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Effect of ambient storage on the microbial characteristics of traditional dried anchovies (*Encrasicholina punctifer*)

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The aim of this study was to evaluate the safety of traditional dried anchovies by characterizing their microbial flora. Dried anchovies were stored at ambient temperature for three months and total aerobic bacterial (TAB), *Staphylococcus aureus*, Enterobacteriaceae, histidine decarboxylating bacterial (HDB), lysine decarboxylating bacterial (LDB) and ornithine decarboxylating bacterial (ODB) counts were determined. Dried anchovies had an initially load of log 4.58, log 3.8, < log 1, < log 1, log 4.03 and log 3.7 cfu/g for TAB, *S. aureus*, Enterobacteriaceae, HDB, LDB and ODB, respectively. During ambient storage, the bacteria load did not change significantly ($p > 0.05$). In total, 184 bacterial isolates representing 15 genera and 18 species were isolated and identified. A high diversity of pathogens and non-pathogens were found. *Acinetobacter lwoffii* dominated the flora (22%) followed by *S. aureus* (19%), *Moraxella* spp. (13%) and *Enterobacter cloacae* (10%). Ornithine decarboxylating bacteria were the most frequently isolated of the amino acid decarboxylating bacteria (18%) followed by LDB (15%) and HDB (13%). All bacterial species and groups listed were found throughout the storage period. This study showed that anchovies were heavily contaminated with pathogens not traditionally associated with foodborne disease which represent a risk factor for the safety of dried anchovies.

Key words: Ambient storage, decarboxylating bacteria, dried anchovies.

INTRODUCTION

Anchovies are small coastal pelagic fishes found in most marine environments and during most seasons of the year. They have been used to develop many traditional fish products such as salted, pickled, smoked and marinated anchovies (Shiriskar et al., 2010; Ozogul et al., 2010; Rabie et al., 2011; Czerner et al., 2011). In the Sultanate of Oman, anchovies are caught by trawling net, generally handled under unhygienic conditions, traditionally dried under the sun for three to five days, packed in sacks and stored at ambient temperature, 25 to 30°C for a few months before consumption. As a traditional product, the characteristics of this fish, such as water activity and microbial load, vary due to fluctuation in processing conditions which could affect the stability and safety of the product.

Anchovies are associated with scombroid-poisoning and have been found to contain biogenic amines such as histamine, putrescine and cadaverine. A high incidence

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Abbreviations: TAB, Total aerobic bacterial; **HDB,** histidine decarboxylating bacterial; **LDB,** lysine decarboxylating bacterial; **ODB,** ornithine decarboxylating bacterial; **TAL,** thin agar layers; **TSA,** tryptone soya agar.

of biogenic amines forming bacteria has been found in dried anchovies (Shakila et al., 2002; Yeh et al., 2004; Pons-Sanchez-Cascad et al., 2005; Vosikis et al., 2008). Traditional fishing, which is practiced in many developing countries, has been found to produce low microbiological quality products heavily loaded with pathogens such as *Staphylococcus aureus*, fecal *Streptococcus* spp. and *Clostridium* spp. (Ali et al., 2011).

Drying reduces the water activity of fish which limits the growth of many microorganisms but this effect depends on the endogenous microflora of the fresh fish. For example, Gram-positive bacteria are often more sensitive to heat than Gram-negative bacteria (Rahman et al., 2004). Drying at 60 to 140°C caused a significant decrease in the total bacterial counts of tuna (Rahman et al., 2004). Drying methods have also been found to affect the microbial quality of fish. For instance, a high incidence of mesophiles and total coliforms was found in traditionally sun-dried fish as compared to that dried under controlled conditions at 50°C (Selmi et al., 2010).

Studies on drying of fish (Shakila et al., 2002; Rahman et al., 2004; Selmi et al., 2010) did not entail speciation of the microbial flora of the dried fish to provide information on the endogenous flora and to elucidate the effect of drying on specific genera or species. In addition, post-harvest contamination due to unhygienic handling and cross-contamination of dried fish, which is common in many developing countries, increases the incidence of potential pathogens such as Staphylococci and Enterobacteriaceae in the product.

Although, dried anchovies are traditionally processed and consumed without any heat treatment in many Arabian Gulf countries, information on the endogenous bacteria of this product is limited. The effect of ambient storage on the microbial and chemical changes in many fish products such as salted mackerel, smoked-dried herring, dried sausages and salted tiger fish have been documented (Plahar et al., 1991; Srikar et al., 1993; Oksuz et al., 2008; Ahmed et al., 2010). The effect of ambient storage on the microbial characteristics of dried anchovies is however not fully understood. This study aimed to microbiologically characterize dried anchovies by enumerating and identifying total bacteria, Enterobacteriaceae, biogenic amine forming bacteria and *S. aureus* and also to determine the effect of ambient storage on the counts and types of these bacteria.

MATERIALS AND METHODS

Dried anchovies

Six kilograms of dried anchovies (*Encrasicholina punctifer*) were purchased from the Al Seeb local market in Oman. The anchovies had previously been traditionally dried under the sun on the sand for 3 to 5 days. Dried fish was brought to the laboratory within 30 min of purchase. Immediately, 500 g portions of fish were weighed and dispensed into polyethylene bags and stored at 25 ± 2°C up to 11 weeks. Samples were analyzed at 2-week interval. At each sampling occasion, three bags of 500 g dried anchovies were used for bacterial enumeration and water content determination.

Determination of water content

Five grams of ground dried anchovies were dried in a 300 plus series atmospheric oven (Gallenkamh, UK) at 105°C to a constant weight for about three hours (Helrich, 1990). Water content was expressed as the difference in the weight of sample before and after drying.

Bacterial enumeration

Total bacteria

Dried anchovies were chopped and ground for one minute using a Warning Commercial Blender (USA). Ground dried anchovies (25 g) were blended with 225 ml 0.1% peptone (Oxoid, UK) in 0.85% bacteriological NaCl (Oxoid, UK) for one min in a Colworth stomacher 400 (UK). After serial dilution, 0.1 ml of each dilution of the dried anchovies suspension was spread on tryptone soya agar (TSA) (Oxoid, UK) supplemented with 2% NaCl and incubated at 32°C for three days (Al Bulushi et al., 2008).

Biogenic amine forming bacteria

Histidine decarboxylating bacteria, lysine decarboxylating bacteria (LDB) and ornithine decarboxylating bacteria (ODB) were enumerated using a differential plating medium developed by Niven et al. (1981) with slight modifications. This medium is composed of 0.5% tryptone (Oxoid, UK), 0.5% yeast extract (Oxoid, UK), 2.7% L-histidine dihydrochloride (Sigma, USA), 0.5% NaCl, 0.1% $CaCO_3$ (BDH, UK), 2% agar (Oxoid, UK) and 0.006% bromocresol purple (Janseen, Belgium).

The pH was adjusted to pH 5.3 and the medium was autoclaved at 115°C for 10 min to minimize the effect of heat on the amine acids. Plates were incubated aerobically at 30°C for two and four days. Purple colonies with a purple halo on the yellow background were considered as histidine decarboxylating bacteria. To enumerate lysine decarboxylating bacteria and ornithine decarboxylating bacteria, histidine dihydrochloride was replaced by 2.7% L-lysine monohydrochloride (Sigma, USA) or L-ornithine monohydrochloride (Sigma, USA), respectively, and 0.01% pyridoxine hydrochloride (Sigma, USA) was added as a coenzyme (Frank et al., 1985).

Staphylococcus aureus

To recover *S. aureus* cells injured by drying, Baird-Parker agar base (RPF) was overlaid with two thin layers of TSA. The dried anchovy suspension was then inoculated on the surface of the thin layers. Thin agar layers (TAL) was used when injured bacteria were enumerated using selective media and TAL was found to significantly increase recovery of injured *S. aureus* (Wu, 2001, 2008). Plates were incubated aerobically at 35°C for one and for two days. Grey-black shiny colonies were considered as *S. aureus*. *S. aureus* was further confirmed using the Staphytect plus system (Oxoid, UK).

Enterobacteriaceae

Enterobacteriaceae were enumerated on violet red bile glucose agar (Oxoid, UK) following the TAL technique as it was described earlier. Plates were incubated aerobically at 32°C for one and two days and round, purple-pink, 1 to 2 mm diameter surrounded by

Table 1. Changes in water content and bacterial counts in dried anchovies during ambient storage.

Time (week)	Water (%)	Bacterial counts, log cfu/g					
		TAB	HDB	LDB	ODB	*S. aureus*	ENT
0	12.5 ±1.40[a]	4.5 ± 0.35[a]	<1	4.0 ± 0.65[a]	3.7 ± 0.76[a]	3.8 ± 0.52[a]	< 1
1	12.5 ± 1.56[a]	4.7 ± 0.32[a]	4.0 ± 0.61[a]	4.1 ± 0.20[a]	4.4 ± 0.15[a]	3.4 ± 0.28[a]	< 1
3	12.3 ± 2.36[a]	5.0 ± 0.07[a]	4.6 ± 0.19[a]	5.0 ± 0.05[a]	4.7 ± 0.19[a]	3.5 ± 0.27[a]	< 1
5	12.2 ± 0.25[a]	4.7 ± 0.36[a]	–	4.3 ± 0.54[a]	3.9 ± 0.50[a]	3.6 ± 0.35[a]	< 1
7	12.4 ± 0.44[a]	4.2 ± 0.41[a]	–	4.5 ± 0.55[a]	3.5 ± 0.52[a]	4.0 ± 0.81[a]	< 1
9	11.5 ± 0.44[a]	4.7 ± 0.60[a]	3.7 ± 0.66[a]	4.1 ± 0.06[a]	4.1 ± 0.06[a]	–	< 1
11	11.4 ± 0.29[a]	5.0 ± 0.09[a]	3.2 ± 0.39[a]	4.3 ± 0.36[a]	–	3.9 ± 0.23[a]	< 1

Each mean was compared with that of 0 week. Means with different alphabetical superscript are significantly different (p< 0.05), n = 3. TAB: total aerobic bacteria, HDB: histidine decarboxylating bacteria, LDB: lysine decarboxylating bacteria, ODB: ornithine decarboxylating bacteria, ENT: Enterobacteriaceae, –: Undetected.

purple haloes which were considered as Enterobacteriaceae.

Bacterial isolation and identification

Five colonies from plates containing 10 to 100 colonies were selected randomly from each medium using Harrison's disc (Harrison, 1938, cited by Harrigan, 1998). The colonies were purified twice in TSA supplemented with 2% NaCl and 2% agar and stored on beads (Abtek, UK) at -55 to -60°C until identified. Before identification, the Gram reaction was determined for all isolates by 3% KOH (BDH, UK) test (Halebian et al., 1981) and bacteria were identified to the genus and species levels by VITEK 2 compact (bioMérieux, France) according to the instructors of the manufacture using GP and GN cards and software version of 4.1.

Statistical analysis

Bacterial numbers were reported as log $_{10}$cfu / g. A one-way ANOVA was used to evaluate the effect of ambient storage on the water content and bacterial count, whereas Two-Sample T-Test and Tukey Simultaneous Test were used to evaluate the difference between the counts of biogenic amine forming bacteria. These tests were conducted in Minitab release 14 software (Minitab Inc., USA), and level of P<0.05 was considered statistically significant. Data were presented as the means and standard deviations of three to four determinations.

RESULTS

Effect of ambient storage on water content and bacterial counts of dried anchovies

Dried anchovies contained 12.5% water at the beginning of the storage period (Table 1) and during ambient storage, water content did not change significantly (P>0.05). Initial TAB counts on dried anchovies were log 4.58 cfu/g (Table 1), a number which is lower than the log 5.0 cfu/g recommended for good quality foods (ICMSF, 1986). During ambient storage, the TAB did not change significantly (P>0.05). The dried anchovies were contaminated with *S. aureus* and the count of this pathogen in the product was initially log 3.88 cfu/g (Table 1) which did not change significantly (p>0.05). The counts of LDB, ODB and HDB were log 4.03, log 3.75 and < log 1 cfu/g, respectively, at the beginning of storage (Table 1). Cadaverine decarboxylating bacteria, ODB and HDB did not change significantly (p>0.05) in this study.

Diversity in bacterial types during ambient storage

In total, 184 bacterial isolates representing 15 genera and 18 species were isolated and identified using the VITEK 2 compact and Staphytect plus systems (Table 2). *Acinetobacter lwoffii* dominated the flora (22%) followed by *S. aureus* (19%), *Moraxell* spp. (13%) and *E. cloacae* (10%). Pathogens accounted for 53% of the flora with *A. lwoffii*, *S. aureus* and *E. cloacae* dominating (Table 3). *S. aureus* and *A. lwoffii* were present at most sampling times, whereas *E. cloacae* showed a low incidence towards the end of storage.

Among the amino acid decarboxylating bacteria, ODB and LDB showed a higher incidence than HDB strains. The incidence of ODB, LDB and HDB was 18, 15 and 13%, respectively (Table 4). *A. lwoffii* dominated the bacteria which decarboxylated histidine and lysine.

DISCUSSION

The water content of dried anchovies was lower than 15%, the level which is normally found in dried fish (Urch, 1984). Water content varied among individual anchovies as seen in Table 1. This variation may be attributable to the adverse relationship between water and lipid content (Shearer, 1994; Zaboukas et al., 2006). Another possible explanation for such variation was the effect of fluctuations on traditional drying conditions. Selmi et al. (2010) reported a significant difference (p<0.05) between traditionally sun dried and electrically dried *Atherina lagunae*. The relative stability of the water content was expected in this study since dried anchovies were stored at 25 ± 2°C which is insufficient to lower relative humidity.

Table 2. Number of bacterial isolates in dried anchovies during ambient storage.

Bacteria	Storage (week)							Number (%)
	0	1	3	5	7	9	11	
Alloiococcus otitis	1	-	-	-	1	-	1	3 (2)
Enterobacter spp.	-	2	-	-	-	-	1	3 (2)
Enterobacter cloacae	10	2	4	1	-	2	-	19 (10)
Pantoea spp.	3	1	-	1	-	-	-	5 (3)
Moraxella spp.	3	4	1	3	5	1	7	24 (13)
Micrococcus spp.	1	2	-	-	-	-	-	3 (2)
Staphylococcus spp.	1	-	-	-	-	-	-	1 (0.5)
Staphylococcus aureus	5	5	5	5	5	5	5	35 (19)
Staphylococcus saprophyticus	1	-	-	-	-	-	-	1 (0.5)
Staphylococcus warneri	-	1	2	-	-	-	1	4 (2)
Staphylococcus xylosus	-	-	-	-	1	-	2	3 (2)
Comamonas testosteroni	3	1	-	-	-	-	-	4 (2)
Kocuria varians	1	-	-	-	-	7	1	9 (5)
Kocuria rosea	-	-	-	-	1	2	-	3 (2)
Kocuria kristinae	-	-	-	-	1	1	2	4 (2)
Streptococcus pneumoniae	-	1	-	-	-	-	-	1 (0.5)
Streptococcus thoraltensis	-	-	2	-	-	-	1	3 (2)
Acinetobacter spp.	-	3	1	1	-	-	2	7 (4)
Acinetobacter lwoffii	-	3	4	4	5	9	16	41 (22)
Sphingomonas paucimobilis	-	-	1	1	1	-	2	5 (3)
Pseudomonas aeruginosa	-	-	1	-	-	-	-	1 (0.5)
Leclercia adecarboxylata	-	-	1	2	-	-	-	3 (2)
Serratia odorifera	-	-	-	1	-	-	-	1 (0.5)
Leuconostoc pseudomesenteroides	-	-	-	-	1	-	-	1 (0.5)
Total					184			

–: Undetected.

Based on the water content of 12.5% and water activity of 0.57, it may be that traditional drying caused cell injury and the low water content inhibited the growth of bacteria in the current study which explains this low microbial load. Many bacteria, including those involved in spoilage, require a water activity above 0.9 for growth and it is therefore expected that the growth of these bacteria would be inhibited by the water activity of dried anchovies (Jay et al., 2005).

The numbers of total aerobic bacteria in dried anchovies closely coincided with the log 4.75 cfu/g found in sun dried fish from an Indian market, but was lower than the log 6.82 cfu/g found in traditionally dried *A. lagunae* (Selmi et al., 2010; Prakash et al., 2011). This relative stability of the bacterial count could be explained by low water activity of dried anchovies. The effect of the water content of the product on its microbial load is further supported by the low microbial count of log 1.54 to log 2.31 cfu/g in a traditional Maldivian seafood product where water activity was found to be 0.55 to 0.8 (Naila et al., 2011).

S. aureus is a heat resistant pathogen and its resistance increases at water activities between 0.85 and 0.99 and is generally highest in complex organic media (Stewart, 2003). It also produces enterotoxins at these water activity levels.

It could therefore be expected that traditional sun drying had little effect on the growth of *S. aureus* as the water activity of dried anchovies at 0.57 was very low for toxin production (Stewart, 2003). The numbers of *S. aureus* was similar to those found on sun dried freshwater fish which showed poor microbiological quality and on which numbers exceeded the recommended level for this pathogen (Ali et al., 2011). Insignificant changes in the count of *S. aureus* during the ambient storage could be explained by the viability of *S. aureus* at low water activity.

The incidence of prolific biogenic forming bacteria belonging to the Enterobacteriaceae such as *Morganella morganii*, *Proteus vulgaris*, *Enterobacter aerogenes*, *Enterobacter cloacae* as well as biogenic amine forming bacteria such as *Staphylococcus epidermidis* have been widely found in anchovies (Hernandez-Herrero et. al., 1999; Nawong et. al., 2005; Pons-Sanchez-Cascado et

Table 3. Incidence of pathogens in dried anchovies during ambient storage.

Bacteria	Storage (week)							Number (%)
	0	1	3	5	7	9	11	
Enterobacter cloacae	10	2	4	1	-	2	-	19 (10)
Staphylococcus aureus	5	5	5	5	5	5	5	35 (19)
S. *saprophyticus*	1	-	-	-	-	-	-	1 (0.5)
Streptococcus pneumoniae	-	1	-	-	-	-	-	1 (0.5)
Acinetobacter lwoffii	-	3	4	4	5	9	16	41 (22)
Pseudomonas aeruginosa	-	-	1	-	-	-	-	1 (0.5)
Total					98			

–: Undetected.

Table 4. Incidence of amino acids decarboxylating bacteria in dried anchovies during ambient storage.

Bacteria	HD	LD	OD
Enterobacter spp.	-	-	3
Enterobacter cloacae	4	3	3
Acinetobacter spp.	-	4	-
Acinetobacter lwoffii	8	10	5
Sphingomonas paucimobilis	1	1	-
Kocuria varians	3	-	5
Kocuria kristinae	-	1	2
Kocuria rosea	-	-	3
Moraxella spp.	4	6	7
Micrococcus spp.	2	-	-
Staphylococcus saprophyticus	1	-	-
Streptococcus thoraltensis	1	-	-
Streptococcus pneumoniae	-	-	1
Pantoea spp.	-	1	1
Comamonas testosteroni	-	1	2
Leclercia adecarboxylata	-	1	1
Total	24	28	33

HD: Histidine decarboxylation, LD: lysine decarboxylation, OD: ornithine decarboxylation, –: undetected.

al., 2005). Biogenic amines formers such as *E. cloacae* survive a water activity of 0.48 and *E*. aerogenesis is a prolific histamine former in dried fish products with water activity ranging from 0.63 to 0.92 (Ibrahim, 2001; Min et al., 2002; Huang et al., 2010). Ornithine decarboxylase and lysine decarboxylase activities have been found in products with water activity as low as 0.7 (Eerola et al., 1997; Periago et al., 2003; Rodrigues et. al., 2003).

Previous studies (Ibrahim, 2001; Min et al., 2002; Huang et al., 2010) indicated that reducing water activity increased the viability of biogenic amine formers without eliminating them which explains the count of biogenic amine bacteria in the current study. In addition, anchovies are exposed to temperature abuse and unhygienic handling during catching and drying and these conditions may have contributed to increase amino acids decarboxylating bacterial load in our study.

The relative stability of amino acid decarboxylating bacterial counts provides evidence for their decarboxylation potential in low water activity medium such as dried anchovies during ambient storage. This potential could be further enhanced by the activities of ornithine decarboxylase and lysine decarboxylase at a water activity of 0.7 (Eerola et al., 1997; Periago et al., 2003; Rodrigues et. al., 2003). Biogenic amines were not quantified in the current study.

The ability of VITEK and VITEK 2 compact to identify *A. lwoffii* and *E. cloacae* has been evaluated in some studies (Bourbeau and Heiter, 1998; Funke and Funke-Kissling, 2004; Nakasone et al., 2007). The VITEK system was found to be a promising and reliable tool to identify these bacteria in these studies. In addition, VITEK has been used to identify *E. cloacae* from salted and dried salted cod (Rodrigues et al., 2003) which confirmed the ability of the system to identify environmental microflora.

E. cloacae has been widely found in many foods such as salted mackerel, mustard pickles, raw beef, fresh anchovies, salted and dried salted cod and infant milk formula (Rodrigues et al., 2003; Pons-Sanchez-Cascado et al., 2005; Tsai et al., 2005; Kung et al., 2006; Shaker et al., 2007; Kwon and Kim, 2008). The possible sources of *E. cloacae* in dried anchovies in the current study were sanitary sources as this bacterium has been isolated from wastewater, fecal materials, the hands and nails of food handlers, treated sewage and human intestines (Dumavibhat et al., 1989; Jimenez et al., 2003; Filipkowska, 2003; Ibenyassine et al., 2007; Krzyminska et al., 2010). These sources may have contributed the *E. cloacae* in dried anchovies in addition to the fact that fresh anchovies are handled under unhygienic conditions and dried directly on the sand near the sea.

E. cloacae have aminogenic activity and are capable of producing some biogenic amines such as histamine, cadaverine and putrescine (Pons-Sanchez-Cascado et al., 2005; Kung et al., 2006; Lavizzari et al., 2010). The ability of *E. cloacae* to form histamine, cadaverine and putrescine provides a risk factor of scombroid food poisoning in dried anchovies since anchovy is one of the fish associated with incidences of such poisoning in many

studies (Vosikis et al., 2008; Murai et al., 2009; Al Bulushi et al., 2009).

A. lwoffii has been isolated from soil, human hands and skin, and fresh water and could indicate a role for cross-contamination in dried anchovies in the current study where the product is exposed to these sources during handling (Berlau et al., 1999; Gouzalez et al., 2000; Aiello et al., 2003; Kasana et al., 2008). Histamine formation by *A. lwoffii* has been found in some studies but other biogenic amines that are associated with scombroid food poisoning have not been reported which indicates that this bacterium is not associated with such poisoning unless histamine potentiators such as cadaverine and putrescine are produced by other flora (Kim et al., 2001; Hostacka and Klokochikova, 2002, Al Bulushi et al., 2009).

E. cloacae has been found to produce leukotoxins and enterotoxins which are able to lyse erythrocytes and leukocutes of blood cells and cause cell apoptosis in *in vitro* studies (Barnes et al., 2001; Paraje et al., 2005; Akhtarieva et al., 2009; Akhtarieva et al., 2010). Similarly, *A. lwoffii* was found to be opportunistic pathogen that can cause bacteremia in immunocompromised hosts (Ku et al., 2000; Regalado et al., 2009; Felfoldi et al., 2010). These findings provide a risk factor in the safety of dried anchovies in Oman especially if the product is consumed without any heat treatment and raises the need for further studies to elucidate the possible association of dried anchovies in food poisoning.

Conclusions

In general, traditional drying and ambient storage controlled water content and bacterial growth in dried anchovies over a three month period but high levels of contamination with *A. lwoffii*, *S. aureus* and *E. cloacae* from human and non-human sources during catching, sun drying and handling after processing were observed. The high incidence of pathogens not traditionally associated with food sources and amino acids decarboxylating bacteria during the storage provides potential hazards and risk factor for foodborne disease and scombroid food poisoning. The authors of this study recommend that fresh anchovies should be handled under hygienic conditions and dried using appropriate technologies.

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