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How to Fortify a Fish Burger with Probiotic Microorganisms

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Abstract

In this study, the suitability of fish as substrate to be fortified by a probiotic bacterial strain was investigated. *Lactobacillus rhamnosus* GG (LGG) was used to the aim of the work. The study was first focused on the optimization of LGG microencapsulation by water-in-oil emulsion technique to avoid bacterial viability loss during cooking. Once the best microencapsulation conditions were individuated, the amount of microencapsulated probiotic to be added to the burger was increased to assure desired levels of viable LGG in the cooked fish burger. To assess the efficiency of the adopted fortification method, acid lactic bacterial count was monitored during every step of microcapsules preparation process as well as in both cooked and uncooked enriched fish samples. In order to evaluate the final acceptance of the fortified burger, the sensory properties of fortified fish burger were also assessed. Results demonstrated that proper microencapsulation conditions together with appropriate concentration of microencapsulated LGG into fish formulation would allow realizing probiotic-fortified burgers, also prized from a microbiological and sensory point of view with a consequent longer shelf life.

Keywords: Microencapsulation; *Lactobacillus rhamnosus* GG; Fish burger; Fish fortification

Introduction

Growing public awareness of diet-related health issues has fueled demand for foods with distinct health-promoting effects. Functional foods are products that have been enriched with added nutrients or other substances that are considered to provide health benefits over and above their nutritional value. Among functional foods, products with probiotic microorganisms represent the example most diffused. The contribution of probiotic bacteria, mainly *lactobacilli*, to provide health effects is well documented and numerous investigations have underlined the benefits deriving from a regular intake of foods fortified with this kind of microorganisms [1-5]. Probiotics are widely used, mainly in fermented dairy products such as milk drinks and yoghurts, beside fruit juices or drinks [6-8]. In addition, there are examples of probiotic sausages with *Lactobacillus rhamnosus* strains, dried fruits vacuum-impregnated with *Lactobacillus casei* subsp. *rhamnosus*, oat-based cereal bar including *Bifidobacterium lactis*, table olives enriched with *L. rhamnosus*, *Lactobacillus paracasei*, *Bifidobacterium bifidum* and *Bifidobacterium longum* [8]. The *Lactobacillus rhamnosus* GG in particular, is a well attested clinical bacterial strain widely used as probiotic culture in dairy food [9,10]. It is able to reduce the severity of diarrhea associated with rotavirus in infants, antibiotic-associated diarrhea in children and traveler diarrhea [11,12]. In order to provide health benefits, probiotic bacteria must be present at a minimum level of 10^7 CFU/g [13] into the food product at the time of consumption. Several factors have been identified as critical to microbial cell survival in food products, in particular food product cooking [14]. Approaches investigated to enhance probiotic survival include for example, the microencapsulation and exploitation of the adaptive mechanisms of living cells for survival under stress conditions. To date, the protection of probiotics by microencapsulation in hydrocolloid beads has been investigated for improving their viability in food products and intestinal tract [15]. Additional benefits of microencapsulation include cell protection from bacteriophages, survival during processing and stability during storage [16,17].

Despite the afore-mentioned research, at the best of our knowledge, the suitability of fish products as a substrate to be fortified by means of probiotic bacterial strains has not yet been investigated. The incorporation of probiotic bacteria into ready-to-cook fish, apart from being a novelty, would add functional features to their already high nutritional value. In fact, in addition to proteins and trace elements (particularly selenium), the high nutritional value of fish is mainly due to their lipid composition: fish contain high levels of Polyunsaturated Fatty Acids (PUFAs), mainly Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA), which are recognized to protect against cardiovascular diseases, to prevent or delay the clinical manifestations of certain cancers and to alleviate some affective and psychiatric disorders [18]. Sea bass, in particular, is rich in potassium, phosphorus, iron and vitamins (B group and D-calciferol). It is classified as a low fat fish with high protein content and a low cholesterol amount. Therefore, due to the lack of knowledge about fortification of fish with probiotic bacteria, the goal of this study was to optimize the microencapsulation technique of *Lactobacillus rhamnosus* GG (LGG) to realize a fortified ready-to-cook fish burger. In particular, a water-in-oil emulsion technique was first optimized and once the best conditions were individuated, the probiotic amount in the burger was increased to the desired concentration level. To the aim of the work, the concentration of acid lactic bacteria was monitored in both microcapsules and fortified fish samples. Once the microencapsulation technique was optimized a preliminary sensory test was conducted in order to evaluate the final acceptance of the new burgers and to choose the better formulation. On the basis of these considerations, microbiological and sensory quality indices were monitored on the best formulations individuated, to study the influence of the microencapsulation technique on the product quality during storage.

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Materials and Methods

Preparation of gelatin microcapsules

Gelatin microcapsules were prepared with a water-in-oil emulsion technique. In the specific, firstly 2 g (w/v) of LGG frozen powder (Granarolo, Italy) were dispersed in a 40% (w/v) gelatin suspension (280 bloom) (Farmalabor, Italy) (dispersed phase) and once completely solubilized, the dispersed phase was quickly added to the sunflower seed oil continuous phase in a 25:50 (v/v) ratio. The mixture was stirred and homogenized to form a water-in-oil emulsion and subsequently it was cooled in ice for 15 minutes to allow the formation of microcapsules. For the microcapsules formation, two different approaches were compared: the recovering of the gelatin microcapsules with and without washing and filtering them. Once the best methodology has been chosen, the water-in-oil ratio was changed to 40:40 (v/v) and the amount of LGG frozen powder to be encapsulated was increased to 8 g (w/v).

Fish burger samples preparation

Bass from Adriatic Sea (*Dicentrarchus labrax*) was purchased from fisherman in the Gulf of Manfredonia (Foggia, Italy). The fishes were directly transferred to the laboratory in polystyrene boxes containing ice within 2 h after purchase. Then, fishes were decapitated, eviscerated, fileted and washed. After being cleaned, basses were cut into cubes, minced with a mincer (Everest, Sbarlati and C., s.n.c Rimini, Italy) and worked by hand to get fish burgers of 25 g. These samples were analyzed uncooked and cooked (CNTR-U and CNTR-C). Fortified fish samples were prepared by adding 10% (w/w) of gelatin microcapsules, obtained with and without the washing step, to the raw fish. The samples will be named F-U-Mic10 and F-C-Mic10, F-U-Mic10-W and F-C-Mic10-W, for uncooked and cooked burgers prepared with and without washed microcapsules, respectively. These samples were compared with fish burgers prepared by directly addition of the same percentage of LGG (10% w/w) in the form of frozen powder (F-U-Pow10 and F-C-Pow10). In the second trial, the gelatin microcapsules percentage added to fish burgers was increased to 32% (w/w) (F-U-Mic32 and F-C-Mic32). In this trial, the probiotic powder directly added to raw fish burgers was set to 0.8% (w/w) (F-U-Pow0.8 and F-C-Pow0.8) to give uncooked fish burgers with about 10^8 ufc/g LAB. All samples were analyzed immediately after their preparation. The cooking procedure consisted in a two minutes per side steaming of each fish burger. To improve clarity about the nomenclature, all the investigated samples were labeled as listed in Table 1.

Microbiological analyses

To prove the nutritional characteristics of the fish burgers, the count of viable lactic acid bacteria (LAB) was carried out during the all the phases of the preparation and specifically:

- On the LGG frozen powder
- On the gelatin solution containing LGG powder in realize the microcapsules
- On the probiotic gelatin microcapsules
- On cooked and uncooked fish samples with gelatin microcapsules
- On cooked and uncooked fish samples with LGG powder
- On cooked and uncooked fish samples without any addition

For the LAB count in the frozen powder, in the gelatin solution and in the microcapsules, 1 g of powder or 1 mL of solution or 1 g of microcapsules were diluted with physiological solution (0.9% NaCl). The decimal dilutions were plated on MRS Agar supplemented with cycloheximide (0.1 g L-1106, Sigma) and incubated anaerobically at 30°C for 2-4 days. It is worth noting that to physically destroy the microcapsules, it was necessary to keep them at 37°C for some hours. For the LAB count in fish samples, the fillets (25 g) were diluted with 225 ml of sterile physiological solution (0.9% NaCl) in a Stomacher bag (Seward) and homogenized for 1 min in a Stomacher LAB Blender 400 (Pbi International, Milan, Italy). Subsequently, decimal dilutions

Samples	Abbreviation
Uncooked fish burger without any probiotic addition	CNTR-U
Cooked fish burger without any probiotic addition	CNTR-C
Uncooked fish burger with 10% of LGG in the form of frozen powder	F-U-Pow10
Cooked fish burger with 10 % of LGG in the form of frozen powder	F-C-Pow10
Uncooked fish burger with 0.8% of LGG in the form of frozen powder	F-U-Pow0.8
Cooked fish burger with 0.8% of LGG in the form of frozen powder	F-C-Pow0.8
Probiotic frozen powder	FP
Gelatin solution	GS
Gelatin microcapsules obtained without washing and filtering procedures	Mic
Gelatin microcapsules obtained with washing and filtering procedures	Mic-W
Uncooked microencapsulated fish-burger with 10% of microcapsules	F-U-Mic10
Cooked microencapsulated fish-burger with 10% of microcapsules	F-C-Mic10
Uncooked microencapsulated fish-burger with 10% of washed microcapsules	F-U-Mic10W
Cooked microencapsulated fish-burger with 10% of washed microcapsules	F-C-Mic10W
Uncooked microencapsulated fish-burger with 32% of microcapsules	F-U-Mic32
Cooked microencapsulated fish-burger with 32% of microcapsules	F-C-Mic32
Uncooked microencapsulated fish-burger with 32% of washed microcapsules	F-U-Mic32W
Cooked microencapsulated fish-burger with 32% of washed microcapsules	F-C-Mic32W

Table 1: Experimental samples.

of homogenates were made using the same diluent and the dilutions were plated on MRS Agar (Oxoid), supplemented with cycloheximide (0.1 g L-1106, Sigma) and incubated anaerobically at 30°C for 2-4 days. In order to study the effect of microcapsules addition on the microbiological quality of fish burger over the time, an evaluation of the principal microbial group growth have been done. Twenty grams of sea bass fillets were aseptically removed from each package, diluted with 180 mL of NaCl solution 0.9% in a stomacher bag and homogenized with a Stomacher LAB Blender 400 (Pbi International, Milan, Italy). Subsequently, decimal dilutions of homogenates were made using the same diluent and the dilutions were plated on appropriate media in Petri dishes.

The media and the conditions used were: Plate Count Agar (PCA) incubated at 30°C for 48 h for aerobic plate count (APC) [19]; Pseudomonas Agar Base (PAB), with added Cephaloridine Fusidin Cetrimide (CFC) supplement, incubated at 25°C for 48 h for Pseudomonas spp.; pour plated Iron Agar (IA), incubated at 25°C for three days, for hydrogen sulphide-producing bacteria (HSPB); spread plated chilled IA, supplemented with 5 g/l NaCl and incubated at 15°C for 7 days, for psychrotolerant and heatlabile aerobic bacteria (PHAB); violet Red Bile Glucose Agar (VRBGA) incubated at 37°C for 18-24 h for the enumeration of *Enterobacteriaceae*. The conditions used during the counts of HSPB and PHAB were those suggested by the Nordic Committee on Food Analyses, with regard to fish and fishery products [20]. For fresh water and marine species, the microbiological limit recommended (MAL^{APC}) by the [19] for APC at 30°C is 7 log/g or log/cm^2, as regard hydrogen sulphide-producing bacteria (MAL^{SH}) and *Enterobacteriaceae* (MAL^{Enter}), a cell load equal to 6 log CFU/g and 5 log CFU/g, respectively is required to spoil chilled fish [21] indicates a significant degradation. All samples were analyzed in duplicate. All media were supplied from Oxoid (Milan, Italy). All analyses were performed in duplicate on two different samples.

Sensory analyses

The sensory evaluation was conducted according to the guidelines of Codex Alimentarious Commission [22]. The trained panel consisted of 10 panelists (students and researchers of the Department of Agricultural Sciences, Food and Environment of the University of Foggia). The panel training consisted of 6 different sessions (2/day, 2 h/session) during which different samples were examined to define the evaluation technique and to familiarize with the off-odor, the texture, and the color attributes of anchovies. During this stage, triangle tests were performed to evaluate the reproducibility of the judge's answer and their capability in discriminating among samples. During the test sessions, burger samples were coded by a letter and presented individually to each panelist in plastic cups covered with a lid in random order. The sensory quality of the fish burgers under investigation was evaluated both in the optimization phase and during storage; it was determined using a scale ranging from 0 to 9 (where 0=very poor and 9=excellent). Panelists were asked to base their decision evaluating color, odor and texture attributes. When the attribute was defined unacceptable the score declined to or under 5 (SAL) that was set as the threshold for acceptability. In addition, on the same scale, samples overall quality was also asked to be evaluated, as an average of the three sensory attributes evaluated by each assessor.

Shelf life calculation

Shelf life was determined as the lowest value among the considered quality indices. To calculate the microbial acceptability limit (MAL), which was intended as the storage time at which the viable cell concentration of total mesophilic bacteria reached the threshold of 10^7 colony-forming units (CFU) g^{-1}, the viable cell concentration of hydrogen sulphide-producing bacteria (MAL^{SH}) and *Enterobacteriaceae* (MAL^{Enter}), resulted equal to 10^6 and 10^5 colony-forming units (CFU) g^{-1}, respectively. The reparametrized Gompertz equation was used [21,23]. In order to determine the sensory acceptability limit (SAL) in terms of overall quality, the same modified version of the Gompertz equation was used to fit the sensory data, as also reported elsewhere [23]. A score equal to 5 represented the threshold for sensory acceptability.

Statistical analysis

Experimental data were compared by one-way Anova analysis. A Duncan's multiple range test, with the option of homogeneous groups ($P<0.05$), was used to determine significance among differences. To this aim, Statistica 7.1 for Windows 152 (StatSoft Inc., Tulsa, OK, USA) was used.

Results and Discussion

A new method to produce fortified ready-to-cook fish burgers was proposed. In particular, a water-in-oil emulsion-based microencapsulation technique to protect probiotic microorganism viability from cooking process was investigated. The study consisted in the optimization of the technique to entrap the LGG and then in the improvement of the final fish burger probiotic level without changing its sensory properties. To assess the efficiency of the adopted fortification method LAB viable count was analyzed at each step of the fish burger production process and the sensory properties of the fortified burger were also assessed. In the following, results related to microbial and sensory evaluation were reported and discussed separately. Once the microencapsulation technique was optimized, a sensory evaluation was carried out to choose the better formulations as a compromise between the functional properties and the sensory perception. On the basis of these considerations, microbiological and sensory quality indices were monitored on the best formulations individuated, to study the influence of the microencapsulation technique on the product quality during storage.

LAB viable count

Gelatin microcapsules, after being recovered by washing and filtering, were immediately added to the raw fish meat at 10% (w/w). Table 2 describes the enumeration of LAB viable cell load (log cfu/g) during all the steps of microcapsules formation (FP, GS and Mic) and on raw and cooked fish burgers (F-U-Mic10-W and F-C-Mic10-W). In order to understand if the microencapsulation technique enhanced probiotic survival, the above-mentioned fortified samples were compared with similar burgers where LGG was directly added in the form of frozen powder (F-U-Pow10 and F-C-Pow10) and with fish burgers without any probiotic addition (CNTR-U and CNTR-C). As it can be inferred from data listed in Table 2, there was about two log cycles LAB decrease from the probiotic frozen powder (10.78 log cfu/g) to the final gelatin microcapsules (8.59 log cfu/g) and about one log cycle decrease respect to the gelatin probiotic solution (9.39 log cfu/g). Considering that no thermal treatments have been applied during the microcapsules preparation, these log cycle differences between the probiotic frozen powder and the gelatin solution and between the frozen powder and the final microcapsules, could be caused only by a dilution effect, determined by the water added to form the gelatin solution and the water added to wash the final microcapsules. The addition of gelatin microcapsules to fish turned out to be quite effective in the burgers functionalization because uncooked samples revealed

a LAB viable count of 7.47 log cfu/g while the cooked samples 6.47 log cfu/g. The effectiveness in the probiotic protection is more evident when these samples are compared with the same ones where the probiotic was added in the form of frozen powder (F-U-Pow10 and F-C-Pow10). In this case in fact, while the uncooked samples recorded a high LAB count (9.08 log cfu/g), the cooking procedure caused four log cycles LAB decrease (5.33 log cfu/g). This finding can be explained by the protection exerted by the microencapsulation towards probiotic heat sensitivity, compared to the powder that was directly exposed to the heat stress of cooking [24]. As one would expect, samples without any probiotic addition did not show LAB enumeration [25].

Even though the results with the first microencapsulation technique were interesting, LAB count in cooked samples was not enough to reach the imposed limit to define a food product as a probiotic source (10^7 CFU/g). Therefore, to solve this problem the microencapsulation technique was slightly changed by only passing the microcapsules through a strainer in order to facilitate their separation, instead of washing them with water. After production, the microcapsules were added to the raw fish at 10% (w/w) to realize new fortified burgers. As it can be seen in Table 3, without washing the microcapsules, only 1 log cycle loss was recorded between the initial gelatin solution and the final microcapsules, in this way the dilution effect determined by the water added to wash the microcapsules was solved. This recovery has also enhanced the probiotic count in the raw fish burger (F-U-Mic10) where a probiotic concentration of 8.05 log cfu/g was found and in the sample after cooking (F-C-Mic10) where the LAB count accounted for about 7.36 log cfu/g, thus within the limit to define a food product as functional. It is also worth noting from the same Table 3 that results obtained without washing were very significant if compared with the uncooked burgers with the frozen powder, where cooking caused a great loss of LGG. Specifically, the F-C-Pow10 samples recorded a LAB count of 5.37 log cfu/g, thus revealing again a relevant probiotic loss during cooking. On the basis of these results, the microencapsulation technique without washing represented the best approach between the two methods tested to improve the probiotic retention. In order to further enhance the probiotic amount in the fish burger at the time of consumption, both the amount of frozen powder to be encapsulated (8 g w/w instead of 2 g w/w) and the quantity of gelatin microcapsules to be added to the fish formulation were increased. The results of this experimental step were reported in Table 4. As shown in this table, the increment of gelatin microcapsules in fish revealed a great LAB count in the enriched burgers. The concentration in the F-U-Mic32 sample was found around 9.07 log cfu/g and so one log cycle higher than the previous trial (see F-U-Mic10 in Table 3). Even though one log cycle LAB loss was found in the cooked sample (F-C-Mic32), a more than acceptable final LAB concentration was recorded (8.13 log cfu/g), thus suggesting that the fortification conditions were suitable to realize valid products. Also in this case the utilization of frozen powder in the fish formulation was found not useful because a drastic probiotic loss after cooking was found (from 8.42 log cfu/g in the uncooked sample to 4.03 log cfu/g in the cooked burgers).

Samples	LAB
	log cfu/g
FP	10.78 ± 0.01 i
GS	9.39 ± 0.07 g
Mic-W	8.59 ± 0.06 f
F-U-Mic10-W	7.47 ± 0.03 e
F-C-Mic10-W	6.47 ± 0.03 d
F-U-Pow10	9.08 ± 0.01 h
F-C-Pow10	5.33 ± 0.02 c
CNTR-U	1.47 ± 0.10 b
CNTR-C	2.0 ± 0.00 a

[a-i]Means in the same column followed by different superscript upper cases are significantly different (p<0.05)

Table 2: LAB count in raw and cooked fish burgers with 10% (w/w) of washed gelatin microcapsules.

Samples	LAB
	log cfu/g
FP	10.63 ± 0.02 i
GS	9.37 ± 0.01 g
Mic	9.05 ± 0.01 f
F-U-Mic10	8.05 ± 0.01 e
F-C-Mic10	7.36 ± 0.02 d
F-U-Pow10	9.50 ± 0.01 h
F-C-Pow10	5.37 ± 0.07 c
CNTR-U	1.47 ± 0.10 b
CNTR-C	1.0 ± 0.00 a

[a-i]Means in the same column followed by different superscript upper cases are significantly different (p<0.05).

Table 3: LAB count in raw and cooked fish burgers with 10% (w/w) of gelatin microcapsules obtained without washing.

Samples	LAB
	log cfu/g
FP	10.64 ± 0.02 i
GS	9.69 ± 0.01 h
Mic	9.62 ± 0.00 g
F-U-Mic32	9.07 ± 0.02 f
F-C-Mic32	8.13 ± 0.02 d
F-U-Pow0.8	8.42 ± 0.03 e
F-C-Pow0.8	4.03 ± 0.01 c
CNTR-U	1.47 ± 0.10 b
CNTR-C	1.0 ± 0.00 a

[a-i]Means in the same column followed by different superscript upper cases are significantly different (p<0.05).

Table 4: LAB count in raw and cooked fish burgers with 32% (w/w) gelatin microcapsules.

Sensory quality

Considering that to the aim of food fortification, the sensory characteristics cannot be neglected being the main factors responsible for product acceptance [26], the samples developed in this study were also judged for sensory properties. Figure 1 describes the evolution of the overall quality at the time of sample preparation of raw and cooked fish burgers with gelatin microcapsules added at 10% (F-U-Mic10 and F-C-Mic10) and 32% (w/w) (F-U-Mic32 and F-C-Mic32). These samples were compared with raw and cooked burgers without any probiotic addition (CNTR-U and CNTR-C). It was decided to show only the overall quality attribute since the other sensory descriptors recorded a similar trend. As it can be seen for uncooked samples with microcapsules at both 10% and 32% (w/w) (F-U-Mic10 and F-U-Mic32) a slight lower overall quality respect to the CNTR-U sample was found, due to the addition of evident microcapsules in the fish burger that affected product appearance, this negative effect was more

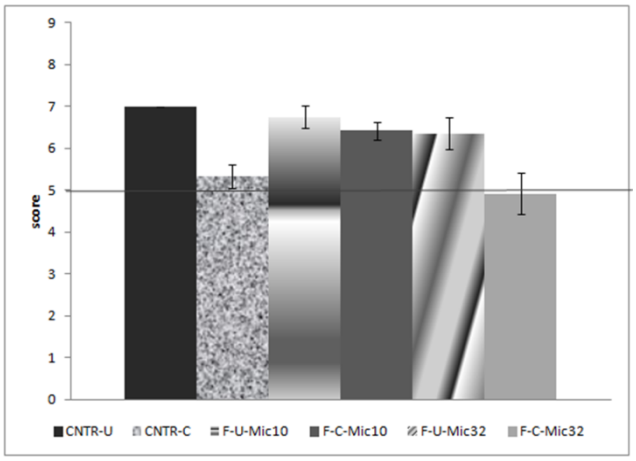

Figure 1: Overall quality of fortified fish burger samples during the optimization phase.

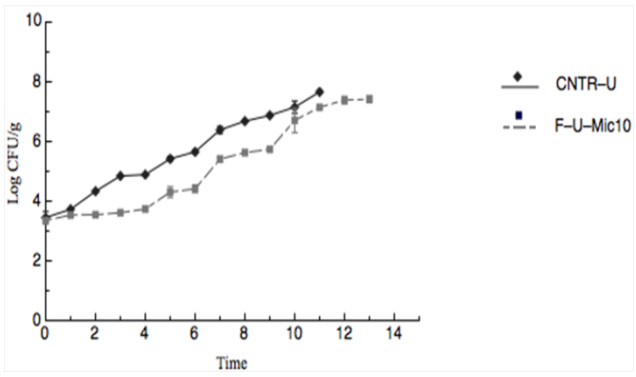

Figure 2: Evolution of aerobic plate count in fortified fish burgers stored at 4°C.

evident for the F-U-Mic32 sample because of the high amount of microcapsules added to the product. This inconvenient could be easily solved with further technological options able to mask the gelatin microcapsules in the uncooked samples. From the same figure, it is interesting to see that F-C-Mic10 fortified cooked fish burger was more prized than the control samples, thus demonstrating that the addition of LGG did not alter the typical sensory characteristics and improved the final consistence of the cooked fish burger which resulted more juicier and less dry probably for the microcapsules gelatin consistence. While F-C-Mic32 sample did not reach the same positive result and was judged less positive respect to the F-C-Mic32 and to the CNTR-C sample because of the excessively tender consistence probably due to the higher amount of microcapsules added on the fish burger.

Therefore in conclusion, the fish burger with 10% microcapsules recorded the best score of overall quality for the ideal texture perception after cooking. In fact, texture was perceived too hard in the CNTR-C sample, most probably due to the water loss during cooking, and too soft in sample with the highest concentration of microcapsules (F-C-Mic32). Other studies dealing with probiotic addition to food also reported an improvement of sensory properties of food. In particular, Hekmat [27] studied the functionalization of yogurt with the addition of *L. reuteri* RC-14 and *L. rhamnosus* GR-1. Angiolillo [28] developed a symbiotic Fiordilatte cheese with LGG and FOS and demonstrated that in addition to the nutritional value of the fresh cheese, an improved sensory quality was also found. On the basis of the sensory evaluation the better formulation chosen for the subsequent shelf life test the was the F-U-Mic10 compared to the CNTR-U sample, because it was a compromise between the functional properties and the good final sensory characteristics.

Fish burger quality during storage

Figure 2 shows the evolution of aerobic bacteria as a function of the storage time. For fresh water and marine species, the microbiological limit recommended by the ICMSF [19] for this microbial group is 7 log cfu/g. As it can be inferred from the figure, CNTR-U sample revealed an immediate growth, reaching the microbial limit after 9.08 days and so faster respect to F-U-Mic10 sample which reached the microbial limit 2 days later. Therefore, the addition of probiotic microcapsules were found to have a little antimicrobial activity in addition to their primary functional activity. Angiolillo [28] have previously found that the addition of probiotic lactic acid bacteria could have an antimicrobial activity besides their primary probiotic function. However, aerobic bacteria could not be considered the only microbial population which determines the microbial deterioration because spoiled marine fish products are characterized by a smell of rotten fish due to the production of H_2S off-odors and flavors [29,30] which are mainly related to specific spoilage organisms (SSOs). The plating medium used in a standard microbial count could affect the number and types of bacteria isolated because of differences in nutrient and salt requirements (as well as in terms of growth temperature) of the various SSOs [31]. For this reason, counts of hydrogen sulphide-producing bacteria (HSPB) and psychrotolerant and heat labile aerobic bacteria (PHAB) and *Enterobacteriaceae* were also performed. As regard hydrogen sulphide-producing bacteria (HSPB) and psychrotolerant and heat labile aerobic bacteria (PHAB) (data not shown) neither of the two samples reached the microbial limit during the entire storage period remaining at a cell concentration of 5.5 (CNTR-U) and 2.5 log CFU/g (F-U-Mic10) for SHPB and at a cell concentration of 6.7 and 3.63 log CFU/g, but while CNTR-U sample revealed an immediate growth, the F-U-Mic10 sample recorded a longer lag phase, starting to grow only at 10 day for SHPB and at 7 day for PHAB.

A similar trend was found for *Enterobacteriaceae* population (Figure 3) the CNTR-U sample recorded an immediate growth, reaching the microbial limit at 8.17 day while the F-U-Mic10 showed a longer lag phase until the 10 day, overlapping the limit only at day 10.48 and then 2.3 days later respect to the CNTR-U sample. The lactic acid bacteria count was monitored for the entire storage period (data not shown) and while for the CNTR-U sample this population remained at values of 3.96 log CFU/g, for the functional sample with microcapsules F-U-Mic10, lactic acid bacteria never dropped below the value of 8.14 log CFU/g, proving that the microcapsules treatment was effective in the functionalization of fish burgers since the lactic acid bacteria count remained stable for the entire storage period. From the above-mentioned microbial date it can be suggested that the addition of gelatin microcapsules with LGG turned to be effective not only for the burger functionalization but at the same time extended their microbial quality.

From a sensory point of view, the incorporation of compounds such as probiotics should not affect consumer acceptance. The addition of probiotics to obtain functional products and the effect of this addition to food has been barely studied [32]. The taste of these nutraceutical ingredients has been regarded as an important factor since several authors found that probiotics cause the acidification of the substrate or production of undesirable metabolites [33].

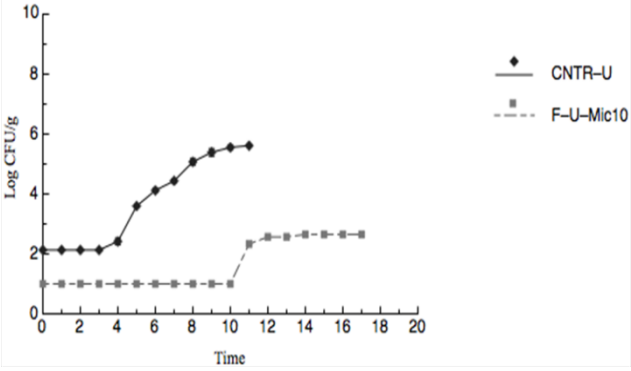

Figure 3: Evolution of *Enterobacteriaceae* count in fortified fish burgers stored at 4°C.

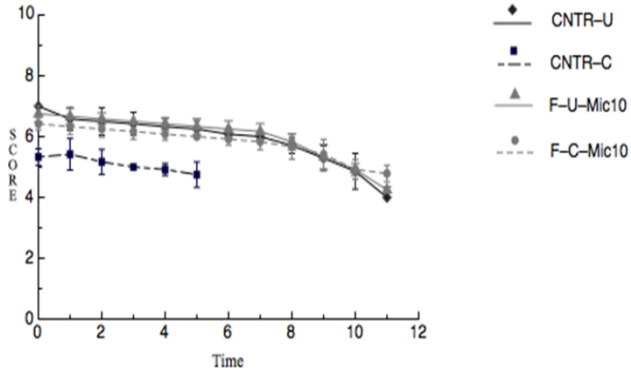

Figure 4: Overall quality of fortified fish burger samples for shelf life evaluation.

Fish burgers are necessarily linked to the concept of freshness of fish matrix and then probiotic addition must be conducted in such a way to not alter the sensory characteristics that are usually associated with neutral odor, good texture and without any discoloration [22]. It is important that treatments applied to functionalize also allow maintaining the appearance (i.e., color and integrity) and the flavor characteristics, being the first factors that consumers perceive as product quality [26]. Figure 4 shows the evolution of the overall quality for uncooked and cooked samples: CNTR-U, CNTR-C, F-U-Mic10 and F-C-Mic10 for the entire observation period. It was decided to show only the overall quality attribute since the other sensory descriptors recorded a similar trend. The curves were obtained by fitting the modified Gompertz equation to the experimental data, whereas the horizontal dashed line is the sensory threshold. As it can be seen, there was a similar decrease trend of the overall quality for the CNTR-U sample and F-U-Mic10 sample that were considered not acceptable at day 9.80 and 9.83 respectively, because of the altered color caused by probable oxidative reactions. The main differences were found on cooked samples, in fact the CNTR-C sample recorded a low score since the first day because of the excessively hard consistence caused by the the water loss during cooking. On the contrary, the F-C-Mic10 sample showed the better cooking performance, which in turn coincided with an higher overall quality score because of the more tender and juicy consistence of the sample with microcapsules even after cooking and for the entire observation period. The improvement of sensory characteristics was in agreement with other studies dealing with probiotic addition to food that reported an improvement of sensory properties of food. In particular, Hekmat [27] studied the functionalization of yogurt with the addition of *L. reuteri* RC-14 and *L. rhamnosus* GR-1. Angiolillo [28] developed a symbiotic Fiordilatte cheese with LGG and FOS and demonstrated that in addition to the nutritional value of the fresh cheese, an improved sensory quality was also found.

Therefore, sensory evaluation confirmed the considerations of the microbial quality: the addition of LGG microcapsules was effective in the functionalization of fish burgers improving at the same time the microbial and sensory quality. The fish burger shelf life is listed in Table 5 for each sample tested in this study. It was calculated as the lowest value between MAL and SAL [23]. As it can be emphasized from data, the microbial quality limited the CNTR-U shelf life in fact for this sample the shelf life was set to 8.17 days and coincided with the time at which sample reached the microbial limit for the *Enterobacteriaceae* population. The F-U-Mic10 recorded a longer shelf life (9.83) as a consequence of the better microbial quality; in this case in fact shelf life was limited by the sensory quality. It is evident that the microcapsules addition to the fish burgers helped not only to functionalize this food matrix but also to improve the microbial quality and as a consequence the shelf life of about 1.6 days.

Conclusions

The goal of this study consisted in the realization of a probiotic fish burger. To this aim, gelatin probiotic microcapsules were compared with direct use of probiotic in the form of frozen powder, in order to choose the best way to add the microorganism during the fish burger preparation. The gelatin probiotic microcapsules obtained by means of an optimized water-in-oil emulsion technique improved the microorganism retention respect to the use of the free cells, particularly after cooking. The increment of gelatin microcapsules to fish (from 10% to 32% w/w) further enhanced LAB retention in the final cooked fish burger. Both the samples fortified with 10% and 32% of LGG microcapsules may be considered as probiotic source because LAB count over the imposed limit was found (10^7 CFU/g). From the subsequent shelf life test it was highlighted that the addition of microcapsules at 10% w/w was effective not only to functionalize fish burger but also to improve their microbial and sensory quality and as a consequence, prolonging their shelf life.

Samples		Microbial quality (day)				Sensory quality(day)	Shelf life
		MAL^{APC}	MAL^{SH}	MAL^{pH}	MAL^{Enter}	$SAL^{O.Q.}$	
T=4°C	CNT-U	9.08 ± 0.23 a	>10	>10	8.17 ± 0.20 a	9.80 ± 0.26 a	8.17 a
	F-U-Mic10	11.08 ± 0.25 b	>12	>12	10.48 ± 0.16 b	9.83 ± 0.11 a	9.83 b

Table 5: Shelf life (days) of fish burger samples as the lowest value between microbial acceptability limit (MALAPC) (days ± SD), and the sensory acceptability limit (SALO.Q.) (days ± SD).

References

1. Cross ML (2002) Microbes versus microbes: Immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. FEMS Immunol Med Microbiol 34: 245-253.
2. FAO/WHO (2002) Working group report on drafting guidelines for the evaluation of probiotics in food. London, Ontario, Canada, pp: 1-11.
3. Imasse K, Tanaka A, Tokunaga K, Sugano H, Ishida H, et al. (2007) Lactobacillus reuteri tablets suppress Helicobacter pylori infection-A double blind randomised placebo-controlled cross-over clinical study. Journal of the Japanese Association of Infectious Diseases 81: 387-393.
4. Nomoto K (2005) Review prevention of infections by probiotics. Journal of Bioscience and Bioengineering 100: 583-592.
5. Shah NP (2007) Functional cultures and health benefits. International Dairy Journal 17: 1262-1277.
6. Vinderola CG, Prosello W, Ghiberto TD, Reinheimer JA (2000) Viability of probiotic (Bifidobacterium, Lactobacillus acidophilus and Lactobacillus casei) and non probiotic microflora in Argentinian Fresco cheese. Journal of Dairy Science 83: 1905-1911.
7. Phillips M, Kailasapathy K, Tran L (2006) Viability of commercial probiotic cultures (L. acidophilus, Bifidobacterium sp., L. casei, L. paracasei and L. rhamnosus) in cheddar cheese. International Journal of Food Microbiology 108: 276-280.
8. Rivera-Espinoza Y, Gallardo-Navarro Y (2010) Not dairy probiotic products. Food Microbiology 27: 1-11.
9. Korpela R, Moilanen E, Saxelin M, Vapaatalo H (1997) Lactobacillus rhamnosus GG (ATCC 53103) and platelet aggregation in vitro. International Journal of Food Microbiology 37: 83-86.
10. Pimentel-González DJ, Campos-Montiel RG, Lobato-Calleros C, Pedroza-Islas R, Vernon-Carter EJ (2009) Encapsulation of Lactobacillus rhamnosus in double emulsions formulated with sweet whey as emulsifier and survival in simulated gastrointestinal conditions. Food Research International 42: 292-297.
11. Vanderhoof JA, Whitney DB, Antonson DL, Hanner TL, Lupo JV, et al. (1999) Lactobacillus GG in the prevention of antibiotic-associated diarrhea in children. The Journal of Pediatrics 135: 564-568.
12. Marteau PR, de Vrese M, Cellier CJ, Schrezenmeir J (2001) Protection from gastrointestinal diseases with the use of probiotics. The American Journal of Clinical Nutrition 73: 430-436.
13. Lee YK, Salminen S (1995) The coming to age of probiotics. Trends in Food Science and Technology 6: 241-245.
14. Castro HP, Teixeira PM, Kirby R (1997) Evidence of membrane damage in Lactobacillus bulgaricus following freeze-drying. Journal of Applied Microbiology 82: 87-94.
15. Rao AV, Shiwnarain N, Maharaj I (1989) Survival of microencapsulated Bifidobacterium pseudo longum in simulated gastric and intestinal juices. Canadian Institute of Food Science and Technology Journal 22: 345-349.
16. Anal AK, Singh H (2007) Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. Trends in Food Science & Technology 18: 240-251.
17. Corona-HRI, Alvarez PE, Lizardi MJ, Alma IRR, de la RLA, et al. (2013) Structural Stability and Viability of Microencapsulated Probiotic Bacteria: A Review. Comprehensive Reviews in Food Science and Food Safety 12: 614-628.
18. Sidhu KS (2003) Health benefits and potential risks related to consumption of fish or fish oil. Regulatory Toxicology and Pharmacology 38: 336-344.
19. International Commission on Microbiological Specifications for Foods (ICMSF) (1986) Sampling plans for fish and shellfish, Microorganisms in Foods 2. Sampling for Microbiological Analysis: Principles and Scientific Applications, 2nd (edn.). University of Toronto Press, Toronto, Canada, pp: 181-196.
20. Nordic Committee on Food Analysis (NCFA) (2006) Aerobic count and specific spoilage organisms in fish and fish products. NMKL, Espoo, Finland.
21. Gram L, Trolle G, Huss HH (1987) Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures. Int J Food Microbiol 4: 65-72.
22. FAO/WHO (1999) Distribution of the Report of the 23rd Session of the Codex Committee on Fish and Fishery Products (ALINORM 99/18). Appendix 2, pp: 30-52.
23. Conte A, Gammariello D, Di Giulio S, Attanasio MM, Del Nobile MA (2009) Active coating and modified-atmosphere packaging to extend the shelf life of Fior di Latte cheese. Journal of Dairy Science 92: 887-894.
24. De Angelis M, Gobbetti M (2004) Environmental stress responses in Lactobacillus: A review. Proteomics 4: 106-122.
25. Solanki HK, Pawar DD, Shah DA, Prajapati VD, Jani GK, et al. (2013) Development of Microencapsulation Delivery System for Long-Term Preservation of Probiotics as Biotherapeutics Agent: A Review. BioMed Research International, ID 620719.
26. Kemp SE (2008) Application of sensory evaluation in food research. International Journal of Food Science and Technology 43: 1507-1511.
27. Hekmat S, Reid G (2006) Sensory properties of probiotic yogurt is comparable to standard yogurt. Nutrition Research 26: 163-166.
28. Angiolillo L, Conte A, Faccia M, Zambrini AV, Del Nobile MA (2013) A new method to produce synbiotic Fiordilatte cheese. Innovative Food Science and Emerging Technologies 22: 180-187.
29. Birte FV, Kasthuri V, Masataka S, Lone G (2005) Identification of Shewanella baltica as the Most Important H2S-Producing Species during Iced Storage of Danish Marine Fish. Applied and Environmental Microbiology 71: 6689-6697.
30. Del Nobile MA, Corbo MR, Speranza B, Sinigaglia M, Conte A, et al. (2009) Combined effect of MAP and active compounds on fresh blue fish burger. International Journal of Food Microbiology 135: 281-287.
31. Nickelson J, Finne G (1992) Fish, crustaceans and precooked seafoods. In: Vanderzant C, Splittstoesser DF (eds.), Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association, pp: 875-895.
32. Rojas-Grau MA, Soliva-Fortuny R, Belloso OM (2009) Edible coatings to incorporate active ingredients to fresh-cut fruits: a review. Trends in Food Science & Technology 20: 438-447.
33. Pithava S, Ambalam P, Dave JM, Vyas BRM (2011) Antimicrobial Peptides of Probiotic Lactobacillus Strains. Science against microbial pathogens: communicating current research and technological advances. Mendez-Vilas A (ed.), pp: 987-991.