ORIGINAL ARTICLE

Microbial and nutritional aspects on the production of live feeds in a fish farming industry

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Key words

Fish-farming • Microbial quality • Fatty acid

Summary

Aquaculture is an enterprise in constant development, in particular relating to its effect on the environment and also the quality of its products. It represents a valid alternative to traditional fishing, facing the increasing demand for fish products.
To guarantee to the consumer a product of high nutritional, organoleptic and hygienic quality, it is fundamental to monitor every phase of the fish farming industry, isolating the potential risk points.
For this reason there has been a rapid evolution of productive technique, particularly in the technology, artificial reproduction and feed sectors.
The aim of this research has been the monitoring of the evolution of certain microbial and nutritional quality indexes (total microbial counts and lipid analysis on suspensions of Rotifers and Artemia, used as live feed) in the larval phase of the productive cycle of the farm raised fish, in an intensive system. The study has shown an increment in the total microbial counts in the fish farming industry within the production of Rotifers and Artemia, more evident in the suspensions of Rotifers.
In addition the study has demonstrated that the maintenance phase, in the enrichment protocol, can reduce the EPA and DHA content. The results confirm the importance of microbial and nutritional control of the live feeds before they get supplied to fish larvae.

Introduction

In the last few years the aquaculture industry has assumed a very important role as an alternative to traditional fishing; an actual necessity to limit the environmental impact of commercial fishing, the abolition of some techniques and the restriction of the activity to allow for biological repopulation, has made fish breeding an enterprise in expansion and with an outlook to develop with time [1].
For these reasons, in Italy and in the other European countries, new productive techniques, especially in the artificial reproduction and feed sectors, as well as innovative technology of breeding are developing. Moreover, strategies for the control of the quality, hygiene and the food safety are in rapid evolution.
The production process to rear the marine fish strains, like sea bass, is very complex and the quality of the final product depends on several factors [2]. Therefore, for the optimization of the fish production, the industry must concern itself with all the productive phases, checking the quality of the products in all stages of production (live food, larvae, final product).
In particular, the availability of high quality juvenile stock, together with the environment of breeding, is of great importance, since it can significantly influence the quality of the final product. A larval feed of good quality is a fundamental requirement in order to get a commercial fish of good quality [3]. The successes that occur all over the world in the induced reproduction of sea strains are due mainly to the possibility to offer live prey to the larvae, in particular Rotifers (*Brachionus plicatilis*) and Artemia (*Artemia salina*) [4].
The production of live feeds in hatcheries represents a very critical phase in the breeding of many different species of fish because, it is technically possible to produce a feed of a high nutritional value, its hygienic quality is very low [5-7].
Many bacteria are able to surpress the growth of Rotifers and Artemia or to provoke an unexpected mortality [8]. In other cases the harmful effects occur in the larval stages, shown in low growth rates and low survival rates [9, 10]. Although the greater part of the bacteria is not pathogenic for Rotifers and Artemia, the risk of a potential accumulation end/or transfer of the bacteria along the trophic chain still exist.
The Rotifers represent the principal vectors of the bacteria [11, 12]. In fact, even when the breeding of Rotifers is carried out while respecting the hygienic norms [13, 14], the elevated culture density (billion of Rotifers) and the food used for the growth of the live feeds constitute an elevated organic load, which is quickly colonized by bacteria [15-18].
Usually the aerobic bacterial population varies from 10^7 to 10^{12} ufc/g in the live feeds and from 10^4 to 10^7 CFU/ml in the culture medium [14].
The Rotifers can concentrate until 10^5 CFU/individuos in their intestine and such bacterial accumulation could originate from the diet rather than from inside multiplication [15]. In the culture medium the bacterial concentration remains around 10^7 CFU/ml.

Under conditions of low food quality, the bacteria, fed on by the Rotifers, could compensate for the food deficit [19]. The *Vibrio anguillarum* causes a crash in Rotifer crops [20, 21].
Balompapueng et al. [22] discovered bacterial strains such as *Flavobacterium*, *Aeromonas* and *Vibrio sp*. resulted toxic for the Rotifer population. Comps and Menu [23] hypothesized that the infectious diseases (viral and mycotic) were in related to the low productivity of Rotifer cultures and to an abnormal mortality rate.
At the moment, most of the rearing systems use culture stock to produce live feed for their larvae. From a microbiological point of view, these systems can be very unpredictable, especially because after the rearing water disinfection there isn't any microbial control in those initial stages of the Rotifer culture.
In this context, the hygienic management of the larval rearing systems represents an issue of extreme importance, for it can strongly influence the larval survival and the hygienic quality of the final product.
The feeding of the larvae of rearing fish strains with live feeds represents a delicate and critical phase, especially considering the nutritional health of the fish. The use of live feed guarantees the availability of food with high nutritional characteristics, particularly under the lipidic profile. The lipids represent a nutritional class that is very important for the fish. In fact the lipids develop the energetic, functional and plastic faculties of the fish. In particular, the polyunsaturated fatty acids (PUFA) above all the highly unsaturated fatty acids (HUFA) (with a high number of carbon atoms), especially series ω3, are very important for the growth and reproduction of the adult fish; besides this they increase the survival rate of the larval forms [24-29]. In terms of this, the essential fatty acids eicosapentaenoic (20:5 ω 3 or EPA) and docosahexaenoic (22:6 ω 3 o DHA) are very important, since they represent the precursors of fatty acids and of molecules of high biological interest. Many organisms are able to convert the linolenic acid (18:3 ω 3) in EPA and DHA, however, sea fish are apparently incapable of it (Watanabe 1982) and therefore the presence of HUFA is fundamental in their diet. Although it is true that the freshwater fish are able to carry out this conversion, even for them the presence of EPA and DHA in the feed is important, probably because such an ability is not particularly efficient in maintaining optimal levels of growth. The larval forms seem to have a greater need for HUFA, probably because they have an elevated metabolism. It is very difficult to quantify exactly, the necessary quantity of HUFA, as it varies in relation to the larval stages, to the rearing strains and to the type of zooplankton [28, 30-32]. Since the nutritional quality of the zooplankton is not always suited to the demands of the rearing animals, techniques of enrichment are often used.

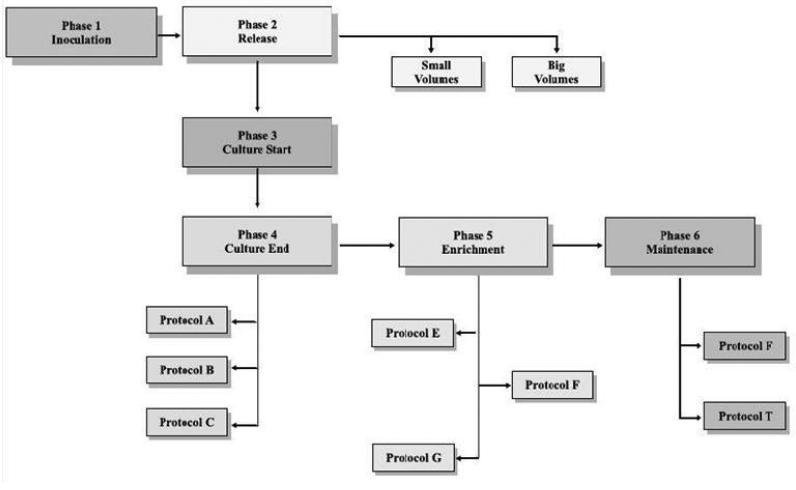
The Rotifers and the Artemia are fed with integrated diets using different protocols in accordance with the necessities and costs [33].
The aim of the present experimental work has been to estimate and to compare different protocols of enrichment employed within an intensive rearing system of bass and bream, in order to discover which is the safer, from a microbial point of view, to be employed within the phases of production of live feeds. Moreover, the nutritional characteristics, particularly the lipidic profile, were studied in order to understand the influence of different used protocols on the live feeds quality.
For this reason the microbial charges in Rotifer and Artemia samples, which came from this rearing system, were evaluated.

Materials and methods

Sampling

The microbial investigation on live samples of Rotifers and Artemia was preceded by a preliminary phase (about six months) of standardization of the methods.
The experimental plan used for this investigation is shown in the Figure 1, for the Rotifers, and in the Figure 2, for the Artemia.
The Rotifers were initially fed with crops of phytoplankton, yeasts and specific commercial products, and enriched with compounds with a high content of polyunsaturated fatty acids, 12 hours before their administration to the fish larvae.
This is a complex production that begins with a sterile algal monoculture in small volumes, culminating in the productions of Rotifers, in great volumes. To the final crop, different protocols of treatment were applied (symbolically indicated s in Fig. 1 with A, B and C) that correspond to common products used in commerce (Selco, HD Culture Selco - ® INVE - and Rotimac™ – BioMarine Aqua Fauna Inc.Hawborne,California, USA). In the next phase three different enrichment

Fig. 1. Experimental plan used for the microbiology analysis on the Rotifers.



protocols were tested, EASY DHA, FITO and DHA PROTEIN SELCO - ® INVE - (indicated with E, F e G in Figure 1). For the maintenance phase two different treatments were analyzed; a thermal treatment and a treatment with phytoplankton (T and F in Fig. 1).
The production of Artemia, consisted firstly of an incubation period, then the hatching of the cysts, followed by an enrichment phase, which used three different protocols (SPARI, SERRANI and SUPER SELCO - ® INVE - respectively indicated in Figure 2 with SP, SE and SS). The maintenance phase was performed through thermal treatment in a refrigerator for 12 hours.
The analyses were conducted in the month of November. Both the typologies of live feed Total Microbial Counts (TMC) were determined in three sample unit.
The collections for the microbial investigations were performed in each phase and for each protocol of treatment. The samples were taken using sterile containers, transported in refrigerated containers (4°C) and subjected to analysis within 24 hours of their collection.

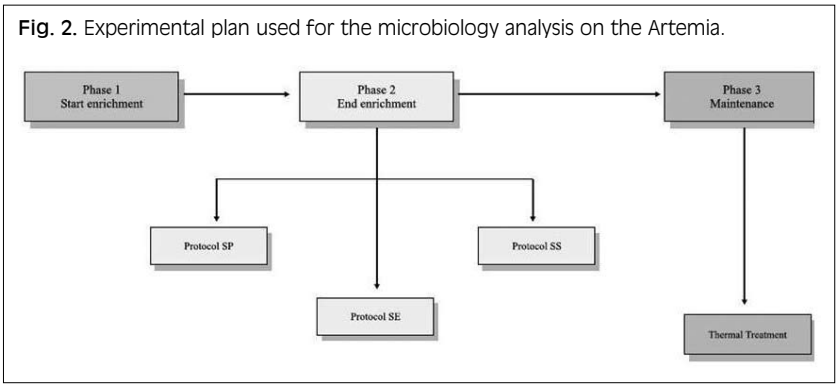


Fig. 2. Experimental plan used for the microbiology analysis on the Artemia.

Total Microbial Count (TMC) Evaluation

This parameter represents the cultivable viable microbial biomass. This analysis assesses the total number of viable microbial cells in 1 ml of sample and gives a general assessment of the hygienic quality of a considered sample. The assessment was carried out on suspensions of water + Rotifers (approximately 200 individuals per millilitre) or Artemia (approximately 1000 individuals per millilitre).
For each suspension scalar dilutions from 10^{-1} to 10^{-7} were prepared. Each dilution, with the original suspension, was seminated in double on Plate Count Agar (PCA, Biokar Diagnostic cod. BK 144HA, Beauvais, France) with 2% NaCl. The plates were incubated at 25°C for 48 hours.

Analysis of lipids

Four different samples of Artemia were analyzed, the first three were subjected to three different enrichments (SP, SE, SA) while the fourth sample, SE after the enrichment, was subjected to the maintenance phase for 12 hours at 4°C. Also for the Rotifers, four different samples were analyzed, the first three were subjected to three different enrichments (E, F, G) while the fourth sample was subjected to the maintenance phase for 8 hours at 4°C and then to the enrichment.
The analysis of lipids was conducted treating the samples with a mixture of chloroform/methanol [34] and the lipid extract was then submitted to the following transesterification through heat treatment (90°C for twenty minutes) with benzene and bore trifluorure in metabolic solution. With the resultant mixture of esters of fatty acids the gas-chromatographic analysis was conducted through gas-chromatography Hewlett Packard GC System, HP6890 with column HP 5890. The method used for the separation was a programmed ramp of which the oven column started at a value of 150°C for 4 minutes, increasing by 4°C every minute to reach a final temperature of 250°C which was maintained for 30 minutes. The total duration of the chromatographic run, being about 60 minutes. The temperature of the injector and detector was fixed at 250°C.
Helium was used as the transport gas in the column (mobile phase) with a constant flow of 1 ml/minute. The identification of the fatty acids was carried out through the comparison of the retention times of the peaks that emerged through the gas-chromatography, relating the our sample to a known standard. The standard mixture used was the "Mixture 37" (Supelco - Bellefonte, PA).
The abundance of the single fatty acids has been expressed as a percentage of the total identified fatty acids, through integration of the peaks revealed by the flame ionization detector with an integrator connected to the gas-cromatograph.

Statistical Analysis

The data were elaborated statistically using the software "Statgraphics® Plus".
For the TMC the mean, median, standard deviation, maximum and minimum were calculated. Besides this, to individualize the eventual differences and significance between the different phases and different treatments the variance analysis was subjected to an ANOVA test and a Student-Newman-Keuls post-hoc test, at a 95% confidence level.
The data from the analysis was plotted in a Box-and-Whisker diagram, that illustrates the distribution in quartiles, the maximum, the minimum, the mean and the median of the values relating to the parameter TMC in each phase or treatment.

Results

Total Microbial Count (TMC) Evaluation of Rotifers

The data, expressed as decimal logarithms of the TMC, recorded in the different phases of the work of the Rotifers, have been statistically elaborated and shown in the Figure 3, plotted in a Box-and-Whisker diagram.

Fig. 3. Box-and-Wisker Plots of TMC in the different phases of Rotifers production. Phases: 1 = Inoculation; 2 = Release; 3 = Culture Start; 4 = Culture End; 5 = Enrichment; 6 = Manteinance.

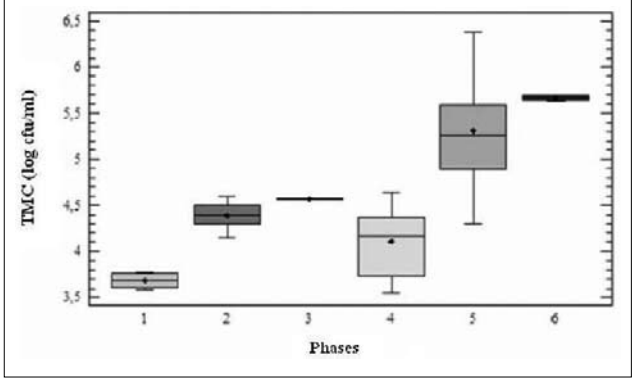
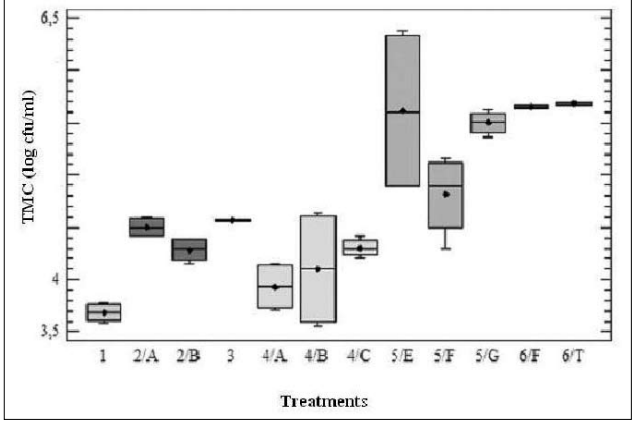


Fig. 4. Box-and-Wisker Plots of TMC in the different treatments, for each phases of Rotifers production. Phases: 1 = Inoculation; 2 = Release; 3 = Culture Start; 4 = Culture End; 5 = Enrichment; 6 = Maintenance. Protocols of treatment: A = Selco; B = HD Culture Selco - ® INVE; C = Rotimac™ – BioMarine Aqua Fauna Inc.Hawborne,California, USA; E = EASY DHA; F = FITO; G = DHA PROTEIN SELCO - ® INVE; T = thermal.



The TCM shows an increasing mean trend with initial values (phase 1) equal to 3,68 Log cfu/ml and final values (phase 6) of 5,67 Log cfu/ml.
The analysis of the variance (ANOVA) shows a significant difference between the TCM of the various phases at a 95,0% confidence level ($p < 0,05$). To determine the statistically significant mean, the Multiple Range Test was used, in accordance with the Student-Newmann-Keuls procedure. The significantly different TMC values have been underlined between phase 1, the group composed of the phases 2, 3 and 4 and the group composed of the phases 5 and 6. In terms of the data relating to the various treatments used in each phase (Fig. 4), statistically significant differences ($p < 0,05$) have been found only between the treatments of the phase 5. In particular the treatment 5/F (FITO Enrichment Protocol) shows TMC values significantly lower than the treatments 5/E (EASY DHA Enrichment Protocol) and 5/G (DHA PROTEIN SELCO Enrichment Protocol).

Total Microbial Count (TMC) Evaluation of Artemia

The data, expressed as decimal logarithms of the TMC, recorded in the different phases of the work of the Artemia, have statistically been elaborated and are shown in Figure 5, plotted in a Box-and-Whisker diagram.
The TCM shows an increasing mean trend with initial values (phase 1) equal to 5,35 Log cfu/ml and final values (phase 3) of 5,98 Log cfu/ml.
The analysis of the variance (ANOVA) records a significant difference between the TCM of the various phases at a 95,0% confidence level ($p < 0,05$).
Also in this case the Multiple Range Test in accordance with the procedure of Student-Newmann-Keuls was used to determine the statistically significant mean. Each phase has shown significantly different TMC mean values, at a 95% confidence level ($p < 0,05$). The analysis of the variance and the Multiple Range Test applied to the different treatments in each phase (Fig. 6), highlights that only the treatment with SP (SPARI Enrichment Protocol) shows TMC values statistically higher ($p < 0,05$) compered to those found in SE (SERRANI Enrichment Protocol) and SS (SUPER SELCO Enrichment Protocol).

Fig. 5. Box-and-Wisker Plots of TMC in the different phases of Artemia production. Phases: 1 = Start Enrichment; 2 = End Enrichment;; 3 = Manteinance.

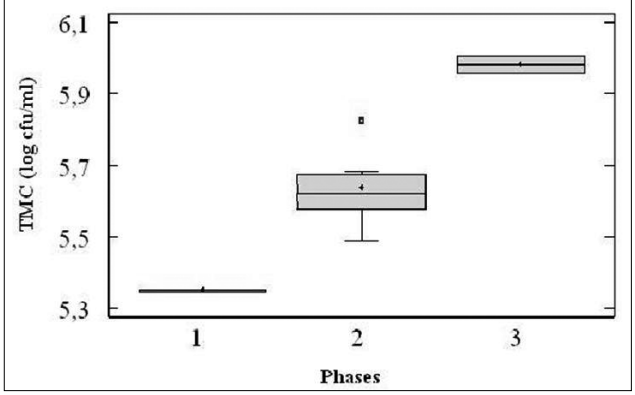
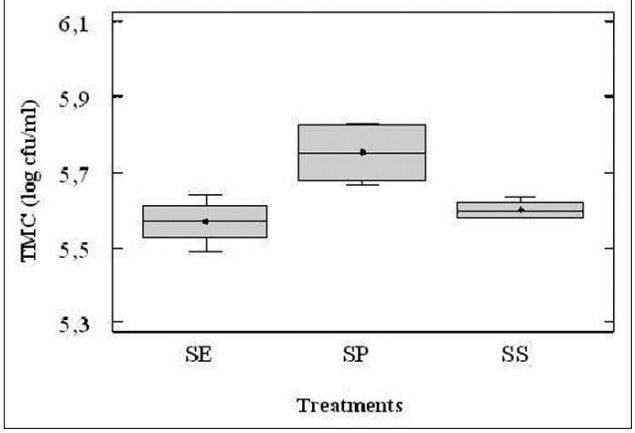


Fig. 6. Box-and-Wisker Plots of TMC in the different treatments, for each phases of Artemia production. Protocols of treatment: SE = SERRANI; SP = SPARI; SS = SUPER SELCO - ® INVE.



Evaluation of the fatty acids profile

The analysis of lipids conducted on the samples of the rotifers (Figure 7 and Figure 8) has not shown particular differences in the fatty acids profile following the protocols of enrichment, except a minor percentage of acid docosahexaenoic (DHA) in treatment F. The results obtained by the analysis of the Artemia (Figs. 9, 10) show a higher percentage of saturated fatty acids (SFA) relative to the treatment SE although it's not far from the other treatments in the polyunsaturated fatty acids profile (PUFA) recording other than having an elevated quantity of eicosapentaenoic acid (EPA). The SP enrichment protocoll has shown a higher percentage of PUFA, especially in the DHA quantity.

Very different data was obtained from the maintenance protocol: in some cases similar percentages were recorded before and after the treatment, in others, an increase of the saturated fatty acid content was observed for the Rotifers, while for the Artemia it was the monounsaturated fatty acid quantity. A diminution in the EPA quantity was observed after the maintenance phase both for the Rotifers and for the Artemia. A reduction of the DHA content was also recorded after the same phase, but only for the Rotifers.

Discussion and conclusions

Both in the case of the Rotifers and of the Artemia a progressive increase of the total microbial charge was recorded in the various phases of production, always starting from values that were on average low. This result was more evident for the Rotifers, whose charge moved from 3.68 Log cfu/ml to 5.67 Log cfu/ml, in comparison to the Artemia that from an initial charge of 5.35 Log cfu/ml a final charge of 5.98 Log cfu/ml was recorded. In accordance with the results recorded in literature, we can say that the values found don't highlight any particularly critical situations.

In fact, usually the density of aerobic bacteria in the culture water ranges from 1 x 10^4 (4 Log) to 1 x 10^7 (7 Log) cfu/ml [12, 35]. In particular the Rotifers culture water the bacterial concentration are in the order of 10^7 cfu/ml [36].

The highest increase of microbial charge found in the production of the Rotifers could possibly be attributed to the higher number of phases involved in the same production process.

The results related to the various protocols adopted in the Rotifer production industry showed statistically significant differences ($p < 0,05$) between the treatments of the enrichment phase. Especially the enrichment with phytoplankton (Protocol F) recorded TCM values significantly lower than the EASY DHA (Protocol E) treatments and than the DHA PROTEIN SELCO (Protocol G) treatment.

Analogous studies on samples of Artemia have pointed out that the enrichment with the commercial product DHA SELCO, containing the antimicrobial substances, represents a valid alternative to enrichment with phytoplankton; nevertheless it's clearly specified that its antimicrobial properties are inferior to those of the phytoplankton [37]. The nutritional benefits of the algae, on the other hand, is extensively documented and their potential

Fig. 7. Fatty acids profile of Rotifers in the different protocols of enrichment.

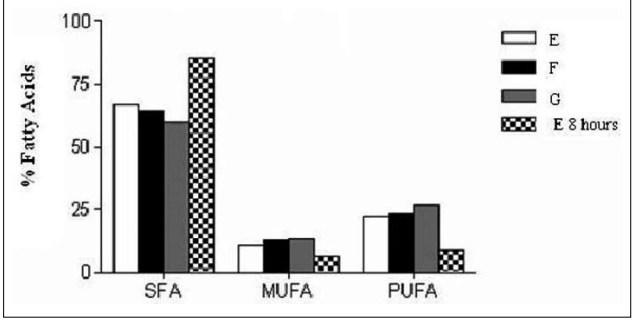


Fig. 9. Fatty acids profile of Artemia in the different protocols of enrichment.

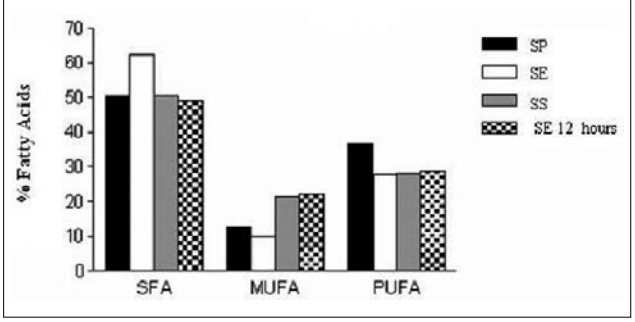


Fig. 8. Percentage of acids eicosapentaenoic (EPA) and acid docosahexaenoic (DHA).

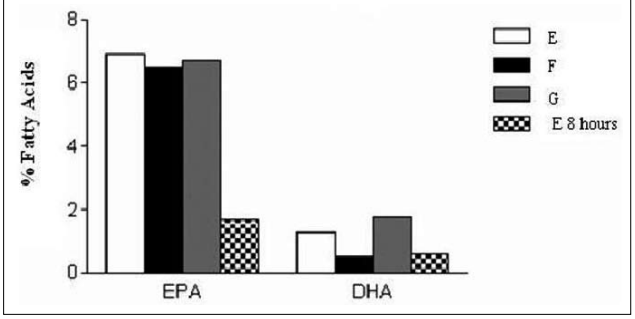
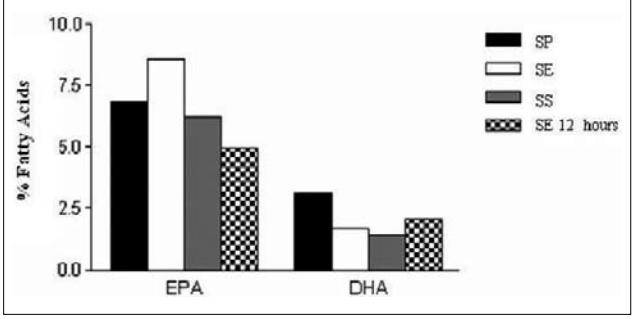


Fig. 10. Percentage of acids eicosapentaenoic (EPA) and acid docosahexaenoic (DHA).



employment as agents of biocontrol in aquaculture has been recognized [38]. From the analysis of the results related to the various treatments employed in the experimentation of the Artemia production has been shown that only the treatment with SP (SPARI enrichment protocol) has TCM values, significantly higher ($p < 0,05$) than to those found with SE (SERRANI enrichment protocol) and SS (SUPER SELCO enrichment protocol).
For Rotifers the different food enrichment protocols don't seem to determine particular differences in the fatty acids profile; some differences have been found however for Artemia.
The maintenance protocols at 4°C seem to bring up some interesting indications, above all relative to the profile of essential fatty acids, EPA and DHA.

As documented in literature [39, 40], the reduction in EPA and DHA observed suggests that the maintenance phase in the enrichment protocol is particularly delicate. It also points out that the content of EPA and DHA could represent a good indicator of the quality and efficiency of the integration practices that the live feeds, destined for use as feed in aquaculture, are submitted.
Comparing the chemical composition of these commercial products (fatty acids, phosphorus, DHA/EPA, vitamins, antioxidants, ecc.) substantial differences don't emerge. For this reason and for a better interpretation of the results it would be interesting for these aspects to be investigated in greater depth in the future research.

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