Vol. 14(8), pp. 374-379, August, 2020
DOI: 10.5897/AJMR2020.9372
Article Number: C32CF7164395
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Evaluation of the microbiological quality of "mabokés" smothered fish and, proteolytic activity of bacteria of the genus *Bacillus* in Brazzaville, Congo

Cyr Jonas Morabandza[1], Tarcisse Baloki Ngoulou[2*], Valentin Dibangou[2], Saphia Jemylah Galekoua Ndjiwa Empilo[1], Armel Faly Soloka Mabika[2], Christian Ngo-Itsouhou[2], Rachel Moyen[2] and Gabriel Ahombo[2]

[1]Normal Superior School, Marien NGOUABI University, BP.69. Brazzaville, Republic of Congo.
[2]Cellular and Molecular Biology Laboratory, Faculty of Sciences and Techniques, Marien NGOUABI University, BP.69 Brazzaville, Republic of Congo.

Received 9 June, 2020; Accepted 7 July, 2020

In order to assess the health risks related to the consumption of smothered fish "mabokés" sold in the markets of Brazzaville, samples of smothered fish sold in three markets in Brazzaville were analyzed for their microbiological quality. The different samples were cultured for isolation on solid media using conventional microbiological methods. The bacteria isolated were identified on the basis of cultural, morphological and biochemical characteristics. At the end of this analysis, 79 bacteria were isolated, of which 29 (36.70%) at site 2, 25 (31.65%) at site 1 and 25 (31.65%) at site 3. Of the 79 strains, 46 (58.22%) were Gram-positive bacteria and 33 (41.78%) were Gram-negative bacteria. These strains consisted of 34 (43.03%) bacteria of the genus *Bacillus*, 12 (15.18%) bacteria of the genus *Staphylococcus* and 33 (41.79%) *Enterobacteria* including 15 bacteria of the genus *Shigella*. The enumeration results show that the average value of the total flora varies from $2.90.10^8 \pm 1.5.10^7$ CFU/ml (Site 2) to $5.55.10^9 \pm 2.10^7$CFU/ml (Site 3), that of *Bacillus* between $1.30.10^7 \pm 1.41.10^6$ CFU/ml (Site 2) and $7.85.10^7 \pm 1.02.10^6$ CFU/ml (Site 3), for *Staphylococcus* between $1.44.10^6 \pm 2.02.10^6$CFU/ml (Site 1) and $6.17.10^6 \pm 8.25.10^6$CFU/ml (Site 2). The average value of *Enterobacteriaceae* ranges from $70.10^4 \pm 2.4.10^4$ CFU/ml (Site 2) to $9.15.10^5 \pm 1.29.10^4$ CFU/mL (Site 3) and from 0 (Site 3) to $5.65.10^4 \pm 7.85.10^4$ CFU/ml (Site 1) for *Shigella*. Proteolytic enzyme production was observed in 9 out of 12 selected strains. Although these smothered fish have strains with proteolytic activity, the presence of *Staphylococcus* and *Enterobacteriaceae* make this food unfit for consumption.

Key words: Microbiological quality, fish, smothered, proteolytic activity.

INTRODUCTION

Fish is an important nutritional supplement in a diet low in protein, vitamins and minerals (Degnon et al., 2012). Its worldwide consumption is increasing significantly. In 2016, out of the 171 million tons of fish produced in the

*Corresponding author. E-mail: tarcissebaloki@gmail.com.

world, 151 million tons were used for human consumption, 88% compared to 67% in the 1960s (FAO, 2008). To date, more than 400 million Africans regularly consume fish in all its forms (Worldfish, 2017). Congo, because of its hydrographic density, has different species of fish that contribute to the economic livelihoods of the population (Kimbatsa, 2016). The diversity of cultures in the world has resulted in a wealth of recipes and methods of fish preparation. In Congo, in rural areas as well as in urban areas including Brazzaville, fresh fish is eaten in stews, sauces, barbeques, fried in oil, grilled and stewed. Summers called smothered fish "mabokés" are very popular and eaten a lot. In addition to households, fish stews are prepared and sold in markets, on public roads, in restaurants and drinking establishments, and at fish auctions by itinerant women. Any food product intended for consumption and offered for sale must protect consumers from infection or contamination and be of good quality (Assogba et al., 2018). The presence and multiplication of microorganisms makes this type of food unsuitable and therefore presents a health risk that can cause gastroenteritis for the consumer. To date, no microbiological studies have been conducted on this product. This work is part of an effort to assess the microbiological quality of the smothered fish "mabokés" sold in some markets in Brazzaville in order to evaluate the sanitary conditions related to the consumption of these products and to determine the proteolytic activity of some strains of *Bacillus*.

MATERIALS AND METHODS

Sample collection, culture conditions, enumeration

Samples of stewed fish commonly known as "mabokes" in Congo Brazzaville, wrapped in leaves and stewed were purchased from three markets in Brazzaville. Three samples were purchased per market or site. At the time of purchase, the samples were placed in ice box containing ice bread and sent to the laboratory for analysis. The targeted sites were:

Site 1: Moukondo market located more or less in the center of the city,
Site 2: Total market located south of the city of Brazzaville,
Site 3: Moungali market located in the center of the city.

In a test tube containing 9 ml of distilled water, 1 ml of fish soup was added as the stock solution from which the decimal dilutions were made. The inoculations were carried out on Petri dishes in six different culture media including: PCA for the total count of microorganisms, Mannitol salt agar for the enumeration of bacteria of the genus *Staphylococcus*, *Salmonella -Shigella* Agar for the enumeration of *Salmonella* and *Shigella* bacteria, Methylene eosin blue for the enumeration of *enterobacteria*, Mossel Agar for the enumeration of bacteria of the genus *Bacillus* and Chloramphenicol Sabauraud for the enumeration of yeasts.

After the counting of appeared the colonies, the enumeration was carried out according to the formula (AFNOR, 1997).

$$CFU = \frac{N}{VD}$$

CFU/ml = Colony Forming Units, N = number of colonies, V = used volume of plating, D = dilution

Colony purification and isolation

Purification is one of the most important steps in strain isolation. It was carried out in nutrient agar. Each colony was seeded separately with streaks until distinct and homogeneous colony was obtained. To ensure the purity of the strains, microscopic observation was performed. The characterization of the isolates was started by the application of classical microbiology techniques, based on the search for a certain number of characters (cell morphology by the fresh status under the optical microscope, the type of Gram) (Mabika et al., 2020). Only *Bacillus* isolated and purified on Mossel Agar were stored at 4°C in cryotubes containing the appropriate broth (Luria-Bertani) with 20% glycerol added.

Proteolytic potential of some *Bacillus* strains

To test the production of the caseinolytic enzyme effect of twelve (12) strains of *Bacillus*, we used the techniques modified by Puri et al. (2002). The cells were cultured in Luria Bertani (LB) medium under agitation at 37°C, for 48 h. 1 ml of the culture was introduced into a 1.5 ml eppendorf tube and centrifuged at 6000 rpm for 5 min in a micro centrifuge. The supernatant was recovered. 2 ml of culture was used to measure optical density with the spectrophotometer at a wavelength of 600 nm (Nguimbi and Wu, 2002). In a 250-ml Erlenmeyer flask containing 100 ml of 0.1N PBS, we dissolved 1 g of agarose and heated it until completely dissolved. After cooling the mixture to 55-60°C, we added 10 ml skim milk and homogenized the mixture. It was poured into Petri dishes; after solidification, wells were prepared in the gel. 50 µl supernatant was placed in each well from the centrifugation of the culture. The plates were placed in the oven at 37°C for 12 h. Observation of a clear translucent zone indicates that the strain produces a proteolytic enzyme with a caseinolytic effect (caseinolytic protease) (Nguimbi et al., 2014; Mabika et al., 2017).

RESULTS

Bacterial isolation

A total of 79 bacteria were isolated from smothered fish. Of these strains, 29 (36.70%) were isolated from site 2 (total market), 25 (31.65%) from site 1 (Moukondo market) and 25 (31.65%) from site 3 (Moungali market) (Figure 1).

Identification

Of the 79 strains, 46 (58.22%) were Gram-positive bacteria and 33 (41.78%) were Gram-negative bacteria (Figure 2). These strains consisted of 34 (43.03%) bacteria of the genus *Bacillus*, 12 (15.18%) bacteria of the genus *Staphylococcus* and 33 (41.79%) *Enterobacteria* including 15 bacteria of the genus *Shigella* (Figure 3). Table 1 shows that *staphylococcus* was mostly isolated from Market 2 samples. *Shigella* spp. were not isolated from the samples from site 3, although higher total flora and *Bacillus* were observed. Yeasts were not isolated from the samples.

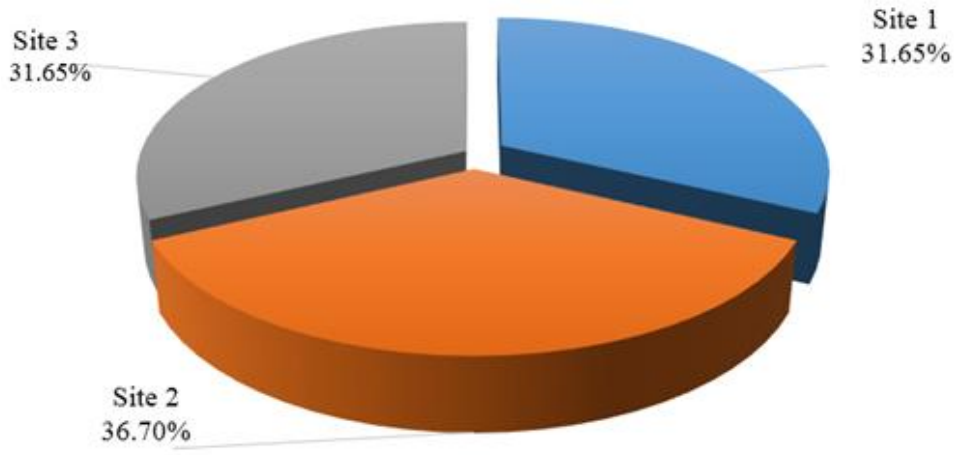

Figure 1. Isolation of bacteria numbers. Site 1: Moukondo market, Site 2: Total market, Site 3: Moungali market.

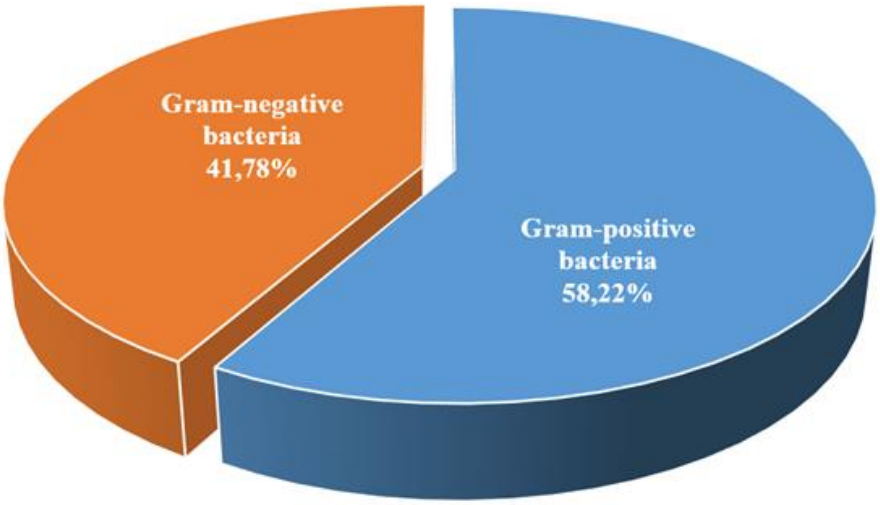

Figure 2. Repartition of isolated bacteria.

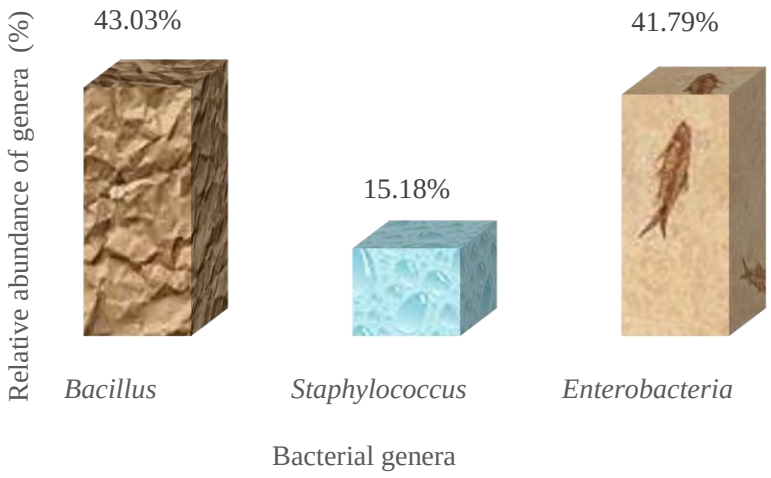

Figure 3. Relative abundance of strains by genera.

Table 1. Average values and standard deviations of microorganisms in CFU/ml.

Microorganisms	Average values ± Standard deviations		
	Site 1	Site 2	Site 3
Total flora	$1.61.10^9 \pm 2.10^8$	$2.90.10^8 \pm 1.510^7$	$5.55.10^9 \pm 2.10^7$
Genus *Bacillus*	$6.07.10^7 \pm 5.5.10^6$	$1.30.10^7 \pm 1.41.10^6$	$7.85.10^7 \pm 1.02.10^6$
Genus *Staphylococcus*	$1.44.10^6 \pm 2.02.10^6$	$6.17.10^6 \pm 8.25.10^6$	$3.45.10^6 \pm 1.06.10^6$
Genus *Enterobacteriaceae*	$9.15.10^5 \pm 1.29.10^4$	$1.70.10^4 \pm 2.4.10^4$	$2.73.10^4 \pm 1.65.10^4$
Shigella spp	$5.65.10^4 \pm 7.85.10^4$	$1.75.10^4 \pm 2.47.10^3$	00

Site 1: Moukondo market, Site 2: Total market, Site 3: Moungali market.

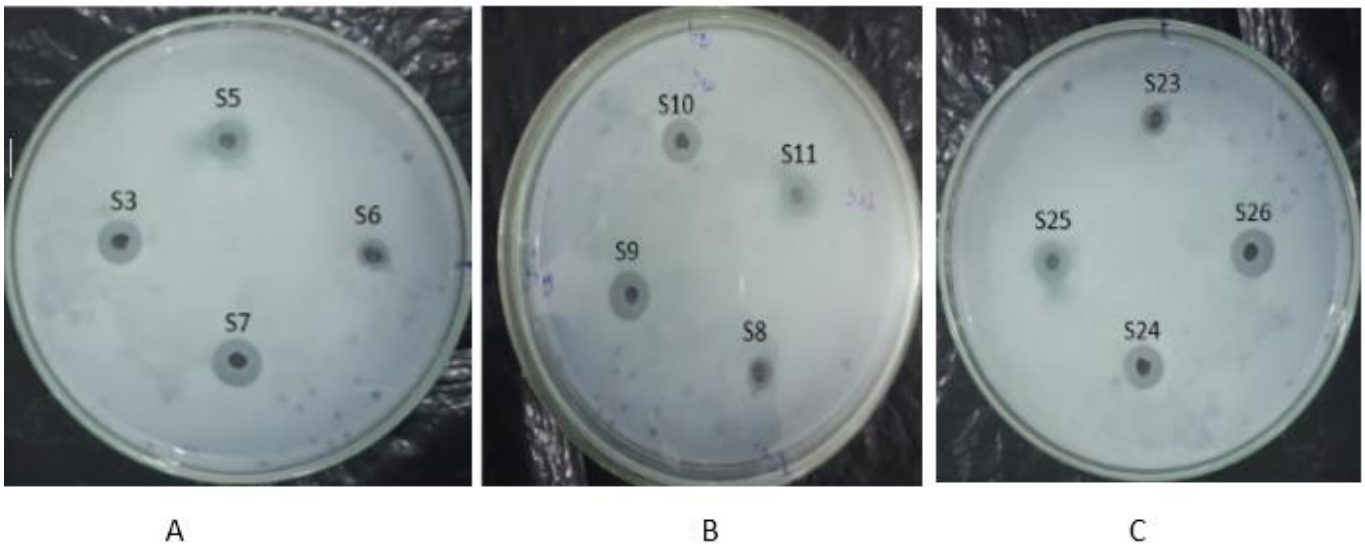

Figure 4. Proteolysis halos of the different strains. A: Site 1 strains; B: Site 2 strains and C: Site 3 strains.

Production of proteolytic enzymes

Detection of the production of a proteolytic enzyme

Figure 4 shows the stains produced by proteolytic enzymes. The smallest clear zones were observed in strains 6, 8 and 23. In the other strains, the diameter of the clear zone was variable.

Evaluation of proteolytic enzyme production

Here, the evaluation of proteolytic enzyme production is done simultaneously with growth, which is expressed as optical density (OD) and enzyme production in clear halo diameter. The values of the optical densities vary from strain to strain and range from 0.623 to 0.914. Strains 5.6, 11 and 26 showed the best growth. With the exception of strains 6, 8 and 23, the production of proteolytic enzymes was observed for the other selected strains. Enzyme production was very high for strains 4, 5, 24 and 26 (Figure 5).

DISCUSSION

The main objective of this study is to assess the microbiological quality of the smothered fish "mabokés" sold in some markets in Brazzaville in order to evaluate the sanitary conditions related to the consumption of these products and to determine the proteolytic production of some *Bacillus* strains. During this study, the average value of the microorganism varies from one site to another. The enumeration results show that the average value of the total flora varies from $2.90.10^8 \pm 1.5.10^7$ CFU/mL (Site 2) to $5.55.10^9 \pm 2.10^7$CFU/mL (Site 3), that of *Bacillus* between $1.30.10^7 \pm 1.41.10^6$ CFU/mL (Site 2) and $7.85.10^7 \pm 1.02.10^6$ CFU/ml (Site 3), for *Staphylococcus* between $1.44.10^6 \pm 2.02.10^6$ CFU/ml (Site 1) and $6.17.10^6 \pm 8.25.10^6$ CFU/ml (Site 2). The average value of *Enterobacteriaceae* ranges from $70.10^4 \pm 2.4.10^4$ CFU/ml (Site 2) to $9.15.10^5 \pm 1.29.10^4$CFU/ml (Site 3) and from 0 (Site 3) to $5.65.10^4 \pm 7.85.10^4$CFU/ml (Site 1) for *Shigella*. Yeasts were absent in all samples.

Variations in the microbial load from one sample to

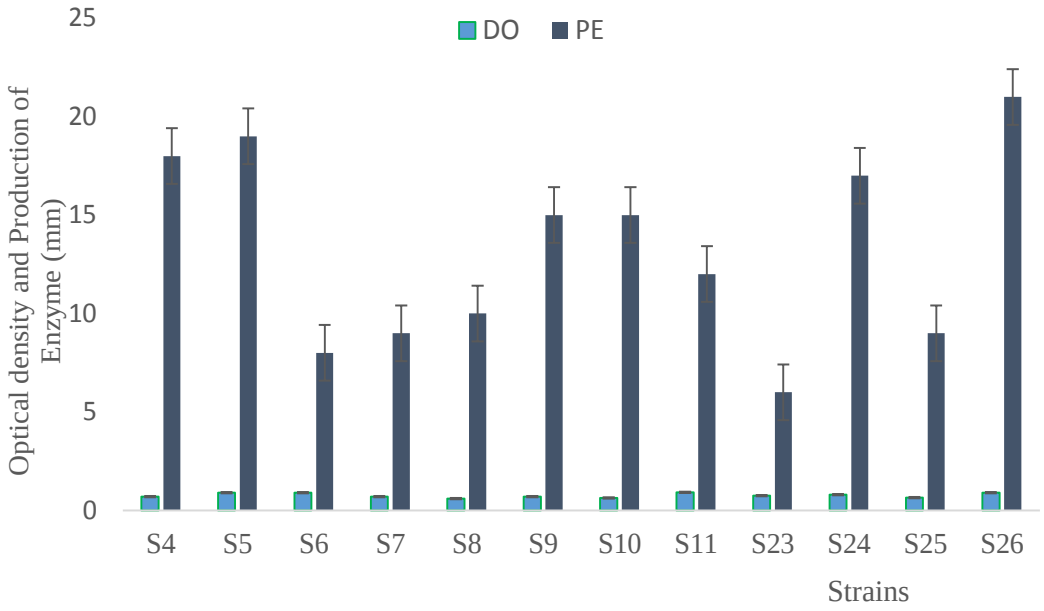

Figure 5. Optical density and proteolytic enzyme production of *Bacillus* strains.

another could be due to preparation and cooking conditions that often express a lack of hygiene that encourages various types of contamination. The presence of microorganisms indicates the alteration of the product in the different samples (Abotchi, 2010). The results on the total flora are similar to those reported in Senegal by Thiam (1993) ($3.4.10^8$ CFU/g) on the microbiological and chemical quality of braised-dried fish (kétiakh) sold on the Dakar market.

A lower average was reported by Watanabe (1974) (2.610^6 CFU/g), working on the technology and hygiene of salt-dried fish processing methods in Africa with special reference to Ghana, Senegal and Zambia. The work carried out by Mokemiabeka et al. (2016) in Congo on a fermented food revealed a total flora variable from one sample to less (68.10^5 CFU/mL) than that reported. Mabika et al. (2020), working on stewed squash sold in markets in Brazzaville, reported lower total flora loads ranging from $1.73.10^7$ CFU/g for the Moukondo Market to $9.43.10^7$ CFU/g (Tsieme Market). *Bacillus* loads ranged from 3.5. 10^6 CFU/g for the total market to 8.96. 10^6 CFU/g for the Tsieme Market. This charge is much lower than the one reported in this work. The presence of *Bacillus* could be explained by the fact that *Bacillus* sporulate and resist cooking temperatures (Ehon et al., 2015). This ability allows this genus to resist even antibiotics and thus opens the way to other more severe pathologies although, the *Bacillus* genus would contribute to the reduction of odours as demonstrated by some work on a mixed population of lactic acid bacteria and *Bacillus* in the microflora of cassava retting (Achi, 2006).

The count of yeasts and moulds in a product is a key indicator of its sanitary quality before it is placed on the market. It is often well-known species that cause undesirable changes in products (Laredj and Waffa, 2017). The absence of yeasts in the samples analyzed from the three sites indicates the freshness of the food. These results are contrary to the results of work on the microbiological quality of smoked fish, which demonstrated the presence of fungal flora (Abotchi, 2010). These results obtained, suggest that cooking as well as the closed environment in which the smothered fish is found, not being exposed to the outside environment, no fungal development is possible. These results also suggest that the batches analyzed were certainly prepared and disposed of on the same day they were acquired. Cleaning with water of the cassava leaves used for packaging of smothered fish could effectively contribute to the improvement of the microbiological quality of this food. The proteolytic (caseinolytic) test shows that 9 out of 12 selected strains showed better degradation of milk casein (Figure 4). This activity was demonstrated in Bacillus strains isolated from Ntoba mbodi and squashes in the Republic of Congo by Mabika et al. (2017, 2018, 2020). It is important to note the presence of proteolytic activity in strains isolated from fish in the smothered state. The genus Bacillus is recognized among bacteria that secrete bioactive substances such as proteases (Nguimbi and Wu, 2002), bacteriocins (Barboza et al., 2009; Mokemiabeka et al., 2016). These results on caseinolytic enzyme production in strains 4, 5, 24 and 26 with diameters between 15 and 21 mm are consistent with those reported by Adinarayana et al. (2003); Nihan and Elif (2011); Ehon et al. (2015); Mabika et al. (2017); Mabika et al. (2018); Ngo-Itsouhou et al. (2019) and Mabika et al. (2020).

Conclusion

This study made it possible to assess the microbiological quality of the smothered fish "mabokés" sold in some markets in Brazzaville in order to evaluate the sanitary conditions related to the consumption of these products and to determine the proteolytic activity of some *Bacillus* strains. Gram postive bacteria were mostly isolated from choking poisons. These include bacteria of the genus *Bacillus*, *Staphylococcus*. Gram-negative bacteria were *Enterobacteria*. The presence of these bacteria in this food indicates that the food is unfit for consumption. It would therefore be advisable to reduce consumption or even stop eating these smothered fish to avoid being exposed to foodborne diseases. On the other hand, the proteolytic test revealed strains of *Bacillus* with a high capacity to produce the caseinolytic enzyme.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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