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ABSTRACT

Kitoza is a traditional meat product from Madagascar manufactured with strips of pork or beef. The process includes a first step of salting and mixing with spices followed by sun-drying or smoking step. As salting and drying select coagulase-negative staphylococci (CNS), our aim was to identify the CNS species in kitoza with the objective in the future of developing indigenous starters. Microbial analyses revealed that the only pathogenic bacterium enumerated was *Staphylococcus aureus*, which was found in 54% of the samples. The level of *Enterobacteriaceae* revealed a rather good hygienic quality of these products. CNS were confirmed in all the samples at high levels ranging from 5 to 7 log cfu/g. Identification of CNS species in a large collection of 829 isolates revealed 9 identified species, 7 for beef and 8 for pork kitoza. There were significant difference in the distribution of CNS species according to the type of meat and the process. *Staphylococcus saprophyticus* was the dominant species for sun-dried or smoked beef and sun-dried pork kitoza (73–75%), while for smoked pork kitoza *Staphylococcus equorum* (26%), *S. saprophyticus* (23%), *Staphylococcus succinus* (23%) and *Staphylococcus epidermidis* (17%) co-dominated. Some CNS could be used as indigenous starters in particular to compete against *S. aureus*.

1. Introduction

Salting, smoking and drying are among the oldest methods of meat preservation. In tropical countries, where the climate and environmental conditions promote the rapid degradation of meat and where there is sometimes a lack of adequate facilities for the storage of raw meat, traditional processing techniques are often based on the use of these single or combined operations. They lead to a wide variety of products such as biltong in South Africa (Petit et al., 2014), kilishi in Nigeria (Kalilou et al., 1998), boucané in Réunion Island (Poligne et al., 2001), charqui in Brazil (Pinto et al., 2002) and kitoza in Madagascar (Santchurn et al., 2012).

Kitoza is a traditional meat product from Madagascar manufactured with strips of either pork or beef. It is produced in rural and urban regions by artisans and families following traditional processes and sold in local markets. In a first step of the process, miscellaneous pieces of pork and beef meats are cut into strips approximately 2–4 cm thick and 20–50 cm long and are salted with coarse salt mixed with spices such as garlic, pepper, and ginger. Then, a sun-drying and/or smoking step is carried out.

Depending on the products, a wide range of water activity (a_w) and salt content have been recorded. They varied from low a_w (0.65–0.68) and high NaCl concentration (5.5–7.9 g/100 g) in dry commercial biltong to higher a_w (0.85–0.89) and lower salt content (3.8–5.6 g/100 g) in moist commercial biltong (Petit et al., 2014). The a_w of beef and pork jerky varied from 0.83 to 0.79 and the a_w of traditional làcon was 0.90 (Lorenzo et al., 2015; Yang et al., 2009). Also, the pH of these products varied from 5.0 to 6.2 (Lorenzo et al., 2015; Petit et al., 2014; Yang et al., 2009). The total microbial viable count is usually high in dried salted meat products, for instance 6 to 9 log CFU/g in biltong or in làcon, and the salt-tolerant microbiota was the dominant population.

Coagulase-negative staphylococci (CNS) are halotolerant and thus one of the dominant microbiota of salted/dried/fermented meat products (Leroy et al., 2015; Lorenzo et al., 2015; Pinto et al., 2002). In fermented sausages, CNS enhance color stability, prevent rancidity by inhibiting oxidation of unsaturated free fatty acids and release aromatic substances (Coppola et al., 1997; Papamanoli et al., 2002; Talon and Leroy, 2006). In jerked beef, a derivative of charqui meat, staphylococci represent the dominant microbiota and the inoculation of *Staphylococcus xylosus* as starter culture leads to products preferred by the sensory panel (Pinto et al., 2002). Meat-associated CNS are able to produce bacteriocins that may contribute to bioprotection against meat pathogens, in particular *Clostridium botulinum* and *Staphylococcus aureus* (Sanchez Mainar et al., 2016). For instance, species usable as starter cultures

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such as *S. xylosus* and *Staphylococcus equorum* have been shown able to produce bacteriocin (Laukovà et al., 2010; Sanchez Mainar et al., 2016). Thus, CNS could contribute both to the sensory qualities and the bioprotection of meat products (Talon and Leroy, 2006).

No data were available about identification of CNS from kitoza and the aim of this study was to identify these bacteria at the species level in kitoza made from beef or pork and either sun-dried or smoked and collected from producers at different sites. These genetic resources can be used to develop indigenous starters that could be applied to product biopreservation. At the same time, a global view of the microbiology of kitoza is given as well as some of the physicochemical properties important for microbial quality.

2. Materials and methods

2.1. Samples

A total of 54 kitoza samples ready for consumption were collected in butcheries, markets or supermarkets in or near Antananarivo (Madagascar). They included 27 samples of beef kitoza (13 smoked, 14 dried) and 27 of pork kitoza (14 smoked, 13 dried).

The kitoza products were manufactured according the two steps described in introduction with a lot of variation in the process. Samples were kept at 4 °C before microbial analyses and stored at −20°Cfor physicochemical analyses.

2.2. Physicochemical analyses

The salt content was measured with the Model 926 Chloride Analyzer (Sherwood Scientific, Cambridge, UK) after 2 h of cold extraction in 0.3 N nitric acid. Water activity was measured at 25 °C with a Fast-lab water activity meter (GBX, Romans, France). The pH was measured with a TitroLine® easy titrator (SI Analytics GmbH, Mainz, Germany) after homogenization of 3 g samples with 27 mL of distilled water for 30 min. These analyses were carried out on the 54 samples in duplicates.

2.3. Microbial analyses

Microbial analyses of the 54 samples were performed on selective media in duplicate. The total counts, yeasts and molds, staphylococci, lactic acid bacteria (LAB), *Enterobacteriaceae,* coagulase positive staphylococci and *S. aureus, Listeria monocytogenes* and *Salmonella* were analyzed as described by Lebert et al. (2007). In particular, the presence of *S. aureus* isolated from Baird Parker agar supplemented with tellurite yolk egg was confirmed by the Pastorex Staph-Plus test, a latex agglutination test for the identification of *S. aureus* (Bio-Rad, Marnes La Coquette, France). For LAB, the MRS media was incubated under anaerobic conditions. In addition, *Bacillus cereus* was numerated on *Bacillus cereus* Selective Agar (Oxoid, USA), incubated at 30 °C for 18–48 h; *Clostridium perfringens* on Tryptose Sulfite Agar (Biokar, France), incubated at 37 °C for 20 h and confirmed with Lactose Sulfite medium (Biokar, France) at 46 °C.

A collection of 829 isolates of staphylococci was constituted by selecting for each sample an average of 15 colonies on countable plates of Mannitol Salt Agar (MSA, Bio-Rad, France, Leroy et al., 2010). These isolates were cultivated on Brain Heart Infusion (BHI, Difco, USA) at 30 °C overnight. They were then stored at −80 °C in BHI broth containing glycerol 20% before identification.

2.4. Identification of the CNS isolates

Firstly, all the isolates were grown on BHI agar (24 h, 30 °C). Amplifications were performed from one colony picked from the agar plate of each isolate with the primers TstaG422 (5′-GGC CGT GTT GAA CGT GGT CAA ATC A-3′) and Tstag765 (5′-TTA CCA TTT CAG TAC CTT CTG GTA A-3′) from the *tuf* gene allowing the identification the *Staphylococcus* genus, as described by Martineau et al. (2001).

Secondly, the isolates belonging to the *Staphylococcus* genus were further identified at the species level by a species-specific oligonucleotide array as described by Giammarinaro et al. (2005). The primers D1 (CCITAYICITAYGAYGCIYTIGARCC) and D2 (ARRTARTAIGCRTGYTCCCAIACRTC) were used to amplify the *sodA* gene by PCR. This PCR product was labeled with digoxigenin, heat denatured and hybridized with the species-specific oligonucleotide probes fixed to a nylon membrane. The hybridized targets were detected with the Dig color detection kit (Roche, Meylan, France).

2.5. Statistical analysis

The statistical analyses were performed with the XLStat program, version 2016 (Addinsoft, Paris, France). Physicochemical parameters and microbial counts of the four types of kitoza were compared using analysis of variance. Tukey's HSD least significant difference was used with a level of significance of 95%. To test for independence between combinations of *Staphylococcus* species and type of kitoza, the Chi-square (χ^2) test was used.

3. Results and discussion

3.1. Physicochemical characteristics

Kitoza belongs to a wide variety of low and intermediate moisture meat products obtained after salting, drying, and sometimes smoking. In our study, the average salt content and the average water activity of the kitoza products varied from 2.64 to 4.15 g/100 g and from 0.83 to 0.96, respectively (Table 1). There were significant differences in water activity ($P < 0.05$) between smoked and dried products, the smoked ones being less dry than the dried ones. Significant differences in salt content were only noticed between pork kitoza samples, the dried ones being more salty than the smoked ones. The water activity of smoked kitoza was of the same order as that of traditional làcon (Lorenzo et al., 2015) while that of dried kitoza was of the same order as that of biltong, a salted dried product (salt content from 3.8 to 7.9 g/100 g) made from beef, kudu, springbok or chicken (0.65–0.89) (Petit et al., 2014). Similarly, the water activity of traditional fermented sausages from Greece, Spain, France or Argentina varies from 0.79 to 0.91 (GarcíaFontán et al., 2007a; García Fontán et al., 2007b; Leroy et al., 2015).

The pH of the beef kitoza samples, either dried (5.67) or smoked (5.87) (Table 1), was in the same range as the pH of fresh beef (5.5 to 5.9; Laurent, 1981). The pH of the smoked pork kitoza (6.08, Table 1) was in the range of the pH of the fresh raw pork meat (5.7 to 6.2; Laurent, 1981), while the pH of the dried pork kitoza (6.45, Table 1) was higher. Thus, in pork samples, the process affected the pH as dried pork products had a significantly higher pH ($P < 0.05$) than the smoked ones (Table 1). Similar pH ranges were found in biltong samples (5.00 to 6.26) (Petit et al., 2014), beef and pork jerky (5.53–6.07) (Yang et al., 2009) and traditional dry cured làcon (5.00 to 6.00) (Lorenzo et al., 2015).

Table 1
Physicochemical parameters of kitoza.

Sample	Water activity Mean ± SD	Salt content (g/100 g) Mean ± SD	pH Mean ± SD
Smoked beef ($n = 13$)	0.94 ± 0.02^{a}	$2.83 \pm 0,81^{a,b}$	$5.87 \pm 0.17^{b,c}$
Dried beef ($n = 14$)	0.86 ± 0.06^{b}	$3.61 \pm 1.21^{a,b}$	5.67 ± 0.18^{c}
Smoked pork ($n = 14$)	0.96 ± 0.02^{a}	2.64 ± 1.13^{b}	6.08 ± 0.55^{b}
Dried pork ($n = 13$)	0.83 ± 0.07^{b}	4.15 ± 1.85^{a}	6.45 ± 0.31^{a}

n: number of samples; SD: standard deviation; ^{a,b,c}: mean values in the same column not followed by a common letter differ significantly ($P < 0.05$).

3.2. Microbiota

Microbial analyses revealed the absence of pathogenic bacteria such as *Salmonella* and only one smoked beef sample out of 54 samples was contaminated by L. *monocytogenes*. Furthermore, *B. cereus* (<2 log cfu/g) and *C. perfringens* (<1 log cfu/g) were not detected. Levels of *S. aureus* were recorded ranging from 2.7 to 3.6 log cfu/g, but with high variability, as attested by the maximum level (4.7 log cfu/g) or by no detection (<2.0 log cfu/g) (Table 2). The number of smoked samples contaminated was inferior to the dried ones: 8 beef and 9 pork smoked samples were below the detection threshold compared with 5 beef and 3 pork dried samples (Table 2). Similar variability in contamination by coagulase-positive staphylococci was noticed for different dried meat products. They were not detected in charqui meats (Pinto et al., 2002) while *S. aureus* was identified in South African biltong (Mhlambi et al., 2010). *S. aureus* and L. *monocytogenes* were able to survive during biltong manufacturing (Naidoo and Naidoo and Lindsay, 2010). The enumeration of *Enterobacteriaceae,* albeit variable, attested the hygienic quality of the final products (Table 2). In particular, for the majority of the smoked products, *Enterobacteriaceae* were under the detection threshold (Table 2).

The enumeration of yeasts and molds was significantly higher in the dried pork kitoza (Table 2). These microorganisms could contribute to the high pH in these products (Table 1) due to their ability to degrade lactate (Berni, 2015; Selgas and Garcia, 2015). Yeasts and molds were found in làcon and in biltong (Lorenzo et al., 2015; Petit et al., 2014). The two co-dominant populations were represented by lactic acid bacteria and coagulase-negative staphylococci, which ranged from approximately 5 to7 log cfu/g (Table 2). The average total count was not significantly different between the samples.

The population enumerated on MSA was largely dominated by staphylococci as all the 829 isolates from MSA were identified as belonging to the genus *Staphylococcus* by using the genus-specific PCR. The presence of salt together with the low moisture content explains the high halotolerant staphylococcal population. This level of staphylococcal population was also found in làcon and in charqui (Lorenzo et al., 2015; Pinto et al., 2002). High levels of LAB were noted in biltong (Petit et al., 2014). In most traditional fermented sausages, staphylococci constituted the second microbiota at the end of the ripening after the lactic acid bacteria and reached a population that ranged from 4.0 to 7.0 log cfu/g (GarcíaFontán et al., 2007a; García Fontán et al., 2007b; Leroy et al., 2015).

The microbial ecosystem of kitoza has been studied by a classical culture-dependent analysis that gives an image dependent on selective media. A culture-independent approach would have revealed great genus diversity, but could have led to the non-identification of certain species as shown by Greppi et al. (2015). In our study, with the aim of characterizing the diversity of *Staphylococcus* species and creating a collection for potential starter development, it was necessary to isolate the strains and identify them by appropriate molecular methods.

3.3. Inventory of the CNS species in beef and pork kitoza

Nine *Staphylococcus* species were identified from a large collection of 829 isolates, including 423 from beef and 406 from pork kitoza samples (Table 3). One to five species could be identified per sample, but in the majority of the samples two species were concomitantly isolated (data not shown). Concerning the diversity of CNS species in beef and pork kitoza, seven species were identified in beef, while eight species were identified in pork with six species in common (Table 3). This CNS species diversity has already been observed in French dry fermented sausages and in the manufacturing environment, from eight species in a collection of 204 isolates in the study of Coton et al. (2010), up to fifteen species in a collection of 676 isolates in the study of Leroy et al. (2010). Seven CNS species have been identified in botillo and five in androlla, two Spanish traditional smoked pork sausages (GarcíaFontán et al., 2007a; García Fontán et al., 2007b). In kadid, a Tunisian traditional salted dried beef product, nine species have been identified (Essid et al., 2007). These CNS species could originate from the animals as they were part of the microbiota of their skin and mucous membranes (Nagase et al., 2001).

In kitoza, four main species were isolated, including *Staphylococcus saprophyticus* (60.8%), *S. xylosus* (11.3%), *Staphylococcus succinus* (9.8%) and *Staphylococcus equorum* (8.2%) (Table 3). It is noteworthy that the four main species found in kitoza constituted the dominant CNS species in foods of animal origin, even if they were in different ratios. Thus, in fermented foods (cheeses and sausages), *S. equorum* represented 28.5%, *S. xylosus* 28.3%, *S. saprophyticus* 12.5% and *S. succinus* 7.7% (Coton et al., 2010). In traditional French sausages, the following ratios were found: *S. equorum* (58.2%), *S. saprophyticus* (11.9%), *S. xylosus* (11.3%) and *S. succinus* (7.7%) (Leroy et al., 2010). These species were prevalent in two Italian traditional sausages, sopressata Ricigliano and Gioi (Mauriello et al., 2004) and in salami (Połka et al., 2015). These four species were also identified in biltong (Mhlambi et al., 2010). In

Table 2
Microbial analyses of kitoza.

Samples	T. count	Entero*	Y/M*	LAB	CNS	*S. aureus**
Smoked beef (n = 13)						
Mean ± SD	6.1 ± 1.6[b]	2.3 ± 1.7[a]	2.8 ± 1.2[b]	5.8 ± 1.6[b]	5.3 ± 1.8[b]	3.1 ± 1.3[a]
Max	8.4	5.0	4.4	8.2	8.1	4.6
Min	4.0	<1.0 (8)	<1.0 (5)	3.5	3.0	<2.0 (8)
Dried beef (n = 14)						
Mean ± SD	6.8 ± 0.9[a,b]	2.9 ± 1.0[a]	3.6 ± 1.0[b]	6.7 ± 1.0[a,b]	6.7 ± 1.0[a,b]	3.6 ± 1.0[a]
Max	8.2	4.3	5.0	8.3	8.1	4.7
Min	5.3	<1.0 (3)	1.6	5.1	4.5	<2.0 (5)
Smoked pork (n = 14)						
Mean ± SD	7.3 ± 1.2[a]	2.3 ± 1.1[a]	3.8 ± 1.3[b]	7.3 ± 1.1[a]	6.5 ± 1.5[a,b]	2.7 ± 0.7[a]
Max	8.9	3.3	6.1	8.3	9.0	3.6
Min	4.8	<1.0 (11)	2.0	4.9	4.0	<2.0 (9)
Dried pork (n = 13)						
Mean ± SD	7.8 ± 0.9[a]	3.7 ± 1.0[a]	5.3 ± 1.5[a]	7.3 ± 0.9[a]	7.2 ± 1.1[a]	3.5 ± 0.8[a]
Max	9.1	4.7	8.6	8.7	9.4	4.6
Min	6.9	<1.0 (4)	3.6	5.5	5.6	<2.0 (3)

n: number of samples; SD: standard deviation; [a,b,c]: mean values in the same column not followed by a common letter differ significantly ($P < 0.05$), results are expressed as log cfu/g (cfu: colony-forming unit); * mean and SD calculated on countable samples (above and equal to detection threshold); () number of samples < 2.0 or <1.0 log cfu/g (detection threshold). T. count: total count; Entero: *Enterobacteriaceae*; Y/M: yeast and mold; LAB: lactic acid bacteria; CNS: coagulase-negative staphylococci.

Table 3
Inventory of the coagulase-negative species in the sun-dried and smoked beef and pork kitoza ($\chi 2 = 427$, $P < 0.0001$).

Staphylococcus	Total	Smoked beef	Dried beef	Smoked pork	Dried pork
Isolates	829	229	194	210	196
S. saprophyticus	504 (60.8)	167 (72.9)	141 (72.7)	49 (23.3)	147 (75.0)
S. xylosus	94 (11.3)	22 (9.6)	19 (9.8)	20 (9.5)	33 (16.8)
S. succinus	81 (9.8)	19 (8.3)	9 (4.6)	48 (22.9)	5 (2.6)
S. equorum	68 (8.2)	6 (2.6)	2 (1.0)	55 (26.2)	5 (2.6)
S. epidermidis	35 (4.2)			35 (16.7)	
S. kloosii	21 (2.5)	1 (0.4)	19 (9.8)	1 (0.5)	
S. sciuri	13 (1.6)	4 (1.7)	4 (2.1)		5 (2.6)
S. warneri	10 (1.2)	10 (4.3)			
S. vitulinus	3 (0.4)			2 (1.0)	1 (0.5)

Numbers in brackets represent the number of isolates as a percentage of the total.

kadid, *S. equorum, S. saprophyticus* and *S. xylosus* were equally distributed in the range of 22 and 26% (Essid et al., 2007).

3.4. Diversity of the CNS species in the sun-dried or smoked beef and pork kitoza

There were significant difference ($\chi 2 = 427$, $P < 0.0001$) in the distribution of CNS species according to the type of meat and the process. The percentages of *S. saprophyticus* (about 73%) and *S. xylosus* (about 10%) were similar in smoked and dried beef kitoza, while they were higher, particularly for *S. saprophyticus* (75%), in dried pork than in smoked pork (23.3%) (Table 3). These two species also constituted an important part of the CNS microbiota in Greek salami and sausage (Samelis et al., 1998; Drosinos et al., 2005), làcon (Vilar et al., 2000), botillo (GarcíaFontán et al., 2007a) and in various Italian sausages (Aquilanti et al., 2007; Coppola et al., 2000; Mauriello et al., 2004). For sucuk, a fermented beef product, *S. xylosus* and *S. saprophyticus* were the dominant species (41.5% and 28.5%, respectively) (Kaban and Kaya, 2008). Taylor (1976) found that *S. saprophyticus* was dominant in biltong from beef.

The percentages of *S. succinus* (22.9%) and *S. equorum* (26.2%) were quite similar and the highest in the smoked pork kitoza, while the occurrence of these two species was low in beef kitoza and in dried pork kitoza (Table 3). These two species were found to be co-dominant in traditional pork sausages (Corbière Morot-Bizot et al., 2006) and identified in salami (Greppi et al., 2015). *S. equorum* was also isolated in Italian (Aquilanti et al., 2007; Blaiotta et al., 2004; Coppola et al., 2000; Mauriello et al., 2004; Villani et al., 2007), and Spanish sausages (García Fontán et al., 2007b) and in biltong (Mhlambi et al., 2010).

S. epidermidis (16.7%) was only found in smoked pork kitoza (Table 3). *S. epidermidis* has already been identified in Greek salami and botillo, two smoked pork sausages (García Fontán et al., 2007b; Samelis et al., 1998). It has also been isolated from Spanish sausages: chorizo, fuet and salchichon (Martín et al., 2006). *S. warneri* was only identified in the smoked beef kitoza samples. *S. epidermidis* and *S. warneri* were the predominant staphylococci found on human skin (Nagase et al., 2001) and could be transferred to meat during the processing. Like dried beef kitoza, biltong was not contaminated by *S. warneri* (Mhlambi et al., 2010). *S. warneri* was found in the Spanish sausages mentioned above and in different Italian sausages (Coppola et al., 2000; Iacumin et al., 2006; Mauriello et al., 2004; Rantsiou et al., 2005). *S. kloosii* was missing in dried pork kitoza while it represented a non-negligible percentage (9.8%) in dried beef kitoza and a minor population in the smoked beef and pork kitoza (Table 3). *S. sciuri* was not found in smoked pork kitoza and *S. vitulinus* represented a minor population only in pork kitoza (Table 3). *S. sciuri* and *S. kloosii* constituted the minor population of a Tunisian salted dried beef meat "kadid" (Essid et al., 2007).

4. Conclusion

Traditional meat kitoza products had similar physicochemical characteristics to other traditional salted, dried meat products. The kitoza products studied did not present hygienic problem and among the potential pathogens researched, only *S. aureus* was enumerated. Staphylococci constitute an important microbial population in kitoza and a high diversity of CNS species was found, *Staphylococcus saprophyticus* being the dominant species isolated from both beef and pork kitoza. The diversity in CNS species varied according to the type of meat—pork or beef, and the process—sun-dried or smoked. Knowledge of the CNS species could lead to the development of competitive indigenous starters that could be applied to product biopreservation and could help to control *S. aureus* as some strains are able to produce bacteriocins (Sanchez Mainar et al., 2016).

As LAB constituted the co-dominant bacteria in these products, it will be interesting in the future to identify the different species and formulate mixed starter cultures composed of CNS and LAB. In a previous study on the acceptance and sensory profiling of kitoza, inclusion of commercial bioprotective cultures composed of one CNS and one LAB confirmed the value of this strategy for these kinds of products (Pintado et al., 2016).

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