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Spray incorporation of *Bacillus subtilis* and *Saccharomyces cerevisiae* with different binding agents into granules for *Oreochromis niloticus*

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Abstract

In aquaculture, probiotics appear as one of the alternatives to the excessive use of antibiotics. They promote good health, growth and improve the quality of fish culture water. One route of administration is incorporation into foods. But the mode of incorporation influences their viability and density. The objective of this study is to evaluate the effectiveness of incorporation of *Saccharomyces cerevisiae* and *Bacillus subtilis* by spraying with three binding agents (sterile salt water 0.9 %, vegetable oil and egg white). After determining the type of interaction existing between the two probiotics, 3×10^9 CFU of each of them was sprayed onto 30 g of granules. Then, the viability of the incorporated probiotics was evaluated over 12 days and after 60 minutes of residence in water. The density of incorporated probiotics showed a significant difference with vegetable oil in *S. cerevisiae* for an average of 3.28×10^7 CFU/g. This density varies from 1.5×10^6 to 2.75×10^6 CFU/g with *B. subtilis* among all binders. For the viability of 12 days, we observe a progressive increase in microbial density with salt water as a binder in *B. subtilis* from 2.4×10^4 to 5.35×10^6 CFU/g and *S. cerevisiae* of 7.43×10^6 to 1.5×10^7 CFU/g. The contact test on water with *B. subtilis* shows a decrease in microbial density from 3.5×10^6 to 1.56×10^6 CFU/g, while with *S. cerevisiae* it favors an increase from 3.07×10^7 to 4, 56×10^8 CFU/g with all binding agents. Sterile salt water 0.9% is the appropriate binding agent for both probiotic strains.

Keywords: Aquaculture; *Bacillus subtilis;* incorporation; probiotic; *Saccharomyces cerevisiae*

1. Introduction

In October 2013, the International Scientific Association for Probiotics and Prebiotics (ISAPP), in view of the current application of probiotics, gave a broader and more relevant definition based on the FAO/WHO 2001 definition. ISAPP defines probiotics as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [1]. In aquaculture, the notion of probiotics also encompasses microorganisms that not only have a beneficial effect on the host, but also improve the quality of the water in which the fish are cultured [1]. The probiotics used in aquaculture are mainly Gram-positive and Gram-negative bacteria, while the non-bacterial group includes bacteriophages, microalgae and yeasts. They can be of the mono-strain (a) type when they contain a strain of a certain

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species, multi-strain (b) when they contain more than one strain of the same species or at least of the same genus and finally multi-species (c), for preparations incorporating strains belonging to one or more genera [2, 3].

Probiotics have been used effectively in aquaculture since the early 2000s and several studies show that probiotics have a positive influence on growth performance, feed utilisation and gut microbiota, as well as improving disease resistance and culture water quality [4, 5]. The main routes of administering probiotics to fish are direct inclusion in the feed, balneation, encapsulation and injection. Inclusion of probiotics in the feed is the most common way of incorporating them; its advantage is that it is easy to administer and dose [2]. To achieve this, probiotics can be added to feed to be administered directly to fish or prepared in the form of pellets/ extrudates [6, 7]. They can also be sprayed directly onto ready-to-use pellets [8]. In this case, the probiotics must be suspended in a substrate before being sprayed onto the pellets [9, 10]. This involves the notion of a 'binding agent'.

Hence the interest in this work, the general aim of which is to incorporate two probiotic strains, *Saccharomyces cerevisiae* and *Bacillus subtilis*, into pellets for *Oreochromis niloticus* (Nile Tilapia). It is divided into two specific objectives, which consist firstly of (i) incorporating the microorganisms *B. subtilis* and *S. cerevisiae* with the three different binding agents: egg white, palm oil and sterile salt water 0.9% (ii) and assessing the viability of the microorganisms incorporated into the food after conservation at room temperature and after a period in water.

2. Materials and Methods

2.1. Isolation of probiotic strains

The probiotic strains were isolated from the wild *O. niloticus* species sampled in the Bandama River (Côte d'Ivoire). Fish sampled were anaesthetised, aseptically isolated digestive tract was then ground, homogenised and serially diluted. The different dilutions were spread on specific agars: MYP: Mannitol Egg Yolk Polymixin (Merck) for *B. subtilis* [11] and Sabouraud added Bromocresol Green for *S. cerevisiae* [12]. Colonies with the morphological characteristics of the strains were then isolated and biochemically identified using API 20 C AUX (Ref 20 210) for yeast and API 50 CHB/E (Ref 50430) for Bacillus (BioMérieux, France). The procedure was tested according to manufacturing instructions.

2.2. Correlation between optical density (OD) at 600 nm and microbial density

Well-isolated colonies of *B. subtilis* and *S. cerevisiae* were cultured at 37 °C for 24 h in Brain Heart Infusion (BHI) and Sabouraud Chloramphenicol Dextrose Broth respectively. From the young cultures, a preculture of 3 hours for *S. cerevisiae* and 4 hours for *B. subtilis* was carried out in the respective broths. The optical density was then read using a spectrophotometer at 600 nm (OD_{600}) against a blank every 1 hour for 6 hours.

For each spectrophotometer reading, a dilution and surface plating on Nutritive Agar for the enumeration of *B. subtilis* and Sabouraud Chloramphenicol Agar for *S. cerevisiae* was carried out. The plates were incubated at 37 C for 24 to 48 hours. Colony counts were carried out on statistically viable plates. Microbial density was obtained using the formula below [13].

$$N = \frac{\sum C}{d(n1 + 0{,}1n2)V}$$

- ∑C: sum of colonies on counted boxes
- V : volume of inoculum (0.1 ml)
- d : dilution retained
- n1: number of petri dish corresponding to the first dilution chosen
- n2: number of petri dish corresponding to the second dilution chosen
- N : number of colonies in colony-forming units per milliliter (CFU/ml)

The equation of the correlation line between OD at 600nm and microbial density was determined using Excel 365 software.

$$\text{Microbial concentration} = a \times \text{OD600} + c$$

- a: coefficient direction of the line of equation
- c: constant

2.3. Study of interaction between *B. subtilis* and *S. cerevisiae*

Precultures of *S. cerevisiae* and *B. subtilis* were prepared from 24-hours microbial cultures in Brain-Heart-infusion broth. An inoculum of 10^7 CFU of each microorganism was prepared from these precultures, then homogenised in 100 ml of BHI to obtain a final concentration of 10^5 CFU/ml.

Batches of three Erlenmeyer flasks, each containing 10^5 CFU/ml of each microorganism (*S. cerevisiae*, *B. subtilis*) were used as growth controls. Another batch of Erlenmeyer flasks containing a mixture of *B. subtilis* and *S. cerevisiae* at a concentration of 10^5 CFU/ml each was used for the antagonistic test between these two microbial strains. The different broths were incubated at 37°C for 8 hours.

Every hour during the 8 hours incubation period, all the Erlenmeyer flasks were read with a spectrophotometer (OD_{600}) and the spreads and incubations were carried out as described in section **2.2**.

2.4. Incorporation of probiotics strains into pellets

B. subtilis and *S. cerevisiae* were incorporated into the food using 3 different binding agents, including 0.9% sterile salt water, palm vegetable oil and egg white.

The probiotic strains *B. subtilis* and *S. cerevisiae* were separately inoculated at 1% in sufficient BHI and incubated at 37 °C for 24 hours. An inoculum of $3{,}10^9$ CFU of each strain was taken from the young culture and centrifuged at 3,000 rpm for 15 min.

The recovered pellet (centrifugation) is suspended in the various binders at 2% of the weight of the feed. The mixture obtained is gradually sprayed on 30 g of granules previously sterilized in an autoclave [14].

The feed inoculated in theory at $1{,}10^8$ CFU/g is left to dry under a laminar flow hood for 5 to 6 hours [15, 16].

2.5. Effectiveness of incorporation

In 5 ml of sterile distilled water, 0.1 g of crushed food from each food incorporated with the probiotic strains was homogenised. Successive dilutions were made. 100 µl of each of the dilutions obtained were then inoculated onto Nutrient and Sabouraud Agars and incubated at 37°C. The colonies that appeared and were counted were expressed as (CFU/g) of feed using the following formula [13]:

$$N' = N/C$$

- N': number of colony-forming units per gram of feed (CFU/g)
- C : total concentration of the stock solution (g/ml)
- N : number of colony-forming units per ml (CFU/ml)

2.6. Viability over time of microorganisms incorporated into pellets

The feeds previously incorporated in probiotics, stored for periods of 0, 4, 8 and 12 days, were crushed and inoculated as previously carried out in **2.5**.

2.7. Microbial density remains in granules after contact time on water

The probiotic-incorporated foods were placed in contact with distilled, sterile water for 0, 20, 40 and 60 minutes. They were then ground and inoculated as described in **2.5**.

2.8. Data analysis

The comparison of the means of microbial densities was performed with the Fisher test with a significance threshold of 0.05 using the XLSTAT 2016 software. The calculations and figures were performed using EXCEL 365.

3. Results

3.1. Correlation study

The ratio of OD_{600} to CFU concentrations of *S. cerevisiae* and *B. subtilis* indicates a significant correlation with R^2 coefficients of 0.96 and 0.97, respectively. The associated correlation equations (CFU/ml) are:

- $C_{(S.\ cerevisiae)} = 1{,}39\times10^7 \times OD_{600} - 8{,}80\times10^5$
- $C_{(B.\ subtilis)} = 2\times10^8 \times OD_{600} - 2\times10^7$

3.2. Interaction between *B. subtilis* and *S. cerevisiae*

The type of interaction between *B. subtilis* and *S. cerevisiae* was determined through the study of the growth kinetics of the two microorganisms cultured together in Brain-Heart-infusion broth. A comparison of these growth kinetics with that of their individual growth (control) in the same culture medium was performed.

Figure 1 shows the growth kinetics curves of the *B. subtilis* (in blue) and *S. cerevisiae* (in orange) controls. The two curves have the same appearance and evolve in the same direction, with the growth curve of the control of *B. subtilis* above that of *S. cerevisiae*.

Figure 2 shows the growth kinetics of *B. subtilis* (in blue) and *S. cerevisiae* (in orange) grown together in the same medium (BCC). Both curves move in the same direction, with that of *B. subtilis* above that of *S. cerevisiae*. However, from the 7th hour onwards, the *B. subtilis* curve decreased until the end of the test, while the *S. cerevisiae* curve continued to increase.

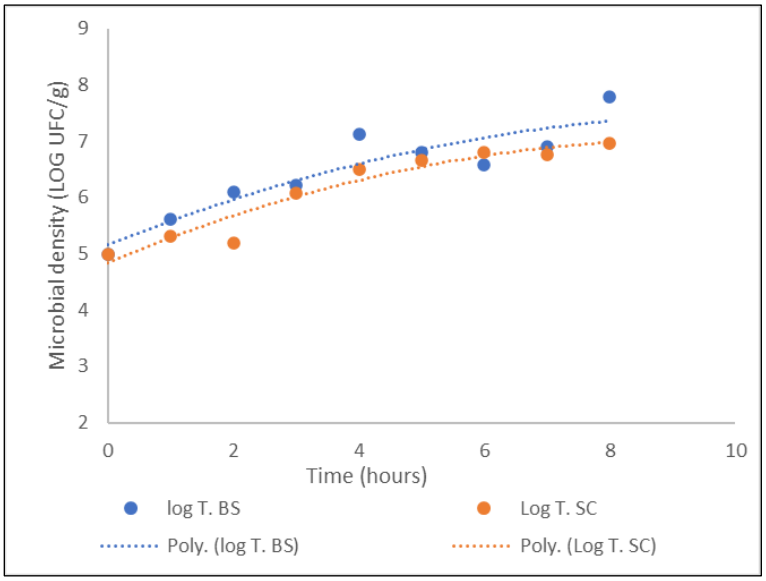


Figure 1 Growth kinetics of *B. subtilis* (blue) and *S. cerevisiae* (orange) controls

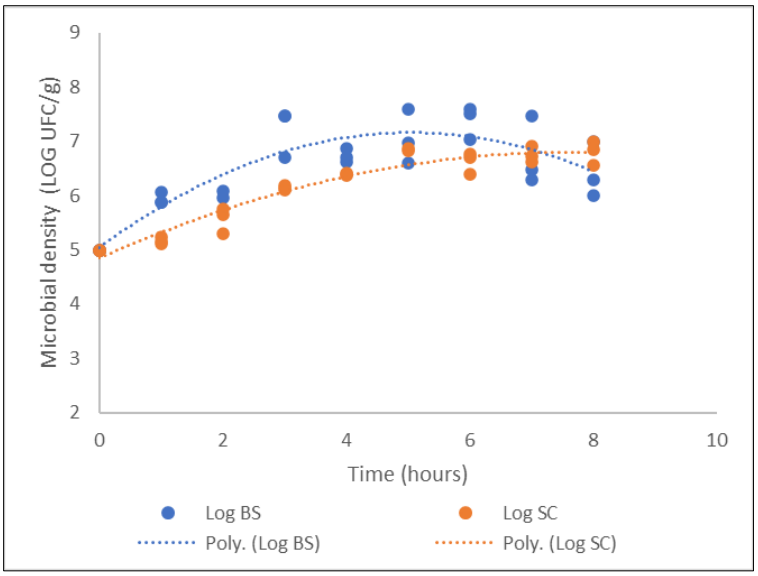


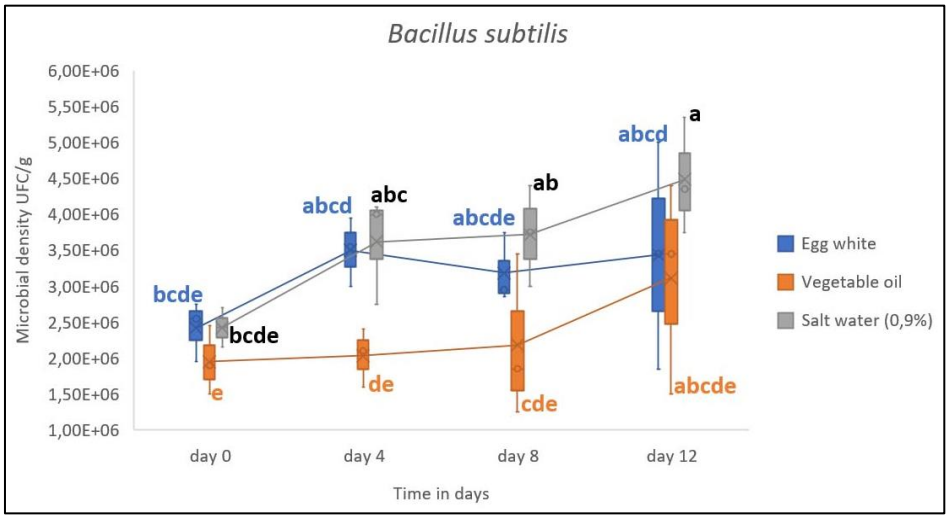
Figure 2 Growth kinetics of *B. subtilis* (blue) and *S. cerevisiae* (orange) grown together

3.3. Evaluation of incorporation efficiency

The quantities of *B. subtilis* incorporated into feed using the different binding agents overlapped regardless of the binding agent used. They ranged from 1×10^6 to 3×10^6 CFU/g of feed, with no significant difference $P<0.05$ between the different binding agents.

For the *S. cerevisiae* strain, the quantities incorporated were higher than those for *B. subtilis* for a range of approximately 5×10^6 to 4×10^7 CFU/g of feed. There was a significant $P<0.05$ difference in density with vegetable oil recording the highest value.

3.4. Viability of incorporated micro-organisms over time



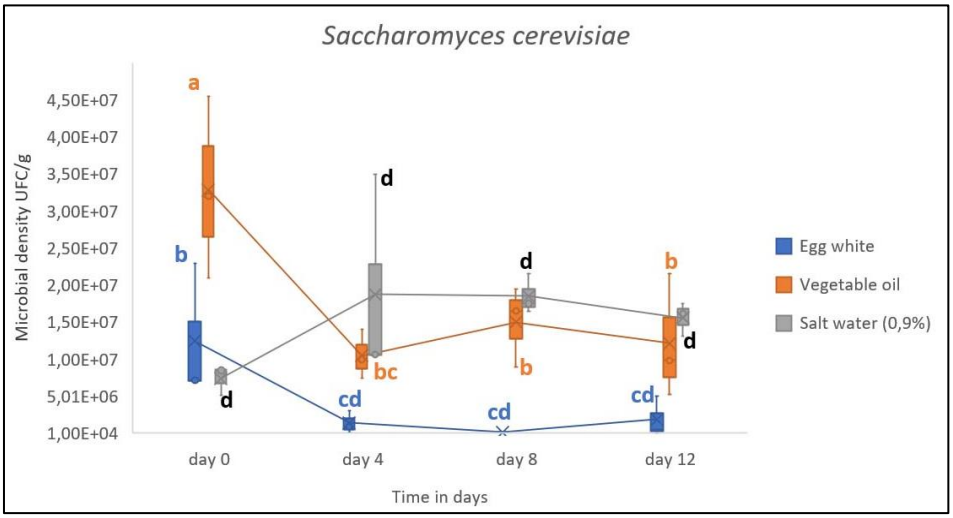
Values with similar letters show no significant difference P < 0,05, Test Fisher

Figure 3 Viability of *B. subtilis* over time with the different binding agents

Figure 3 shows the variation in the density of *B. subtilis* incorporated into foods with the different binding agents over 0, 4, 8 and 12 days. The microbial densities induced by the different binding agents showed no significant difference on the 12th day of sampling. However, with the saltwater binding agent, there is a gradual increase in the microbial density initially incorporated with the shelf life for values from 2.4×10^4 CFU/g to 5.35×10^6 CFU/g.

The same trend was observed with the *S. cerevisiae* strain using sterile salt water as a binder, but with a significant difference ($P < 0.05$) between the binders at day 12, going from an average of 7.43×10^6 CFU at day 0 to 1.5×10^7 CFU at day 12. See figure 4.

Sterile salt water appears here as the best binding agent preservative of the microbial density of the strains incorporated into the food during the 12-day shelf life.



Values with similar letters show no significant difference P < 0,05, Test Fisher.

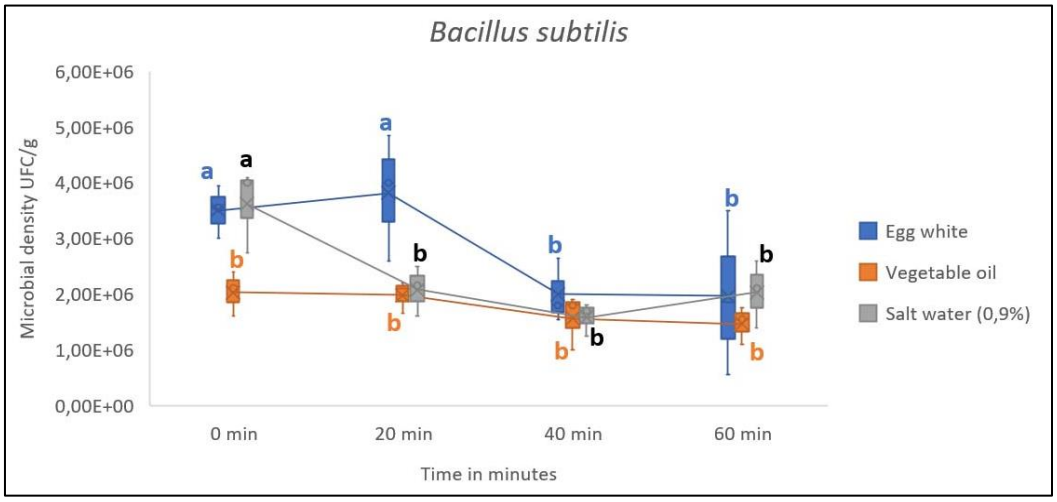
Figure 4 Viability of *S. cerevisiae* over time with the different binding agents

3.5. Microbial density in food after contact with water

The variation in the density of *B. subtilis* in the food after the durations of stay in the water indicates that there is no significant difference between the binders at the end of the test, for a mean value of 1.82×10^6 CFU/g. However, an increase in microbial density was observed with egg white at the 20th min from 3.5×10^6 to 4.85×10^6 CFU/g (figure 5).

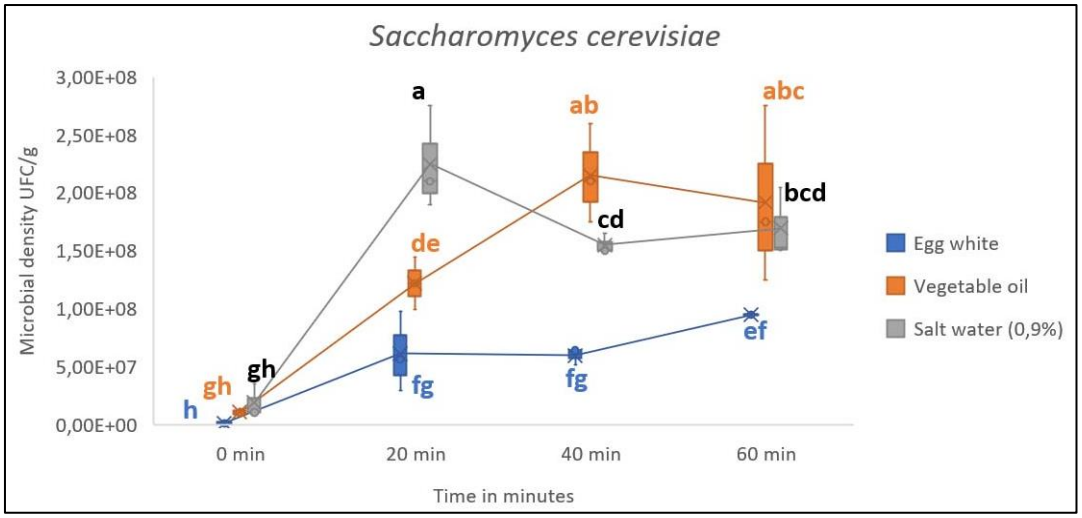
With the *S. cerevisiae* strain, a progressive increase in microbial density from 1.02×10^7 to 4.56×10^8 CFU/g was observed with the different binding agents. A significant increase was observed at each sampling period with vegetable oil. A peak of 2.75×10^8 CFU/g was observed at 20 min with sterile saline water. It should be noted that throughout the test, the microbial density values for egg white remained the lowest (Figure 6).

For water contact tests, the appropriate binding agents for *B. subtilis* and *S. cerevisiae* are egg white and vegetable oil respectively, followed by sterile saline water.



Values with similar letters show no significant difference P < 0,05, Test Fisher

Figure 5 Temporal dynamics of B. subtilis density in granules after contact with water



Values with similar letters show no significant difference P < 0,05, Test Fisher

Figure 6 Temporal dynamics of the density of *S. cerevisiae* in pellets after contact with water

4. Discussion

The incorporation of multi-strain or multi-species probiotics into a food requires an assessment of the type of interaction existing between the germs concerned. In principle, multispecies probiotics are composed of microorganisms that do not show antagonistic activities towards each other but can interact together to confer benefits on the host.

It appears from our investigations that there is an antagonistic interaction between the two germs of interest [17]. By comparing the growth kinetics of the controls with those of the mixture of the two probiotic strains. The presence of *S. cerevisiae* could be responsible for the reduction in *B. subtilis*. In fact, *S. cerevisiae* carries out alcoholic fermentation by transforming the sugars in the medium into ethanol and CO_2. These compounds produced may inhibit the growth of *B. subtilis* in the environment [18, 19]. Also, the temperature/ethanol couple can also act synergistically. Indeed, high ethanol concentrations, with a temperature above 25°C, can completely inhibit bacterial growth [20], hence that of *B subtilis*. Also, the inhibition of bacteria can result from the depletion of nutrients such as oxygen by yeasts during fermentation [20, 21].

The quantities incorporated into the feed with the different binding agents for the two probiotic strains were lower than the desired objective, with a higher incorporation score for *S. cerevisiae*. This can be explained by losses caused during the incorporation treatments: centrifugation, progressive pulverisation and drying. These results are similar to those of Darafsh [22] who prepared experimental diets with a commercial probiotic (D-pro) containing *B. subtilis* and *B. licheniformis* strains at 1.6×10^{12} CFU/g. The additives were sprayed at a concentration of 5 g per kg of feed, which theoretically gives us 8×10^{9} CFU/g of feed. At the end of the treatment, the total number of bacteria revealed the presence of $5.3.10^{6}$ CFU/g of feed in the diets. Nevertheless, the amounts incorporated were within the range of dietary inclusion of probiotics in tilapia. According to Hai [4], a varied range of probiotic dosages from 10^{5} to 10^{9} CFU/g feed is the common way to administer probiotics. Several studies [23-26] have incorporated the *B. subtilis* strain into Tilapia pellets at the following ratios: 5×10^{5}, 5×106, 10^{7} and 5×10^{9} CFU/g diet. Alves incorporated the yeast *S. cerevisiae* at a proportion of 4.2×10^{7} CFU/g of feed. Thus, the dose of probiotics to be incorporated depends on the duration of the treatment and the objectives of the study.

Tests on the viability of the probiotics during the 0-to-12-day shelf life identified the binding agent that maintains viability and microbial density during the approximately two-week shelf life at room temperature. Sterile salt water appears here to be the best binding agent for both strains *S. cerevisiae* and *B. subtilis*. To date, there has not been a comparative study of the incorporation of probiotics with various binding agents. There are many binding agents for probiotics in aquaculture. It can be phosphate buffer, physiological serum, sterile distilled water salted at 0.9%, vegetable oil (soybean, palm), animal oil (cod liver oil), sodium alginate, etc. [27-29]. Regarding the shelf life of foods incorporated in probiotics, it varies from one author to another ranging from 4 days to 2 weeks maximum, with recommended storage in the fridge at 4-5°C for a longer preservation of microbial viability [28, 30, 31].

The various feeds formulated with the binders were brought into contact with water to assess the stability of the microbial density in the feeds incorporated during the feeding of the fish. Our study showed that microbial density increased in foods incorporated with what would be described as every strain-specific binding agent. Thus, with the *S. cerevisiae* strain, values of up to 10^8 CFU/g were obtained. The longevity of viable probiotic cells in foods depends on the strain, reflects the composition of the food, its method of preparation and the storage conditions.

5. Conclusion

This research was carried out with the aim of incorporating *B. subtilis* and *S. cerevisiae*, two probiotic strains, into pellets for Tilapia fish.

The results showed that 0.9% sterile saline water is to be the most suitable binding agent for achieving the desired microbial density in the pellets. This applies to both strains. Sterile salt water retains the density for around two weeks at room temperature. The test of the viability of the probiotics incorporated into the pellets after the contact time on the water made it possible to quantify the leaching of the probiotics once the feed was in the water for the maximum period of one hour. It appears that the initial incorporated microbial densities increased slightly specifically with the use of certain binding agents. Thus, the *S. cerevisiae* strain was gradually increased with vegetable oil as a binder, while *B. subtilis* had more affinity with egg white, followed for both strains by sterile salt water.

The study on the type of interaction between the two probiotic strains revealed that they inhibit each other. Thus, as a perspective, an additional study of antagonism between the two strains must be carried out to identify the tolerance threshold of the two microorganisms put together. As regards the desired microbial density in the granules, it is preferable to choose a dose interval rather than a fixed quantity, given the losses caused by the spraying method.

Author contributions

A M P A: Investigation, Conceptualization, Formal analysis, Writing – Original draft, **J-L A M**: Conceptualization, Supervising, Formal analysis, Writing – Review & Editing; **A R N'D**: Investigation; **F F**: Investigation, Formal analysis, Writing – Original draft; **M E Y-M A** : Investigation; **E R D'O** : Supervising, Writing – Review & Editing; **Y B** : Supervising, Writing – Review & Editing; **A C** : Supervising, Resources.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare no conflict of interest.

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