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SHORT COMMUNICATION

MICROBIOLOGICAL CONTROL OF FROZEN AND THAWED CUTTLEFISH (*SEPIA OFFICINALIS*)

CONTROLLO MICROBIOLOGICO DI SEPPIE (*SEPIA OFFICINALIS*) CONGELATE E SCONGELATE

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ABSTRACT

Sixty-nine batches of frozen and thawed cuttlefish (*Sepia officinalis*) obtained from five Italian EEC-authorised fish farms were analyzed microbiologically. The following parameters were analyzed: total bacterial count, psychrophylic bacterial count, total coliform count, *Escherichia coli* count, *Staphylococcus aureus* count, enterococci count, presence of *Salmonella* spp., *Vibrio parahaemolyticus* and *Vibrio cholerae*. The total bacterial and psychrophylic bacterial counts were high in

RIASSUNTO

Sessantanove campioni di seppie congelate e scongelate (*Sepia officinalis*), prelevati da cinque ditte italiane autorizzate CEE, sono stati sottoposti ad analisi microbiologiche per i seguenti parametri: conta batterica totale, conta batterica psicrofila, conteggio dei coliformi totali, di *Escherichia coli*, di *Staphylococcus aureus* e degli enterococchi, ricerca qualitativa di *Salmonella* spp., *Vibrio parahaemolyticus* e *Vibrio cholerae*. I risultati hanno evidenziato valori elevati nella conta batterica

- Key words: cuttlefish, HACCP, microbiological criteria, *Vibrio parahaemolyticus* -

55% of the batches. *Salmonella* spp. and *V. cholerae* were not detected. *V. parahaemolyticus* was isolated in 8.7% of the batches and although a very small percentage of strains are pathogenic, they represent a potential health risk to the consumer.

totale e nel numero dei batteri psicrofili nel 55% dei campioni. *Salmonella* spp. e *V. cholerae* sono risultati assenti in tutti i campioni analizzati. Nell'8,7% dei lotti esaminati si è isolato *V. parahaemolyticus* che, sebbene siano patogeni in percentuale molto ridotta, può rappresentare un rischio potenziale per la salute del consumatore.

INTRODUCTION

Boneless fish like squid, tattler, octopus, shrimp and cuttlefish are mainly imported from non-European countries, including those of southeastern Asia. Italian EEC-authorized fish farms that distribute frozen and thawed fish must follow the HACCP regulations for monitoring all of the production steps.

Cuttlefish are frequently imported as frozen products and are usually sold as thawed end-products. Thus, the manufacturing process consists of thawing by spraying with water, followed by cutting and "mechanical shaking" of the fish. The latter process involves flattening the fish with a special roller machine to obtain optimal freshness that is particularly appreciated on the fish market.

The aim of the present study was to determine the microbiological quality of frozen and thawed cuttlefish imported into Italy from several non-European countries and prepared according to HACCP food safety regulations.

MATERIALS AND METHODS

From 2000 to 2002, 69 batches of cuttlefish imported from non-European countries, including southeast Asian countries, were collected from five Italian EEC-authorized fish farms, which follow the Hazard Analysis Critical Control Point (HACCP) system in preparing the final thawed product. Of these, 55 batches were imported from southeast Asian countries: 41 from Thailand, 10 from Vietnam, 2 from India and 2 from Malaysia. Fourteen batches came from other non-European countries (4 from Senegal, 1 from Morocco, 1 from Argentina and 8 from non-specified countries).

According to the HACCP system applied in the five Italian farms, the raw frozen cuttlefish, thawed products, water, self-made ice, equipment and workers were all subjected to microbiological testing. The microbiological criteria for cephalopods were set by the analytical laboratory itself, because Italy has no established tolerance limits for these seafood products.

Twenty-one batches of frozen and 34 batches of thawed and flattened cuttlefish imported from southeastern Asian countries were analyzed. Five batches of frozen and nine batches of thawed cuttlefish imported from the other non-European countries were tested.

The microbiological assays required by the HACCP system are the following: aerobic plate count (APC), psychrophilic bacterial count, total coliform count, *Escherichia coli* count, *Staphylococcus aureus* count and enterococci count. In addition, cuttlefish imported from southeast Asian countries and the end-products obtained from them were tested for

Salmonella spp., *Vibrio cholerae* and *Vibrio parahaemolyticus*.

All samples were diluted in 0.1% peptone water (Oxoid, Basingstoke, U.K.) (ISO 6887-1988) and homogenised in a Stomacher blender (PBI International, Milan, Italy). For APC, serial dilutions of the samples were mixed with 15 mL of Plate Count Agar (PCA) (Oxoid) and the plates were incubated at 30°C for 48 h (ISO 4833-1991). The psychrophilic count was determined by spreading sample dilutions onto PCA plates and incubating them at 4°C for ten days. For the total coliform count, the ISO 4832-1991 method was followed. Violet Red Bile Lactose Agar (Oxoid) plates were incubated at 37°C for 24 h. The enterococci count was determined by spreading sample dilutions onto Kanamycin Aesculin Azide Agar (Oxoid) plates and incubating them at 37°C for 48 h; suspected colonies were confirmed by using the method of MOSSEL *et al.* (1978). For the *E. coli* count, serial dilutions were poured onto Violet Red Bile Agar + 4-methylumbellyferil-β-D-glucuronide (Oxoid) plates and incubated at 37°C for 24 h (ISO 6391-1988). Coagulase and DNA-ase positive *S. aureus* were counted according to the ISO 6888-1983 method. Baird-Parker medium with the addition of Egg Yolk Tellurite (Oxoid) emulsion was incubated at 37°C for 48 h. To detect *Salmonella* spp., the ISO 6579-1993 method was followed. Buffered Peptone Water (Oxoid) was used in a pre-enrichment step (37°C for 16-20 h). The enrichment procedure was carried out using Rappaport-Vassiliadis Soya Peptone broth (Oxoid) and Selenite Cystine broth (Oxoid). Rambach Agar (Merck, Darmstadt, Germany) was employed as selective medium. To detect *Vibrionaceae*, samples were enriched in Alkaline Peptone Water (Biolife Italiana, Milano, Italy) at 37°C for 6-8 h. Thiosulfate-Citrate-Bile-Sucrose Kabayashi Agar (Biolife Italiana) plates, incubated at 37°C for 18 h, were used for the selective isolation step.

The following microbiological limits were set by the laboratory: $5.0x10^5$ cfu g^{-1} for APC and psychrophilic bacterial count, $1.0x10^3$ cfu g^{-1} for total coliforms, $1.0x10^2$ cfu g^{-1} for *E. coli* and coagulase and DNA-ase positive *S. aureus*, $5.0x10^2$ cfu g^{-1} for enterococci. *Salmonella* spp., *V. cholerae* and *V. parahaemolyticus* should not be detected in 25 g of seafood product.

The fixed level of $5.0x10^5$ cfu g^{-1} for both total and psychrophilic bacterial counts is very low and does not correspond to sensorially detectable product spoilage, but it was set in order to assure good hygienic conditions of imported fish products. In 1989 the American Food and Drug Administration (FDA, 1989) set a limit of $5.0x10^5$ cfu g^{-1} for the APC of bivalve molluscs.

RESULTS AND DISCUSSION

The APC exceeded the analytical tolerance level in 29 of the 69 (42.0%) batches of frozen cuttlefish, with values ranging from $6.0x10^6$ to $1.0x10^7$ cfu g^{-1} (mean value: $3.1x10^6$ cfu g^{-1}) (Table 1).The values of thawed end-products ranged from $5x10^5$ to $6.5x10^6$ cfu g^{-1} (mean value: $2.3x10^6$ cfu g^{-1}).

The psychrophilic bacterial count was higher than the accepted analytical value in 34 of the 69 (49.3%) cuttlefish batches, with values ranging from $1.2x10^6$ to $7.2x10^6$ cfu g^{-1} (mean value: $3.3x10^6$ cfu g^{-1}) in the frozen cuttlefish and from $1.2x10^6$ cfu g^{-1} to $7.0x10^6$ cfu g^{-1} (mean value: $3.3x10^6$ cfu g^{-1}) in thawed end-products.

The values of psychrophilic counts were higher than the APC values. This could have been due to the incubation temperature (30°C) for the APC which was quite high for the heat-sensitive microflora of marine fish. Another factor could have been the medium selected for bacteria enumeration, *i.e.* Plate Count Agar, which contains little NaCl.

Table 1 - Number and percentage of cuttlefish batches that exceeded the fixed tolerance values.

Analytical tolerance (cfu g^{-1})	Aerobic plate count ($5.0x10^5$)	Psychrophilic bacterial count ($5.0x10^5$)	Total coliforms (1.0×10^3)	*E. coli* ($1.0x10^2$)	*S. aureus* ($1.0x10^2$)	Enterococci ($5.0x10^2$)
Frozen cuttlefish (S.E. Asian countries)	11/21 (52.4%)	10/21 (47.6%)	3/21 (14.3%)	0/21	1/21 (4.8%)	1/21 (4.8%)
Thawed products (S.E. Asian countries)	14/34 (41.2%)	19/34 (55.9%)	1/34 (2.9%)	0/34	0/34	1/34 (2.9%)
Frozen cuttlefish (non-EU countries)	1/5 (20.0%)	1/5 (20.0%)	0/5	0/5	0/5	0/5
Thawed products (non-EU countries)	3/9 (33.3%)	4/9 (44.4%)	0/9	0/9	0/9	0/9

In fact, marine microorganisms require NaCl for growth. A more appropriate incubation temperature for mesophilic psychrotrophic aerobic bacterial count of fish products is 20°C for 3-5 days and a more suitable medium is Tryptic Soy Agar (APHA, 1992; FRANZETTI *et al.*, 2001). Nevertheless, the analytical method applied in this study was suitable for the growth of "human" or "environmental" microorganisms that could contaminate the fish products during harvesting, storage and transport to the Italian fish farms. High levels of these microorganisms are indicators of poor hygienic conditions and/or a poorly maintained cold chain at either end of the fish production chain.

Only a few cuttlefish batches showed total coliform values higher than the tolerance criteria, as reported in Table 1. The irregular coliform values in frozen samples ranged from $2.0x10^3$ to $3.5x10^3$ cfu g^{-1} (mean value: $2.6x10^3$ cfu g^{-1}), while the coliform value was $2.2x10^3$ cfu g^{-1} in the thawed cuttlefish.

All of the samples were characterised by an *E. coli* count of <5 cfu g^{-1}. In one thawed cuttlefish sample, obtained from frozen products imported from south-eastern Asia, the coagulase and DNA-ase positive *S. aureus* count was $1.0x10^2$ cfu g^{-1}. The highest value tolerated for enterococci ($5.0x10^2$ cfu g^{-1}) was exceeded in only two samples. The majority of the samples, 55 of 69 (79.7%), were characterised by values <50 cfu g^{-1}. *Salmonella* spp. and *V. cholerae* were not detected in any of the analyzed samples. In contrast, *V. parahaemolyticus* was isolated in six out of 69 (8.7%) samples, all of which were either imported from Thailand or were thawed end-products obtained from Thai batches. In particular, *V. parahaemolyticus* was detected in three out of 21 frozen (14.3%) cuttlefish batches and three out of 34 (8.8%) end-products.

The microbiological values of the APC and the psychrophilic count of frozen and thawed cuttlefish reflect the hygienic conditions of the fishery, and of the subsequent manipulation, storage, transport, and processing of the seafood product. The high values of both total bacterial and psychrophilic bacterial counts demonstrate that the frozen and thawed cuttlefish analysed in this study may not have been processed under conditions that complied with GMP (Good Manufacturing Practices). On the other hand, total coliforms, *E. coli,* coagulase and DNA-ase positive *S. aureus* and enterococci did not present a real hygienic or health problem.

Among the foodborne pathogens, only *V. parahaemolyticus* was detected in frozen cuttlefish imported from Thailand

and in thawed cuttlefish produced in Italy with frozen batches from Thailand. All the samples contaminated by *V. parahaemolyticus* had high psychrophilic and APC values.

Consumption of either raw or cooked seafood contaminated with *V. parahaemolyticus* has been associated with acute gastroenteritis (CHAKRABORTY *et al.,* 1997). At least four haemolytic components exist in *V. parahaemolyticus*: a thermostable direct hemolysin (TDH), a TDH-related hemolysin (TRH), phospholipase A, and a lysophospholipase. TDH and TRH are strictly related to pathogenicity, but TDH- and/or TRH-positive strains of *V. parahaemolyticus* represent a very small percentage of the strains found in seafoods (WONG *et al.,* 1993). A recent investigation on enzymatic (urease, lipase, gelatinase and haemolysin) and biological (adhesiveness, cytotoxicity and enterotoxicity) activities and enteropathogenicity in mice of fifty-two *Vibrio* strains isolated from the Adriatic Sea (Italy) demonstrated that pathogenicity of *V. parahaemolyticus* and other halophilic vibrios could be the result of a combination of several virulence factors (BAFFONE *et al.*, 2001).

Raw fish is an important vehicle for transmitting *V. parahaemolyticus* to the consumer especially in countries where marine fish is eaten raw, such as in Japan, where *V. parahaemolyticus* gastroenteritis is very common (RODRICK, 1991). In societies where seafood is eaten cooked, *V. parahaemolyticus* could survive if the fish is undercooked. The bacterium is very sensitive to heat and well-cooked products present no risk to the consumer. Another important way that *V. parahaemolyticus* is transmitted to humans is by cross-contamination of cooked foodstuffs with raw seafood or contamination in the processing environment (DESMARCHELIER, 2000). Since cuttlefish in Italy are normally well-cooked, cross-contamination would be the most likely way that foodborne pathogens could be transmitted to the consumer.

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