100)	0 (0)	0 (0)	15 (88.2)	0 (0)	2 (11.8)
CXM	8 (100)	0 (0)	0 (0)	5 (29.4)	2 (11.8)	10 (58.8)
CTX	8 (100)	0 (0)	0 (0)	5 (29.4)	6 (35.3)	6 (35.3)
CN	3 (37.5)	1 (12.5)	4 (50)	9 (52.9)	0 (0)	8 (47.1)
CD	4 (50)	1 (12.5)	3 (37.5)	11 (64.7)	2 (11.8)	4 (23.5)
LZ	8 (100)	0 (0)	0 (0)	17 (100)	0 (0)	0 (0)
TZP	7 (87.5)	0 (0)	1 (12.5)	4 (23.5)	0 (0)	13 (76.5)
OX	8 (100)	0 (0)	0 (0)	10 (58.8)	0 (0)	7 (41.2)
SP	4 (50)	2 (25)	2 (25)	7 (41.2)	7 (41.2)	3 (17.6)
MY	4 (50)	3 (37.5)	1 (12.5)	11 (64.7)	3 (17.6)	3 (17.6)
PRL	6 (75)	0 (0)	2 (25)	12 (70.6)	0 (0)	5 (29.4)
TOB	6 (75)	0 (0)	2 (25)	11 (64.7)	0 (0)	6 (35.3)
NET	5 (62.5)	1 (12.5)	2 (25)	10 (58.8)	0 (0)	7 (41.2)
E	5 (62.5)	1 (12.5)	2 (25)	10 (58.8)	3 (17.6)	4 (23.5)
SXT	8 (100)	0 (0)	0 (0)	8 (47.1)	3 (17.6)	6 (35.3)
C	8 (100)	0 (0)	0 (0)	11 (64.7)	0 (0)	6 (35.3)
CIP	7 (87.5)	0 (0)	1 (12.5)	5 (29.4)	0 (0)	12 (70.6)
P	7 (87.5)	0 (0)	1 (12.5)	16 (94.1)	0 (0)	1 (5.9)
AML	7 (87.5)	0 (0)	1 (12.5)	13 (76.5)	0 (0)	4 (23.5)

AUG = amoxicillin/clavulanic-acid, CRO = ceftriaxon, CFM = cefixim, CXM = cefuroxim, CTX = cefotaxim, CN = gentamycin, CD = clindamycin, LZ = metronidazole, TZP = piperacillin/tazobactam, OX = oxacillin, SP = spiramycin, MY = lincomycin, PRL = piperacillin, TOB = tobramycin, NET = netilmicin, E = erythromycin, SXT = trimethoprim/sulfamethoxazole, C = chloramphenicol, CIP = ciprofloxacin, P = penicillin G, AML = amoxicillin, R = resistant, I = intermediate, S = sensitive. Strains categorized as "S" are those for which the probability of therapeutic success is strong in the case of a systemic treatment with the recommended dosage provided in the summary of the product characteristics (SmPC), written by the French Agency Health Safety of Health Products (AFSSAPS). Strains categorized as "R" are those with a high probability of treatment failure regardless of the type of treatment and antibiotic's dose used. Strains categorized "I" are those with therapeutic success is unpredictable.

the environment (Messano et al., 2013). Indeed, Staphylococci (*S. aureus,* and even methicillin-resistant *S. aureus*, MRSA) have been detected with high-speed instruments during dental therapy (Kimmerle et al., 2012). Since *S. aureus*, and also occasionally MRSA, can be detected in the dental environment (Petti and Polimeni, 2012), contamination seems to be caused mostly by contact with hands. Our socio-economic data showed that low-income patients were the most frequently represented group in this study (57.7%). This result may be explained by the fact that most of these participants (n=50; 96.2%) have poor oral hygiene (p=0.0001), and that they lack the required financial resources to obtain timely treatment. This poor level of hygiene also appears to be linked to a general disregard for oral hygiene practices. The present study showed, however, that despite having poor oral hygiene, the 19-40 age group engaged in a consistent brushing regimen; with daily brushing of 2 to 3 times a day. Lack of effectiveness and irregularly brushing of the mouth could however explain this prevalence.

Microbiological analysis showed that 17 strains of *Staphylococcus* (32.7% of the samples) and 8 strains of *Streptococcus* (15.4% of the samples) were isolated from the total of 52 samples that were collected. Others have also reported the involvement of *Staphylococcus* and *Streptococcus* in cellulitis of dental origin (Miloundja et al., 2011), including some studies in Cameroon (Njifou et al., 2014; Kityamuwesi et al., 2015). Eight *Staphylococcus* species, especially *Staphylococcus warneri* were isolated in this study. As a common saprophyte of human epithelia, *Staphylococcus warneri* is frequently isolated from saliva, dental plaques and nasal swabs. Indeed, it represents the third most prevalent coagulase-negative *Staphylococcus* species after *S. epidermidis* and *S. hominis* (Ohara et al., 2008). In light of the progressive refinement of identification techniques over the last three decades, *S. warneri* has increasingly emerged as a new pathogenic species that is capable of causing serious infections, usually in association with the presence of

implant materials (Campoccia et al., 2010). The mouth, by virtue of its constant temperature, and the presence of many food fragments and metabolites, is an ideal culture medium for these bacterial species. Thus, poor oral hygiene readily permits the multiplication of oral microbiota (Lam et al., 2012).

All *Streptococcus* strains were fully resistant to trimethoprim-sulfamethoxazole, chloramphenicol, oxacillin, cefixim, cefuroxim, cefotaxim and ceftriaxon. The *Staphylococcus* species were largely resistant to cefixim (88.2%), piperacillin (70.6%), penicillin G (94.1%) and amoxicillin (76.5 %). All isolates (*Streptococcus*, *Staphylococcus*) were resistant to metronidazole (100%). Similar to what has been reported in previous studies (Oberoi et al., 2015), the *Streptococcus* and *Staphylococcus* isolates in this study were highly resistant to β-lactam antibiotics. β-Lactam antibiotics are a major class of antibiotics that are used widely in clinical practice. Development of antibiotic resistance in bacteria is a natural phenomenon, but high-level resistance is exacerbated by the overuse of antibiotics (Oberoi et al., 2015). Furthermore, resistant strains appear to be the dominant forms, and this is the result of selection pressure following exposure to the antibiotic (Oberoi et al., 2015). *Staphylococcus* and *Streptococcus* strains are opportunistic pathogens, commensal on the human body. Yet, this study revealed that these strains are not only involved in cellulitis of dental origin in Burkina Faso, also they exhibit multi-resistance to common antibiotics. This indicates that this burgeoning problem needs to be given due consideration by healthcare policymakers.

Metronidazole is an anti-parasitic and antibiotic agent that is used to treat infections caused by parasites and obligate anaerobic bacteria (Audu et al., 2012). The resistance to metronidazole reported in the strains isolated in this study may be due to the fact that these strains are not obligate anaerobes. 'Natural resistance' is a chromosomal property, present in all strains of the same species or the same bacterial genus, which influences sensitivity towards an antibiotic. *Streptococcus* is naturally resistant to sodium azide, crystal violet, nalidixic acid, polymixims and aminoglycosides (low level natural resistance). Natural resistance in *Staphylococcus* is rare, although natural resistance to quinolones does occur. The isolates from this study (*Staphylococcus* and *Streptococcus*) were resistant to metronidazole, which is an antibiotic and antiparasitic agent that belongs to the nitroimidazoles group. It could be possible that the strains exhibited natural resistance to low-doses (for example, 5 µg) of metronidazole.

Conclusion

The present study further showed that all cases of cervicofacial tumefactions should receive thorough medical attention. Cellulitis of dental origin is caused by the proliferation of aerobic and anaerobic bacteria present in the oral flora, and it is generally initiated by decay or pulpar necrosis. In this study, it was observed that *Staphylococcus* strains were the most frequently involved in acute cellulitis, albeit with acceptable levels of antibiotic susceptibility. On the other hand, although *Streptococcus* strains were less often involved, they were resistant to a greater diversity of antibiotics. Fortunately, accurate diagnosis combined with efficient antibiotherapy and surgical treatment (avulsion of the causal tooth and purulent collection's drainage) enables healthcare professionals to achieve a cure in most cases.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Effect of an autochthonous starter culture, including lactococci and *Geotrichum candidum* strains, on the ripening of a semi-hard goat's milk cheese

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Four batches of Armada semi-hard goat cheese were elaborated from pasteurized milk inoculated with a natural starter culture, constituted of two lactococci strains, combined with a *Geotrichum candidum* strain. The four *G. candidum* strains tested as co-starter were selected for their proteolytic and lipolytic activities. The effects of an autochthonous starter on physico-chemical, microbiological and sensorial characteristics during the ripening of this cheese were evaluated. The depth of proteolysis was very low, which confirms the presence of low-level aminopeptidase activity. Fungal population was involved in lipolysis. The cheeses elaborated with *G. candidum* strains developed a desirable flavour characteristic of goat cheese and a creamy texture. Cheeses from batch I (that included the strain of *G. candidum* with high lipolytic activity and low proteolytic activity) presented a hardness profile that differed from the others, as it was the batch with the highest scores at thirty days of ripening and was even the best evaluated at the end of ripening because of their odour, fresh balanced pleasant taste and creamy smooth texture.

Key words: Cheese, starter culture, *Geotrichum candidum*, lactic acid bacteria.

INTRODUCTION

The autochthonous microbiota of traditionally made cheeses elaborated from raw milk gives them their particular characteristics, and thus constitute an excellent source of new strains of microbes with phenotypic and genotypic diversity, which could be of technological interest (Rademaker et al., 2007). This microbial diversity is responsible for the production of compounds giving rise to flavour during ripening (Coolbear et al., 2008). The activity of the starter which is made up of lactic acid bacteria and could include strains producing aroma, could be reinforced by adding a secondary microbiota that will contribute to the ripening process of the cheeses. Non-starter yeast and molds (NSYM) population in cheese is very diverse and its role in the ripening is often

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underestimated (Lavoie et al., 2012).

The importance of using the filamentous yeast-like fungus, *Geotrichum candidum* as a co-starter in dairy products is well known because of its various properties related to the development of flavour and texture in semi-hard cheeses (Gaborit et al., 2001). In fact, in recent years, there has been increasing interest in the use of this microorganism, especially in the manufacturing of cheeses from pasteurized milk in order to reproduce the characteristics of cheese made from raw milk (Boutrou and Guéguen, 2005). *G. candidum* was the dominant yeast species in the first and second week of Armada ripening (Fresno et al., 1996; Tornadijo et al., 1998). Then, it could have important contributions to the flavour and texture of this one or similar cheeses. Nonetheless, *G. candidum* strains differ in their biochemical capacity to produce aromatic compounds in dairy products (Spinnler et al., 2001), which require a selection process on the basis of their technological suitability. In an earlier study, several properties of technological relevance were studied in *G. candidum* and the strains with greater technological capacities were characterized at a molecular level (Sacristán et al., 2012, 2013).

The aim of this study was to investigate the contribution of a natural starter composed of strains of *Lactococcus* and *G. candidum* as co-starter on the chemical, microbiological and sensorial characteristics of a goat's milk cheese. The *G. candidum* strain that provides the best sensorial properties was selected to be included as co-starter in the Armada cheese manufacture.

MATERIALS AND METHODS

Cheese manufacture and sampling

All the strains used in the cheese manufacturing were isolated from a traditional Armada cheese elaborated by the artisanal cheese-makers themselves (Tornadijo et al., 1995). Lactic acid bacteria were characterized from a technological point of view including acidifying activity and proteolytic and lipolytic activities and selected in order to obtain a starter (Herreros et al., 2003; 2007). *Lactococcus lactis* subsp. *lactis lactis* (TAUL 1292) was selected because of its acidifying capacity and its proteolytic activity, and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (TAUL 12) for its high dipeptidase activity and its capacity to produce aroma compounds. The *G. candidum* strains were selected for their proteolytic and lipolytic activities (Sacristán et al., 2012).

Four batches of Armada cheese were manufactured by duplicate (8 batches in total) using the traditional method (Tornadijo et al., 1995). The goat's milk used for the manufacture of a total of 32 cheeses (4 cheeses per batch) was pasteurized and inoculated with an autochthonous starter culture composed of *Lactococcus lactis* subsp. *lactis* (TAUL 1292) and *L. lactis* subsp. *lactis* biovar. *diacetylactis* (TAUL 12) at a level of 0.5% for each, and *G. candidum* as co-starter, inoculated at 1%. Batch I included the *G. candidum* Ge-1886 strain with high lipolytic activity and low proteolytic activity. Ge-1903 strain (with low lipolytic activity and high proteolytic activity) was added in batch II. Batch III incorporated the Ge-1889 strain with both lipolytic and proteolytic activity at a high level. Ge-1893 strain (with both lipolytic and proteolytic activities at an intermediate level) was used in batch IV.

Table 1. Autochthonous starter cultures used in the manufacture of the Armada cheeses.

Batches[a]	Autochthonous starter culture strains[b]
Control batch	TAUL 12 + TAUL 1292
Batch I	TAUL 12 + TAUL 1292 + Ge-1886
Batch II	TAUL 12 + TAUL 1292 + Ge-1903
Batch III	TAUL 12 + TAUL 1292 + Ge-1889
Batch IV	TAUL 12 + TAUL 1292 + Ge-1893

[a]All the batches were manufactured from pasteurized goat's milk; [b]All the strains were isolated from the artisanal Armada cheese; *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* TAUL 12 was selected because of its acidifying capacity and its proteolytic activity; *L. lactis* subsp. *lactis* TAUL 1292 was selected for its high dipeptidase activity and its capacity to produce aroma compounds; *Geotrichum candidum* Ge-1886 was selected because of its high lipolytic activity and low proteolytic activity; *G. candidum* Ge-1903 was selected for its low lipolytic activity and high proteolytic activity; *G. candidum* Ge-1889 was selected because of its both high lipolytic and proteolytic activities; *G. candidum* Ge-1893 was selected for its both intermediate lipolytic and proteolytic activities.

The control batch was composed of the Armada cheese elaborated with the LAB strains (Herreros, 2010) (Table 1).

After thirty minutes of the inoculation of the starter cultures, 10 mL of commercial calf rennet (1:10000 strength) were added to every batch (100 L of milk), which was left to coagulate for about 2 h at room temperature (20 to 25°C). The curd was then cut and transferred to cheese-cloths where the whey was drained off over a period of 48 h. The curd was then kneaded in a very rigorous manual operation called "Sobado". Salting was carried out by adding dry salt (1.68%; w/w) to the curd during the second kneading process, and finally the curd was hand-moulded to produce its characteristic square shape. Subsequently, the cheeses were placed on a plate for four days at 10°C and 90% relative humidity. The ripening process took place over 60 days at 11°C and 86% relative humidity.

Milk as well as 2-, 15-, 30- and 60-day-old cheese samples were taken from each batch. Each sample was made up of one cheese and was analysed as specified as follows.

Microbiological analysis

Samples for microbiological analysis of milk and cheese were prepared according to the International Dairy Federation (IDF) standard 122B (IDF, 1992).

Aerobic mesophilic bacteria were enumerated on standard plate count agar (PCAm) (Oxoid, Unipath Ltd., Basingstoke, U.K.) following the APHA method (APHA, 1978) after incubation at 30°C for 48 h. General population of lactic acid bacteria (LAB) were determined on MRS agar (De Man et al., 1960), after incubation at 30°C for 72 h; lactococci were counted on the M17 agar (Biokar, Beauvais, France) incubated at 30°C for 18 to 24 h (Terzagui and Sandine, 1975) and lactobacilli were counted on ROGOSA agar (Oxoid, Unipath Ltd., Basingstoke, U.K.) after incubation at 30°C for 5 days (Rogosa et al., 1951). Yeasts and moulds were counted on oxytetracycline glucose yeast extract agar (OGYEA) (Oxoid, Unipath Ltd., Basingstoke, U.K.) after incubation at 22°C for 5 days (Mossel et al., 1970). Enterobacteriaceae were enumerated on violet red bile glucose agar (VRBGA) (Oxoid, Unipath Ltd., Basingstoke, U.K.) after incubation at 37°C for 18 to 24 h (Mossel et al., 1962).

Table 2. Physico-chemical parameters (average values ± standard deviation) throughout ripening of Armada cheeses elaborated with an autochthonous starter culture.

	Ripening time (days)				Batch	Time
	2	15	30	60		
Moisture [a]	64.60 ± 2.86	50.00 ± 2.43	40.46 ± 3.01	27.55 ± 2.23	**	***
a_w	0.995 ± 0.002	0.972 ± 0.003	0.959 ± 0.005	0.929 ± 0.010	NS	***
Salt [b]	0.54 ± 0.09	3.08 ± 0.29	2.99 ± 0.16	3.28 ± 0.48	**	***
S/M [c]	0.30 ± 0.05	3.08 ± 0.23	4.42 ± 0.46	8.65 ± 1.21	*	***
pH	4.94 ± 0.20	4.61 ± 0.29	4.51 ± 0.30	4.56 ± 0.09	NS	***
TA [d]	2.22 ± 0.24	1.56 ± 0.18	1.12 ± 0.16	0.90 ± 0.14	NS	***
Lactose [b]	5.23 ± 0.98	2.94 ± 0.76	2.21 ± 0.63	1.57 ± 0.48	**	***
FAI [e]	1.96 ± 0.48	5.86 ± 2.73	1.93 ± 0.65	1.74 ± 0.54	NS	***
Fat [b]	56.68 ± 5.47	59.10 ± 3.06	60.64 ± 2.36	59.71 ± 1.63	NS	NS
Protein [b]	32.57 ± 2.48	28.85 ± 0.65	28.98 ± 1.11	28.81 ± 0.86	NS	***

The last two columns are referred to the significant differences between batches and between the ripening time. NS: no significant differences; *: significant differences ($p < 0.05$); **: significant differences ($p < 0.01$); ***: significant differences ($p < 0.001$); [a]Expressed as g 100 g^{-1} of cheese; [b]Expressed as g 100 g^{-1} of dry matter; [c]expressed as g salt 100 g^{-1} of moisture; [d]expressed as g lactic acid 100 g^{-1} of dry matter; [e]expressed as mg KOH g^{-1} of fat.

Cheese physico-chemical analysis

The contents of dry matter (DM), fat, protein, lactose and salt were determined in cheese according to standard methods (IDF, 2004, 2008, 2001, 1967; AOAC, 1990a).

The pH was determined potentiometrically from samples homogenized with 100 mL of distilled water warmed at 45-50°C so as to disperse the fat and then cooled down to 20°C ± 2°C. Water activity (a_w) was measured using an Aqua Lab CX-2 water activity meter (Decagon, WA, USA). The titratable acidity of the cheese and the fat acidity index (FAI) were determined according to standard methods (AOAC, 1990b; IDF, 1969).

Extraction and quantification of pH 4.4 soluble nitrogen (pH4.4-SN), trichloroacetic acid soluble nitrogen (TCA12%-SN) and phosphotungstic acid soluble nitrogen (PTA5%-SN) fractions were carried out using the method described by Bütikofer et al. (1993). All analyses were carried out in duplicate.

Sensory analysis

Cheeses were analysed after 15, 30 and 60 days of ripening by a panel of 20 trained tasters following the standard recommendations (ISO, 2005; 2012). Several parameters, related to appearance (mouldy rind, yellowish rind, white paste, mouldy spotted paste and cracked paste), taste (bitter taste, sweet taste, acid taste, salty taste, metallic taste, spicy taste, astringency, aftertaste and persistence), odour (fresh milky odour, mouldy odour, rennet odour, buttery odour and farmyard odour) and texture (hardness, buttery texture, grainy texture, crumbly texture and sticky texture) were evaluated on a 7-point intensity scale from 1 (dislike extremely) to 7 (like extremely), with 4 being an “acceptable” value. Finally, the cheeses were scored from 1 to 10 on the the basis of overall sensory impression.

Statistical analysis

In order to investigate possible significant differences among batches, the ANOVA/MANOVA analysis using Fisher's least significant difference (LSD) test (Statistica 8.0 computer program: Statsoft, Tulsa, Oklahoma, U.S.A.) was carried out with the confidence intervals set at 95% level and other higher levels (99 and 99.9%). The correlation between changes in the physico-chemical parameters and the log counts of the major microbial groups was also studied by the Pearson’s correlation coefficient, so as to discover what influence the ripening process had on microbial development.

RESULTS AND DISCUSSION

Changes in physico-chemical parameters

Changes in chemical and physico-chemical parameters during the ripening of the different batches of Armada cheese elaborated with an autochthonous starter culture and various selected strains of *G. candidum* as a co-starter are shown in Table 2. The changes throughout ripening time of the physico-chemical parameters which showed significant differences between batches are also shown in Figure 1.

Over the ripening process, there was a notable decrease in moisture and water activity. The final values for moisture even fell below 30%, with significant differences ($p < 0.01$) between batches on different sampling days.

The values for the relationship between salt and moisture (S/M) after two days of ripening were practically identical for all the batches, rising over the whole ripening period, in particular up to fifteen days and after sixty days of ripening. With regard to the influence from the specific batch, significant differences ($p < 0.05$) were noted, especially in samples taken after sixty days. In all cases, the results obtained for S/M ratio at the end of the ripening process were similar to those observed by Herreros (2010).

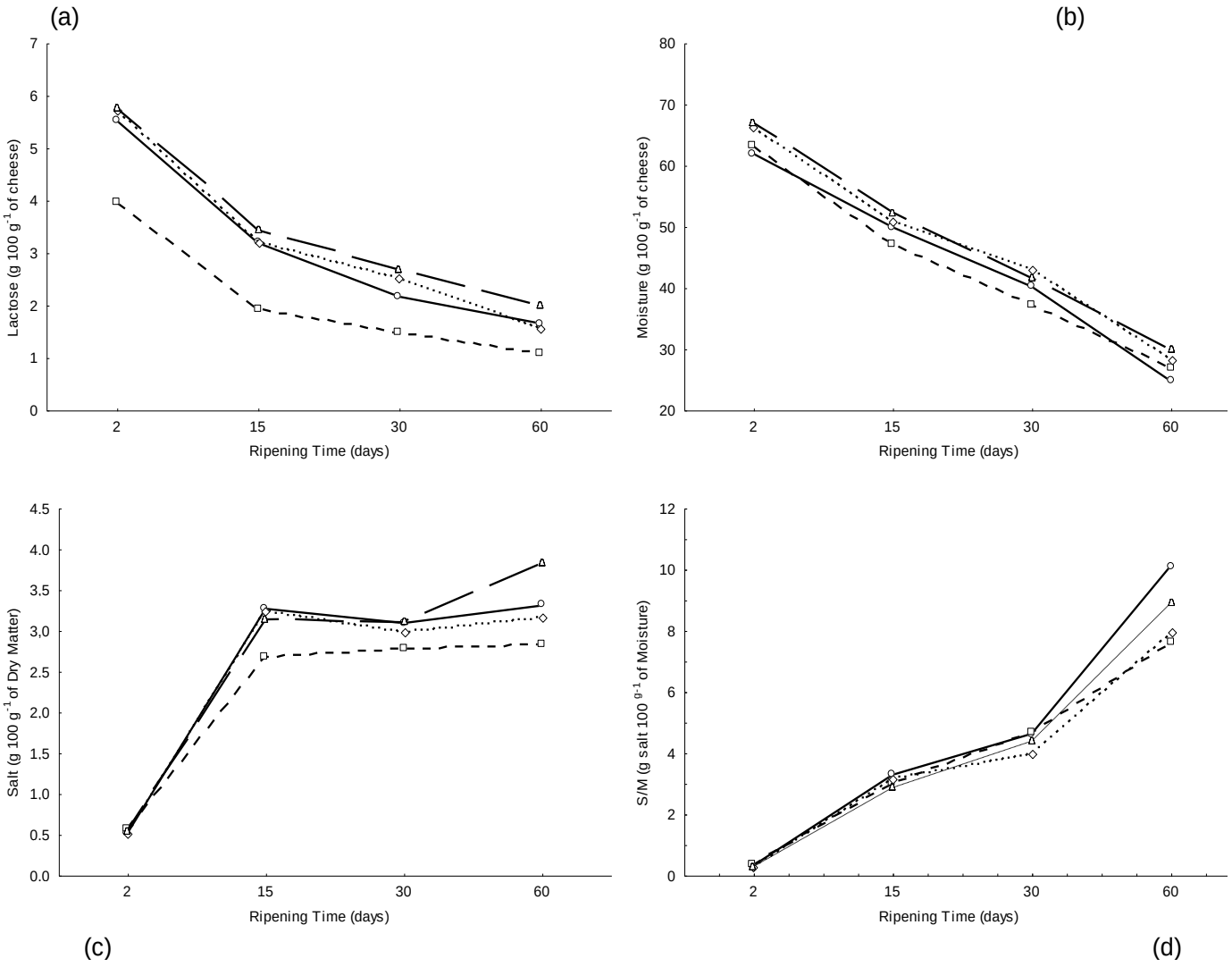

Figure 1. Changes in the physico-chemical parameters which showed significant differences between batches throughout the ripening time of Armada cheeses manufactured using the autochthonous starter cultures. Batches of cheeses were made with: batch I (-○-), Ge-1886; batch II (-□-), Ge-1903; bath III (-◊-), Ge-1889; batch IV (-Δ-), Ge-1893.

The use of *Lactococcus lactis* subsp. *lactis* (TAUL 1292) strain selected for its acidifying and proteolytic activity (Herreros et al., 2003), caused a very marked fall in pH at early stages. The slight increase in pH observed after sixty days of ripening could have been due to the capacity of *G. candidum* to metabolize lactic acid, which would favour the implantation of another type of microbiota (Cosentino et al., 2001; Fadda et al., 2004). Titratable acidity (TA), expressed as g of lactic acid 100 g^{-1} of DM, decreased during the ripening ($p < 0.001$), with no significant differences between batches. With regard to changes in lactose, significant differences ($p < 0.01$) were noted between the different batches. The lactose content in the curds and at the start of the ripening was similar to the content observed in Armada cheeses manufactured from pasteurized milk and the LAB strains (Herreros, 2010). The lactose content dropped sharply up to the fifteenth day of ripening. This decline is attributed to the rapid development of the lactic microbiota included in the starter, favoured by the low salt to moisture ratio (S/M) in two-day-old cheese. These results agree with those obtained by Herreros (2010) in batches made form pasteurized goat's milk and the LAB strains.

The FAI showed no significant differences between batches. However, there were significant differences ($p < 0.001$) as a function of ripening time, with much higher values after fifteen days. The presence of cracks in the cheeses, especially after fifteen days of ripening, favoured the growth of fungi and hence that of lipolysis. After the fifteenth day of ripening, a fall in the FAI was observed until the end of ripening. The lipoprotein lipase in milk is deactivated by pasteurization and the addition

Table 3. Nitrogen fractions content (average values ± standard deviation)[a] throughout ripening of Armada cheeses elaborated with an autochthonous starter culture.

Nitrogen fractions	Ripening time (days)				Batch	Time
	2	15	30	60		
pH4.4-SN	12.18 ± 2.23	12.69 ± 1.85	12.47 ± 1.30	12.29 ± 1.49	NS	NS
TCA12%-SN	2.38 ± 0.64	3.35 ± 0.93	3.43 ± 0.66	3.41 ± 0.48	*	**
PTA5%-SN	0.13 ± 0.15	0.40 ± 0.54	0.68 ± 0.49	0.98 ± 0.55	***	***
Polypeptide N	9.80 ± 2.60	9.34 ± 2.33	9.04 ± 1.70	8.88 ± 1.84	NS	NS
Peptide N	2.25 ± 0.57	2.95 ± 0.71	2.76 ± 0.81	2.42 ± 0.74	NS	NS

[a]Values expressed as g 100 g^{-1} of total nitrogen; pH4.4-SN: pH 4.4 soluble nitrogen; TCA12%-SN: trichloroacetic acid soluble nitrogen; PTA5%-SN: phosphotungstic acid soluble nitrogen; Polypeptide N: Polypeptide nitrogen; Peptide N: peptide nitrogen. The last two columns are referred to the significant differences between batches and between the ripening time. NS: no significant differences; *: significant differences ($p < 0.05$); **: significant differences ($p < 0.01$); ***: significant differences ($p < 0.001$).

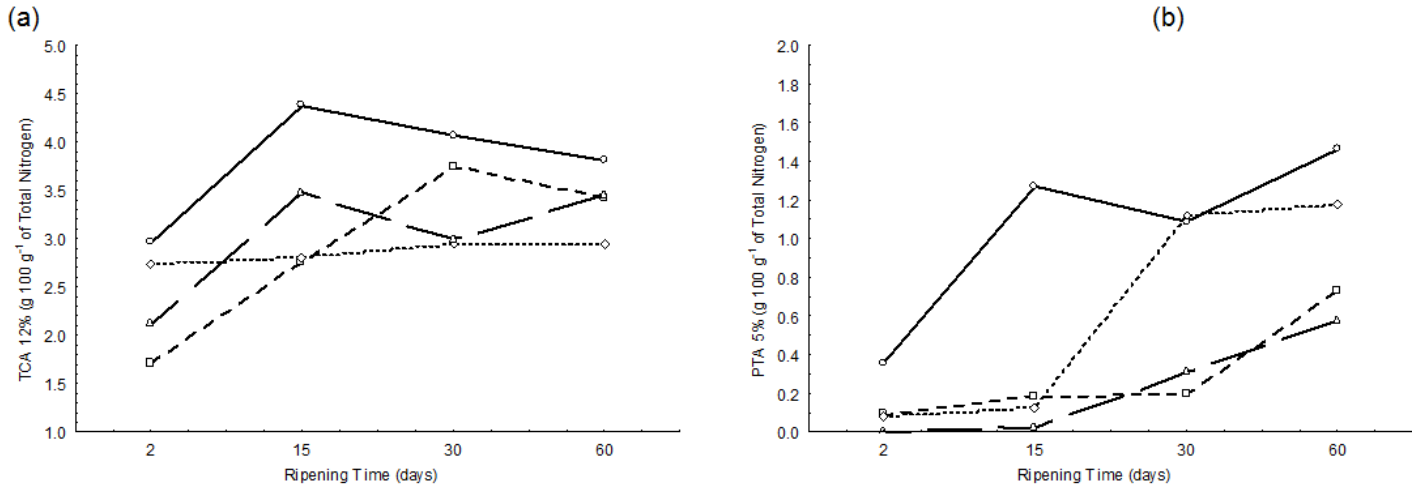

Figure 2. Changes in the nitrogen fractions content which showed significant differences between batches throughout the ripening time of Armada cheeses manufactured using the autochthonous starter cultures. Batches of cheeses were made with: batch I (-○-), Ge-1886; batch II (-□-), Ge-1903; bath III (-◊-), Ge-1889; batch IV (-Δ-), Ge-1893.

of a starter culture triggers off a marked decrease in pH at the beginning of ripening, which could negatively affect the lipases present.

The protein content decreased slightly during the first two weeks of ripening, and then remained stable until the end of the process. Significant differences were then observed with regard to the ripening time ($p < 0.01$), but in contrast there were no significant differences between batches (Table 2). In order to determine the degree of proteolysis in cheese, the changes in different nitrogen fractions were evaluated (Table 3). The nitrogen fractions which showed significant differences between batches were also illustrated in Figure 2.

The formation of pH 4.4 soluble nitrogen during the ripening of the cheese is mainly due to the rennet action on caseins and, to a lesser extent, to the plasmin and acid protease action in milk. No significant differences were found as a function of the batch or ripening time in the batches of Armada cheese elaborated using the various *G. candidum* strains. The pH 4.4-SN values remained stable at about 12.4%, which indicates that only slight proteolysis took place. These results were very similar to those reported by Herreros (2010) for Armada cheeses elaborated from pasteurized milk and the LAB strains. With regard to the trichloroacetic acid soluble nitrogen, significant differences were noted during ripening ($p < 0.01$). The TCA12%-SN increased slightly up to thirty days, but then stabilized. Significant differences ($p < 0.05$) were also observed between the various batches. The TCA12%-SN, measures the depth of proteolysis and it is nevertheless an indicator of proteolytic activity due to the microbiota present in cheese. In the case of phosphotungstic acid soluble nitrogen (PTA5%-SN), significant differences ($p < 0.001$) were found with regard to both ripening time and the various manufactured batches. In all cases, the average

Table 4. Microbial counts (average values ± standard deviation)[a] throughout manufacture and ripening of Armada cheeses made with an autochthonous starter culture.

	Milk	Cheese (days of ripening)				Batch	Time
		2	15	30	60		
PCAm (aerobic mesophilic bacteria)	7.04 ± 0.05	9.91 ± 0.56	9.27 ± 0.29	8.33 ± 0.39	6.11 ± 1.67	NS	***
MRS (lactic acid bacteria)	7.05 ± 0.07	9.76 ± 0.19	9.19 ± 0.19	8.37 ± 0.48	6.21 ± 1.78	*	***
M17 (Lactococci)	6.99 ± 0.27	9.82 ± 0.38	9.38 ± 0.34	8.37 ± 0.33	6.07 ± 0.94	*	***
ROGOSA (Lactobacilli)	0.11 ± 0.30	0.63 ± 0.95	3.37 ± 1.59	5.15 ± 1.17	5.42 ± 0.98	*	***
VRBGA (Enterobacteriaceae)	0.81 ±1.01	1.00 ±1.51	0.17 ± 0.24	0.74 ± 1.40	0.00 ± 0.00	**	**
OGYEA (yeasts and moulds)	2.52 ± 0.30	3.30 ± 0.30	5.88 ± 0.63	4.01 ± 1.91	3.60 ± 0.81	NS	***

[a]Microbial counts are expressed as log CFU g^{-1}; the last two columns are referred to the significant differences between batches and between the ripening time. NS: no significant differences; *: significant differences ($p < 0.05$); **: significant differences ($p < 0.01$); ***: significant differences ($p < 0.001$).

values detected at each sampling point were very low, which confirms the presence of low-level aminopeptidase activity. These results were lower than those found by Herreros (2010) in the same type of cheese elaborated without the addition of the relevant *G. candidum* strains. Polypeptide nitrogen (Polypeptide N), calculated from the difference between pH4.4-SN and TCA12%-SN, decreased over the ripening process, but without any significant differences being noted either in ripening time or in batches. As for peptide nitrogen (peptide N), calculated from the difference between TCA12%-SN and PTA5%-SN, no significant differences were seen either with regard to ripening time or between the batches (Table 3).

Changes in the main microbial group counts

Changes in the microbial counts during the ripening process of the various batches of Armada cheese are shown in Table 4. The microbial counts which showed significant differences between batches are also illustrated in Figure 3.

Most of the microbial groups had an increase of approximately two logarithmic units in two-day-old cheese due to the physical retention of microorganisms in curds, their multiplication during coagulation and drainage and the delay of salting process in this cheese. The highest counts of mesophilic aerobic microorganisms (PCAm) were recorded after two days of ripening, but thereafter there was a gradual decline in counts until at the end of ripening. The concentration of NaCl dissolved in the moisture in cheese, which increased during ripening, has an inhibitory effect on microorganisms (Beresford et al., 2001). All the batches showed a very similar evolution and no significant differences were observed with regard to the batch in the counts of mesophilic aerobic bacteria. Nevertheless, there were significant differences with regard to ripening time ($p < 0.001$). The counts recorded were similar to those in Armada cheese elaborated from pasteurized goat's milk using the same lactic acid bacteria strains as starter culture (Herreros, 2010).

The counts observed on MRS and M17 agar followed a pattern practically identical to that observed on PCAm. On both culture media, significant differences were observed with regard to both ripening time ($p < 0.001$) and batch ($p < 0.05$) up to sixty days of ripening. Counts were also similar to those reported for other goat and cow's cheeses (Arenas et al., 2004; González et al., 2003; Herreros et al., 2007). Counts on M17 agar reached their highest values between two and fifteen days of ripening because *Lactococcus* break down lactose and their counts increase rapidly (Williams et al., 2000). Then, slowly dropped until the end of ripening at which point significant differences were noticeable between batches. A significant positive correlation ($p < 0.01$) was found in the *Lactococcus* on M17 counts with regard to the a_w levels ($r = 0.88$) and moisture ($r = 0.86$) (Table 5).

Lactobacilli count on ROGOSA agar increased progressively as ripening proceeded ($p < 0.001$), with significant differences ($p < 0.05$) being observed between batches. The slower lactobacilli metabolism and its greater capacity to adapt to adverse conditions (acidity, low values for a_w or high NaCl concentrations) related to other LAB could contribute to its increasing predominance as the number of days of ripening increases. In fact, there was a significant negative correlation ($p < 0.01$) between the developments in counts on ROGOSA agar and titratable acidity ($r = -0.52$), a_w ($r = -0.45$) and moisture ($r = -0.45$). On the other hand, a positive correlation ($p < 0.05$) was found between the ROGOSA agar counts and the S/M ratio ($r = 0.39$) (Table 5).

Significant differences were detected in VRBGA medium with regard to the batch ($p < 0.01$) and ripening time ($p < 0.01$). After fifteen days of ripening, the counts for Enterobacteriaceae decreased up to undetectable count, and eventually they completely disappeared owing to the unfavourable growth conditions which gradually

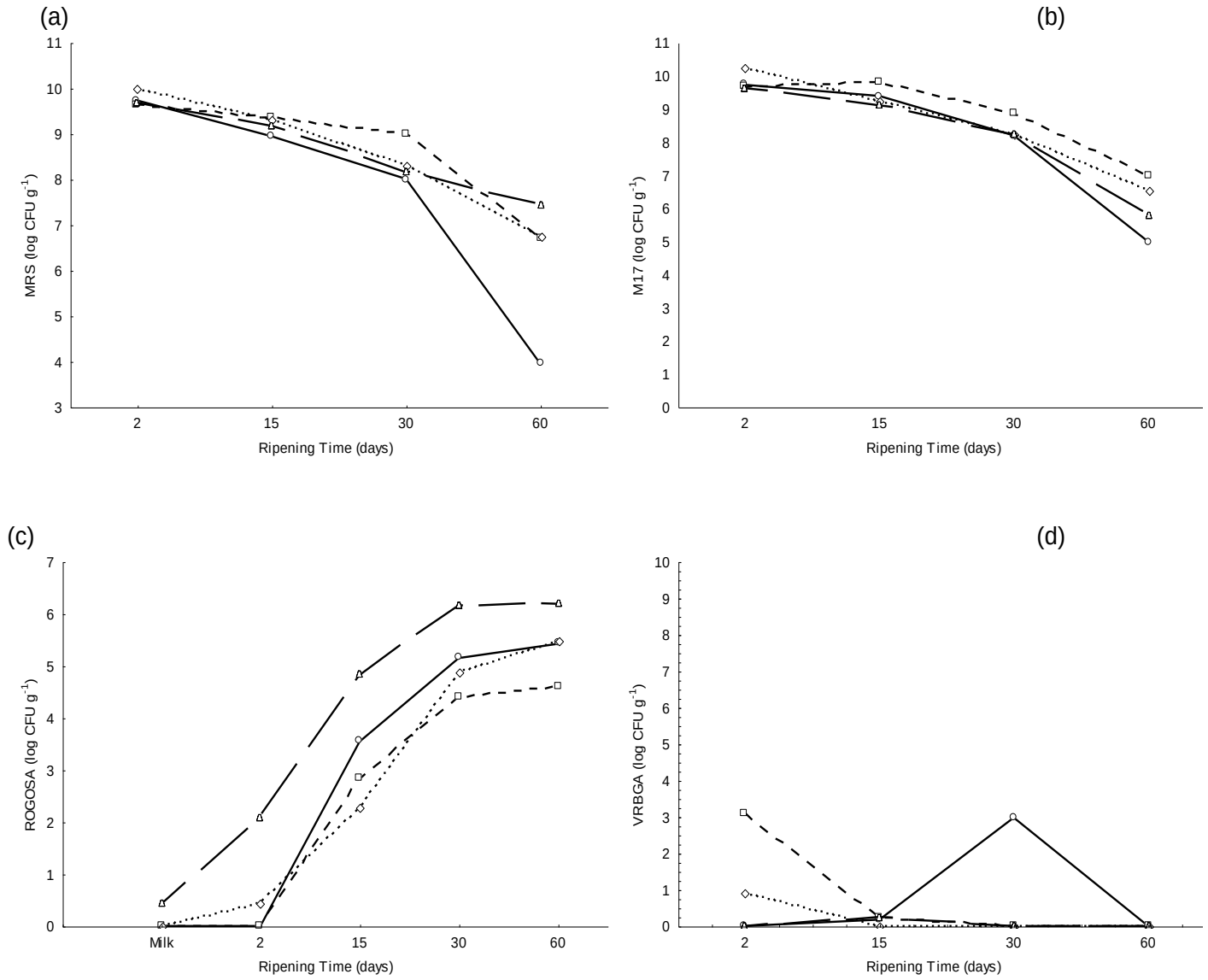

Figure 3. Changes in the microbial counts which showed significant differences between batches throughout the ripening time of Armada cheeses manufactured using the autochthonous starter cultures. Batches of cheeses were made with: batch I (-○-), Ge-1886; batch II (-□-), Ge-1903; bath III (-◊-), Ge-1889; batch IV (-Δ-) and Ge-1893.

prevailed in the cheese as this process develops (Buffa et al., 2001). The detection of Enterobacteriaceae in the cheeses of one of the batches after thirty days of ripening may have been due to later contamination.

Counts for moulds and yeasts on OGYEA medium increased up to fifteen days of ripening, when they reached their highest levels. They then decreased until the end of ripening. The evolution of this microbial group was similar in all batches manufactured. However, there were significant differences ($p < 0.001$) with regard to ripening time. *G. candidum* was the predominant fungal species during the manufacturing and ripening of the cheeses elaborated from the lactic starter culture and the *G. candidum* co-culture. Thus, OGYEA counts constitute an indicator of changes undergone by *G. candidum* during the ripening. The highest counts were reached after fifteen days of ripening, without apparent inhibition of growth. Interactions of *G. candidum* and starter culture were reported by other authors (Šípková et al., 2015). From here on, the counts fell more or less steeply depending on the batch, until after sixty days when they reached values similar to those at the start of the ripening.

Lipolytic activity by LAB is generally very limited. Their lipases act primarily on mono- and di-glycerides previously formed by indigenous lipases of the milk, and their capacity to act on triglycerides is very slight (El Soda et al., 1986). Positive correlation between the values for

Table 5. Correlations between the microbiological and physico-chemical parameters in the manufacture of Armada goat's cheeses.

	Culture medium				
	M17	**MRS**	**ROGOSA**	**OGYEA**	**PCAm**
Moisture	0.86[a]	0.80[a]	-0.45[a]	0.01	0.82[a]
a_w	0.88[a]	0.80[a]	-0.45[a]	0.07	0.83[a]
S/M	-0.92[a]	-0.84[a]	0.39[a]	-0.07	-0.85[a]
TA	0.77[a]	0.73[a]	-0.52[a]	-0.11	0.73[a]
Lactose	0.63[a]	0.58[a]	-0.48[a]	-0.16	0.63[a]
FAI	0.37[a]	0.29	-0.08	0.64[a]	0.31

[a]Marked correlations are significant at $p < 0.05$.

Table 6. Overall sensory impression[a] throughout ripening of the Armada cheeses elaborated with an autochthonous starter culture.

Batches[b]	**Ripening time (days)**		
	15	**30**	**60**
I	6.8 ± 0.22	6.9 ± 0.29	7.3 ± 0.90
II	6.2 ± 0.33	6.1 ± 0.14	6.0 ± 0.40
III	6.2 ± 0.48	6.2 ± 0.39	6.5 ± 0.02
IV	6.5 ± 0.35	7.6 ± 0.20	6.5 ± 0.73

[a] Average values evaluated on a scale running from 1 to 10 by a panel of 20 tasters; [b] The batches were elaborated with four starter cultures: Starter batch I: *Lactococcus lactis subsp. lactis* (TAUL 1292), *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (TAUL 12) and *G. candidum* Ge-1886 strain. Starter batch II: TAUL 1292, TAUL 12 and *G. candidum* Ge-1903 strain. Starter batch III: TAUL 1292, TAUL 12 and *G. candidum* Ge-1889 strain. Starter batch IV: TAUL 1292, TAUL 12 and *G. candidum* Ge-1893 strain.

the FAI and the counts obtained for OGYEA shows the involvement of fungal population in lipolysis (Table 5).

Sensory evaluation

The overall sensory evaluation of the Armada cheeses elaborated by adding an autochthonous starter culture and co-starter culture of *G. candidum* is shown in Table 6. These cheeses developed a notable flavour characteristic of goat cheese, which gives individuality and quality to this type of cheese (Gaborit et al., 2001). In fact, flavour is one of the main sensory attributes of cheese's quality (Zabaleta et al., 2015). The evaluation of the texture and taste parameters in cheeses after sixty days of ripening is shown in Figure 4.

With regard to the overall sensory evaluation of the manufactured cheeses, no significant differences could be seen throughout the ripening period, but there were significant differences ($p < 0.05$) with regard to batches. Batches I and IV received the best scores after sixty and thirty days of ripening, respectively.

Because of the particular techniques used in the manufacturing of these cheeses, in which salting is carried out after two days, kneading stage was required, and a dry grainy texture is a constant in cheeses after fifteen and thirty days of ripening. This was also seen in the cheeses elaborated with pasteurized milk using commercial and autochthonous starter culture in the test carried out by Herreros (2010). Generally, as the ripening process went on, hardness increased ($p < 0.001$) and soft buttery texture decreased ($p < 0.001$), as did the stickiness of the cheese paste in the mouth ($p < 0.05$). Batch I presented a hardness profile that differed from the others, as it was the batch with the softest texture after fifteen and thirty days as well as had the greatest increase in hardness. Stickiness decreased in all the batches, although somewhat irregularly, depending on the batch.

The fresh milky odour characteristic of these cheeses declined slightly during the ripening period ($p < 0.01$), with significant differences observed among batches ($p < 0.05$). Another pleasant attribute of these cheeses was a buttery odour, which increased slightly over the ripening period in all the cheeses. A rennet odour and a farmyard odour were two negative attributes of some of these

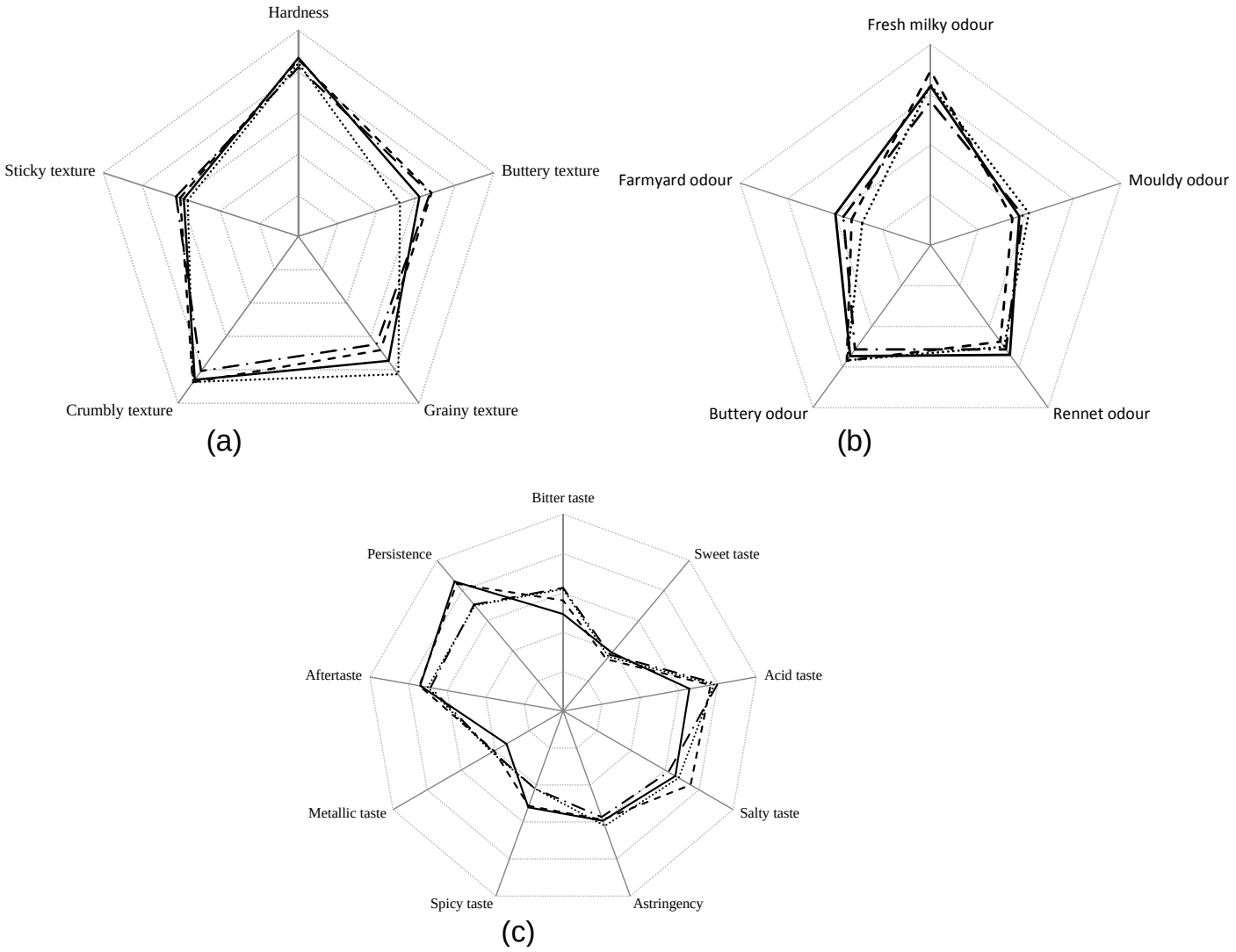

Figure 4. Sensorial evaluation of texture (a), odour (b) and taste (c) parameters of Armada cheese at 60 days of ripening. Parameters evaluated on a 7-point intensity scale. (**-**) Batch I, (**⋯**) Batch II, (**-·-**) Batch III, (**---**) Batch IV.

cheeses, with significant differences ($p < 0.05$) arising as ripening progressed. In general, the two unpleasant parameters were observed more in thirty-day-old cheeses, then decreased after sixty days of ripening.

The cheeses were characterized by a fresh acid taste which decreased somewhat during the ripening period ($p < 0.05$). The desirable odour and aftertaste of goat which developed was a parameter for quality in these cheeses, associated with *G. candidum*. In fact, these cheeses received higher scores than those elaborated from pasteurized milk without the addition of *G. candidum* culture. Astringency was also affected by ripening time ($p < 0.01$), increasing from the fifteenth to the sixtieth day. The spicy taste characteristic of these cheeses also increased during ripening ($p < 0.05$). A further parameter that was affected was the persistency on the palate, which notably increased after sixty days of ripening period ($p < 0.001$), which indicates a greater potency of the mixture of tastes and odours at the end of the ripening process.

With regard to the assessment of batches as a function of ripening time, at fifteen days of ripening the taste was equal, milky and fresh in all batches, if not very intense. In some cheeses it was possible to note a certain mouldy taste, and in those from batch I a typical goat odour. In general, after thirty days the cheeses in batches II and III showed a drier and grainier texture and an unequal and metallic taste, with an unpleasant aftertaste and marked bitterness. In some cases, odour defects were observed, such as a mouldy or rennet odour. At sixty days of ripening, cheeses of the batch III presented a more acceptable texture (stronger buttery texture and less grainy) and a strong buttery odour, but it did not reach high overall scores. Although, cheeses in batch IV

achieved the best scores at thirty days of ripening, then they worsened in texture, becoming grainier and crumblier, as well as having a taste that was excessively acid and astringent. With regard to cheeses of the batch I, they obtained high scores at thirty days of ripening and were even the best evaluated at the end of ripening because of their odour, fresh balanced pleasant taste and creamy smooth texture.

Conclusions

The use of an autochthonous starter culture constituted lactic acid bacteria strains and *G. candidum* as co-culture affected the evolution of chemical and physico-chemical parameters in the case of Armada cheese, in particular lactose, pH and titratable acidity. The impact that this starter culture had on the sensorial characteristics of the Armada cheese was observed from the fifteenth day of ripening. Cheeses elaborated with the autochthonous lactic starter culture and *G. candidum* co-culture had a marked odour characteristic of goat cheese and a stronger buttery and creamy texture, as they did not undergo excessive drying-out and did not develop any residual rancid taste. Cheeses in batch I, elaborated with the Ge-1886 strain (with high lipolytic activity and low proteolytic activity) were given the best assessments for presenting a wider and more intense range of pleasant odours, and a soft buttery and creamy texture.

Conflict of Interests

The authors have not declared any conflict of interests.

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