Amaranth Meal and Environmental *Carnobacterium maltaromaticum* Probiotic Bacteria as Novel Stabilizers of the Microbiological Quality of Compound Fish Feeds for Aquaculture

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Abstract: Fish feed should be characterized by microbiological stability to guarantee the optimal health of farmed fish. The aim of this study was to determine the efficacy of amaranth meal (*Amaranthus cruentus*) and a highly active environmental strain of probiotic bacteria, *Carnobacterium maltaromaticum*, as novel supplements that stabilize the quantitative and qualitative composition of microbiota in compound fish feeds for aquaculture, regardless of storage temperature. The total viable counts of mesophilic bacteria at 28 °C (TVC 28 °C), hemolytic mesophilic bacteria (Hem 37 °C), *Staphylococcus* sp. bacteria, aerobic spore-forming bacteria (ASFB), sulfite-reducing anaerobic spore-forming *Clostridium* sp. bacteria, yeasts, and molds were analyzed in control feed (CF), in feed supplemented with amaranth meal (AF), and in feed supplemented with amaranth meal and *C. maltaromaticum* (ACF), stored at a temperature of 4 °C and 20 °C for 98 days. *Amaranthus cruentus* and *C. maltaromaticum* significantly reduced bacterial counts in fish feeds, regardless of the temperature and duration of storage. The antibacterial and antifungal effects of the tested additives were statistically significant ($p \leq 0.05$). The studied novel supplements contribute to the microbiological safety of compound fish feeds. The tested additives could be recognized as the key ingredients of organic, environmentally friendly fish feeds, which guarantee the high quality of fish intended for human consumption.

Keywords: aquaculture; compound feed; antimicrobial stabilizers; *Amaranthus cruentus*; *Carnobacterium maltaromaticum*

1. Introduction

Feed is one of the main factors that influence fish welfare and the microbiological status of water in aquaculture and freshwater ecology [1]. The nutritional value and microbiological quality of feed determine fish weight gains, and the sanitary and epidemiological safety of aquatic organisms and the aquatic environment [2–6].

Synbiotics containing probiotics and prebiotics enhance the health benefits of feed. They promote the growth and metabolic activity of beneficial microorganisms in the host's gastrointestinal tract without compromising endogenous gut microbiota [7–9]. Probiotics are natural microbiome bacteria that deliver multidirectional beneficial effects for living organisms (humans and animals) at the local and systemic level [2,10]. The role of probiotic feed microbiota in the maintenance of gut homeostasis is increasingly recognized as a critical success factor in fish breeding [6,11–13].

The group of probiotic bacteria includes members of the genus *Carnobacterium* [14–17]. *Carnobacterium maltaromaticum*, which colonizes natural aquatic habitats, is one of the most metabolically active probiotic bacteria in the digestive tract of animals. This bacterial species easily adapts to changes in habitat conditions such as temperature, salinity, and pH, and it delivers health benefits for the host organism [14,18–20] *Carnobacterium maltaromaticum* effectively inhibits the development of pathogenic bacteria and is regarded as a potent immune stimulator in fish [14,21–23].

The growth and activity of probiotic bacteria are influenced by environmental conditions that can be optimized with the use of prebiotics [24,25]. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of bacteria colonizing the gastrointestinal tract [7].

Animal and plant meals are one of the main ingredients of fish feeds [26]. However, plant meals contain anti-nutritional factors, and their applicability in compound fish feeds is limited [27]. One of exceptions is amaranth meal, characterized by a low content of anti-nutritional factors, mainly saponins and phytic acid [28].

Amaranth meal contains lignins and various compounds with antioxidant, antibacterial, antiviral, and fungistatic properties [29–31]. Amaranth seeds are also abundant in other health-promoting substances, such as squalene and fiber [32,33]. In a study by Niewiadomski et al. [34], feed supplemented with 20% of amaranth meal promoted the growth of rainbow trout (*Oncorhynchus myskiss*) and improved the digestibility of dietary nutrients. A microbiological analysis in a pilot study conducted by Potorski and Niewiadomski [35] revealed that amaranth supplementation can prevent excessive growth and proliferation of *Staphylococcus* sp. bacteria, *Clostridium* sp. anaerobic spore-forming bacteria, yeasts, and molds in compound fish feeds. A similar beneficial influence of amaranth meal on selected probiotic strains was also observed by Vieira et al. [36] who demonstrated that amaranth meal stimulated the fermentation ability of ten probiotic strains (*Lactobacillus* spp. and *Bifidobacterium* spp.)

The microbiological composition of fish feeds significantly influences fish health and weight gains. This parameter is particularly important if feeds contain harmful microorganisms that compromise fish health, disrupt digestive metabolism, and compromise the reproduction and survival of farmed fish [4]. Feeds should be characterized by microbiological stability and high quality to guarantee the optimal health status and physiological condition of farmed fish. Nevertheless, not all undesirable microorganisms are eliminated during fish feed production. According to the literature [4,37], the standard extrusion process does not guarantee complete elimination of various microorganisms from fish feeds. Furthermore, the metabolic activity of heterotrophic bacteria that survive in ready-made feeds involves the oxidative degradation of lipids and proteins. As a result, the nutritional value of feeds can be modified by natural feed microbiota or by contamination with exogenous microorganisms. Inadequate storage temperature and prolonged storage can also promote the development and metabolic activity of various groups, genera, and species of heterotrophic microorganisms [38].

Our previous experiment [39], which investigated the effect of *C. maltaromaticum* on heterotrophic microbiota, revealed that probiotic bacteria were the main factor responsible for a decrease in the counts of all analyzed bacterial groups in commercial fish feed. The results of studies conducted by

other authors [40–42] demonstrated that amaranth meal increased the survival and growth rates of probiotic bacteria and improved the microbial stability of foods. The combined use of environmental probiotic bacteria and amaranth meal as stabilizers of the microbiological quality of fish feeds remains insufficiently researched. These facts have prompted the authors to evaluate the effectiveness of a highly active environmental isolate of *C. maltaromaticum* and amaranth meal in stabilizing the microbiological quality of fish feed. The aim of this study was to determine the efficacy of amaranth meal (*Amaranthus cruentus*) and a highly active environmental strain of probiotic bacteria, *C. maltaromaticum*, as novel supplements that stabilize the quantitative and qualitative composition of microbiota in compound fish feeds for aquaculture, regardless of storage temperature.

2. Materials and Methods

2.1. Isolation and Identification of C. Maltaromaticum Probiotic Bacteria

A probiotic strain of *C. maltaromaticum* was isolated from water samples collected from the benthic zone of Lake Legińskie (at a depth of 34 m) located in north-eastern Poland (N = 53°58′51″ N and E = 21°8′4″). The strain had been isolated during a previous study conducted by the Department of Environmental Microbiology of the University of Warmia and Mazury in Olsztyn.

The isolate was identified to species level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF VITEK® MS) at the Department of Microbiology, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, in Mexico City, Mexico. The identification was additionally verified by 16S rDNA (recombinant DNA) sequencing with the BigDye Terminator v3.1 kit in the ABI 3730xl genetic analyzer (Applied Biosystems, Foster City, USA). In addition, 16S rDNA genes were sequenced by PCR with the use of 27F (5′-AGAGTTTGATCATTGGCTCAG-3′) and 1492R (5′-GGTACC-TTGTTACGACTT-3′) primers according to the method described by Gillan et al. [43]. The BLAST program available on the website of the National Center of Biotechnology Information [44] was used to identify DNA sequences. The results of 16S rDNA sequencing are presented in Table S1 (Supplementary Materials).

After the identification process, *C. maltaromaticum* was considered as a probiotic strain based on the hemolysis assay, and its acid and bile tolerance properties, according to the guidelines developed by a joint FAO/WHO working group [45]. The hemolytic activity of *C. maltaromaticum* was determined on tryptone soya agar (TSA; Oxoid, Basingstoke, UK) with 5% addition of defibrinated sheep blood incubated at 37 °C for 48 h [46]. The bile salt tolerance test of the studied strain was performed in MRS broth culture medium (Sigma - Aldrich, Germany) containing 0.5%, 1.0%, or 2.0% bile salts (Oxoid LP0055) according to the procedure proposed by Succi et al. [47]. The *C. maltaromaticum* isolate was tested for acid tolerance based on its growth on medium with varying pH (1.5, 2.5, 3.5, and 4.5), as described by Vijayarama et al. [48].

2.2. Determination of the Metabolic Activity of Probiotic Bacteria Based on the Utilization of Different Carbon Sources

The applicability of the environmental *C. maltaromaticum* isolate for further analysis was determined by analyzing the bacteria's metabolism based on its utilization of various carbon sources. The biochemical activity of the *C. maltaromaticum* probiotic isolate and its potential to compete for nutrients with feed microbiota were estimated using the OmniLog®System (Biolog, USA). A 96-well plate containing various carbon compounds was inoculated with the evaluated bacterial strain. The plate was incubated, and biochemical parameters were read in a microstation reader. The strain's utilization of different carbon compounds as sources of energy was determined based on the intensity of color reactions.

2.3. Compound Feed

The experiment was performed on three types of extruded compound feeds: Control feed (CF) without the addition of amaranth meal, experimental feed containing 20% of amaranth meal (AF), and experimental feed containing 20% of amaranth meal and *C. maltaromaticum* probiotic bacteria (ACF). The composition of each feed is presented in Table 1. All feeds were formulated based on the recommendations of Hart et al. [49] and NRC [50]. The feeds were extruded with a co-rotating twin screw extruder (Metalchem, Poland) equipped with a Ø 4.5 mm pellet stencil. The following extrusion processing parameters were applied: Screw speed—105–125 rpm, cutter speed—50 rpm, head temperature—120 °C, barrel temperature of 130–150° C in 30 s, die diameter—2.0 mm. Compound feeds were enhanced with a mixture of fish oil and soybean oil (5% each). The tested strain of *C. maltaromaticum* was added to the oil mixture. Next, the probiotic oil suspension was added to two experimental feed samples. Tthe oil mixture was pumped into the feed at 0.9 Mpa for 5 min with the use of a vacuum pump. The feed contained 40.0% crude protein, 15.0% crude fat, 3.0% crude ash, 37.0 nitrogen-free extract (NFE), and 5% water.

Table 1. Feeds composition (g·100 g^{-1} dry diet).

Ingredients	Feed Type		
	CF [1]	AF [2]	ACF [3]
Soybean meal	32.00	27.00	27.00
Wheat flour	25.00	10.00	10.00
Amaranth meal	0.00	20.00	20.00
Fishmeal	15.00	15.00	15.00
Hydrolyzed feather meal	15.00	15.00	15.00
Codliver oil	5.00	5.00	5.00
Soybean oil	5.00	5.00	5.00
Vitamin premix [4]	1.00	1.00	1.00
Mineral premix [5]	2.00	1.00	1.00
C. maltaromaticum (CFU·g^{-1})	0.00	0.00	1.5×10^9

[1]—control feed (CF); [2]—feed containing 20% amaranth (AF); [3]—feed containing 20% of amaranth and Carnobacterium maltaromaticum (ACF); [4]—Composition of the vitamin premix (IU·1 kg^{-1} dry diet): Vitamin A—70,000 IU; vitamin D—200,000 IU; vitamin E—17,500 IU; vitamin K—867 IU; vitamin C—28,500 IU; vitamin B1—1067 IU; vitamin B2—2000 IU; vitamin B5—5334 IU; vitamin B6—1334 IU; vitamin B12—400 IU; biotin—200 IU; niacin—12,000 IU; folic acid—800 IU; inositol—20,000 IU; choline chloride—120,000 IU; betaine—75,000 IU; [5]—Composition of the mineral premix (g·1 kg^{-1} dry diet): FeSO$_4$·H$_2$O—4334 g; KI—0.734 g; CuSO$_4$·5H$_2$O—0.267 g; MnO—0.734 g; ZnSO$_4$·H$_2$O—1250 g; ZnO—0.750 g; Na$_2$SeO$_4$—0.034 g; CFU—colony forming unit.

2.4. Experimental Design

The prepared feeds (CF, AF, and ACF) were used in an experiment that lasted for 98 days. The control feed (CF) was divided into two equal parts, and the feed containing 20% amaranth meal (AF) was divided into four equal parts under sterile conditions. Every CF and AF sample was placed in a separate, sterile, and tightly closed vessel made of dark glass. Two samples (CF 4 °C, AF 4 °C) were chill-stored at a temperature of 4 °C, and two samples (CF 20 °C, AF 20 °C) were stored at a temperature of 20 °C throughout the experiment. Cultures of the environmental *C. maltaromaticum* strain were added to the remaining two samples (AF) at 1.5×10^9 CFU·g^{-1} (Table 1). One of the samples containing probiotic bacteria (ACF 4 °C) was chill-stored at 4 °C, and the other sample (ACF 20 °C) was stored at 20 °C for 98 days.

2.5. Microbiological Analyses

All feed samples (CF, AF, and ACF) stored at 4 °C and 20 °C were subjected to microbiological analyses after 7, 14, 21, 28, 35, 42, 49 56, 63, 70, 77, 84, 91, and 98 days of the experiment. The following parameters were determined: Total counts of *C. maltaromaticum* bacteria on tryptone soya agar (TSA; Oxoid, Basingstoke, UK) with the addition of 3% yeast extract and 1.5% (*w/v*) NaCl [14], total counts

of mesophilic bacteria on tryptone soya agar (TSA; Oxoid, Basingstoke, UK) incubated at 28 °C for 48 h (TVC 28 °C), total counts of hemolytic mesophilic bacteria on tryptone soya agar (TSA; Oxoid, Basingstoke, UK) with 5% addition of defibrinated sheep blood incubated at 37 °C for 48 h (hemolytic mesophilic bacteria (Hem) 37 °C), total counts of aerobic spore-forming bacteria (ASFB) on an agar/broth medium (Biocorp, Warsaw, Poland) with glucose incubated at 28 °C for 72 h, counts of *Staphylococcus* sp. bacteria on the Chapman medium (Merck KgaA, Darmstadt, Germany) incubated at 37 °C for 48 h, counts of sulfite-reducing anaerobic spore-forming *Clostridium* sp. bacteria on the Wilson-Blair medium (Merck KgaA, Darmstadt, Germany) incubated at 37 °C for 18 h, and total yeast and mold counts on the Rose-Bengal-Chloramphenicol Agar (RGBC; Merck KgaA, Darmstadt, Germany) incubated at 28 °C for 5 days.

All analyses were performed according to Polish Standard [51]. The potential pathogenicity of Hem 37 °C, *Staphylococcus* sp., and *Clostridium* sp. bacteria was determined based on their hemolytic activity on tryptone soya agar (TSA; Oxoid, Basingstoke, UK) with 5% addition of defibrinated sheep blood incubated at 37 °C for 48 h. Hemolysis was confirmed when a transparent zone was formed around the inoculated colonies [46]. Mean microbial counts were calculated based on the values determined in three replicates of the same sample of compound fish feed. Finally, the counts of all analyzed microorganisms were expressed in $CFU \cdot 1 \; g^{-1}$ of compound feed.

2.6. Statistical Analysis

The mean values, standard deviations, standard errors, and confidence interval (CI = 95%, N = 3) of microbial counts in feeds (CF, AF, ACF) stored at a temperature 4 °C and 20 °C were calculated. The relationships between *C. maltaromaticum* bacterial counts and microbial (TVC 28 °C, Hem 37 °C, ASFB, *Staphylococcus* sp., *Clostridium* sp., yeasts, and molds) counts were determined by Spearman's non-parametric rank correlation test ($p \leq 0.05$). The significance of differences in microbial counts between the analyzed types of fish feed (CF, AF, ACF) stored at different temperatures (4 and 20 °C) and for different periods of time (7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, and 98 days) was determined by one-way analysis of variance (ANOVA). Leven's test was used to assess the homogeneity of variance. The verified hypothesis was rejected when Leven's test produced statistically significant results. The Kruskal–Wallis test, a non-parametric version of the classical one-way ANOVA, was then applied. Statistical analyses were performed in the Statistica 13.3 software package (TIBCO Software Inc., Palo Alto, USA) [52].

3. Results

3.1. Probiotic Properties of Carnobacterium Maltaromaticum

The studied *C. maltaromaticum* isolate was not capable of causing hemolysis, which suggested that the strain was not pathogenic.

The strain tolerated the tested pH values. After 3 h acid exposure, the isolate's survival rate was higher at pH 2.5 (76.1%) than at pH 1.5 (65%), and it reached 82.3% at pH 3.5 and 87.8% at pH 4.5. The bile salt tolerance test revealed a small difference in the survival rates of *C. maltaromaticum*. The highest isolate viability (85.2%) was observed at a 2% concentration of bile salts, whereas the lowest viability (79.5%) was noted at a 0.5% concentration of bile salts; 83.2% of *C. maltaromaticum* bacteria survived at a 1.0% concentration of bile salts (data not shown).

The *C. maltaromaticum* isolate tested in our study could be classified as a probiotic strain based on the results of the above analyses and according to the guidelines developed by a joint FAO/WHO working group [45].

3.2. Metabolic Activity of C. maltaromaticum Probiotic Bacteria

The results of the analyses examining the utilization of various carbon sources by the environmental *C. maltaromaticum* isolate are presented in Figure 1. The analyses performed in the Omnilog Gen III

system (Biolog, Hayward, CA, USA) revealed that the evaluated strain actively metabolized 70 carbon sources. The studied *C. maltaromaticum* strain was capable of growth at pH 5 and 6, and in the presence of 1%, 4%, and 8% NaCl. The tested isolate did not metabolize the following substrates: L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, histidine, D-gluconic acid, and mucic acid. The analyzed strain did not metabolize vancomycin, tetrazolium blue chloride, L-pyroglutamic acid, α-ketoglutaric acid, α-ketobutyric acid, and acetoacetic acid. These results confirmed the very high biochemical activity of the studied environmental probiotic isolate, and suggested its potential to compete for nutrients with feed microbiota.

Negative control	Positive control	D-Maltose	D-Trehalose	D-Cellobiose	Gentobiose
Sucrose	D-Turanose	Stachyose	Dextrin	pH 6	pH5
D-Raffinose	α-D-Lactose	D-Melibiose	1% NaCl	4% NaCl	8% NaCl
N-Acetyl-β-D-Mannosamine	N-Acetyl-D-Galactosamine	N-Acetyl Neuraminic acid	Methyl-D-Glucoside	D-Salicin	N-Acetyl-D-Glucosamine
α-D-Glucose	D-Mannose	D-Fructose	D-Galactose	3-Methyl Glucose	D-Fucose
L-Fucose	L-Rhamnose	Inosine	Sodium Lactate	Fusidic acid	D-Serine
D-Sorbitol	D-Mannitol	Arabitol	myo-Inositol	Glycerol	D-Glucose-6PO$_4$
D-Fructose-6PO$_4$	D-Aspartic acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline
Gelatin	Glycyl-L-Proline	L-Alanine	L-Arginine	L-Aspartic acid	L-Glutamic acid
Histidine	D-Glucuronic acid	L-Serine	Lincomycin	Guanidine HCl	Niaproof
Pectin	Nalidixic acid	L-Galactonic acid	D-Gluconic acid	Methyl Pyruvate	Glucuronamide
Mucic acid	Quinic acid	D-Saccharic acid	Vancomycin	Tween 40	Tetrazolium Blue
p-Hydroxy-Phenylacetic acid	L-Pyroglutamic acid	D-Lactic Acid Methyl Ester	L-Lactic acid	Lithium Chloride	α-Ketoglutaric acid
D-Malic acid	L-Malic acid	Bromosuccinic acid	D-Galacturonic acid	Citric acid	Potassium Tellurite
Tetrazolium Violet	γ-Aminobutryric acid	α-Hydroxybutyric acid	β-Hydroxy-D,L-Butyric acid	α-Ketobutyric acid	Acetoacetic acid
Propionic acid	Acetic acid	Formic acid	Aztreonam	Sodium Butyrate	Sodium Bromate

████████ - positive reaction　│　☐ - negative reaction

Figure 1. The results of a metabolic activity test analyzing the chemical sensitivity of an environmental *Carnobacterium maltaromaticum* probiotic isolate and its ability to utilize different carbon sources (GEN III MicroPlate™). Purple color—metabolic activity of the *C. maltaromaticum* isolate, white color—no metabolic activity of the *C. maltaromaticum* isolate.

3.3. The Quantitative and Qualitative Composition of Bacterial Microbiota in Compound Fish Feeds
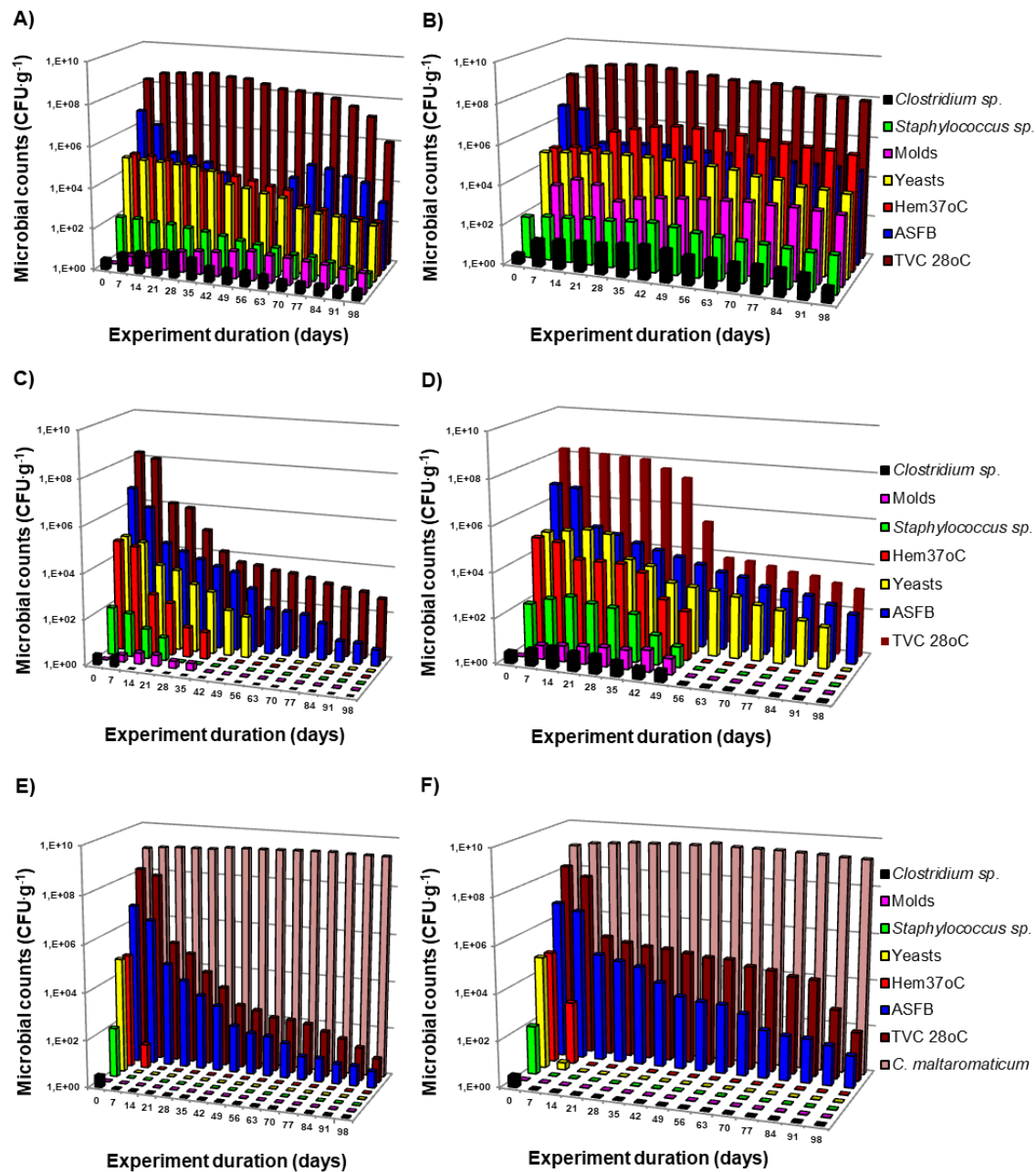
The mean (of three replicates) counts of mesophilic bacteria (TVC 28 °C), hemolytic mesophilic bacteria (Hem 37 °C), *Staphylococcus* sp., *Clostridium* sp., aerobic spore-forming bacteria (ASFB), yeasts and molds in CF, AF, and ACF, and *C. maltaromaticum* bacteria stored at a temperature of 4 °C and

20 °C during the 98-day experiment are presented in Figure 2. The mean values, standard deviations, standard errors, and confidence interval of three replicates of microbial counts are shown in Table S2 (Supplementary Materials). In CF, microbial counts differed by several orders of magnitude, depending on the analyzed microbial group and the temperature and time of feed storage. In CF 4 °C samples, TVC 28 °C and *Clostridium* sp. counts increased several-fold after 14 and 28 days of storage, respectively, relative to initial values. The counts of other microbial groups (Hem 37 °C, *Staphylococcus* sp., yeasts, and molds) in CF 4 °C samples continued to decrease in successive weeks of the experiment. The noted decrease ranged from 10^1 to 10^5 CFU across the analyzed microbial groups, subject to storage time (Figure 2A).

Figure 2. The mean (of three replicates) total viable counts (CFU·g^{-1}) of mesophilic bacteria (TVC 28 °C), hemolytic mesophilic bacteria (Hem 37 °C), *Staphylococcus* sp., *Clostridium* sp., aerobic spore-forming bacteria (ASFB), yeasts, and molds in: (**A**) Control feed (CF 4 °C) stored at a temperature of 4 °C, (**B**) control feed (CF 20 °C) stored at a temperature of 20 °C, (**C**) feed supplemented with 20% amaranth

meal (AF 4 °C) stored at a temperature of 4 °C, (**D**) feed supplemented with 20% amaranth meal (AF 20 °C) stored at a temperature of 20 °C, (**E**) feed supplemented with 20% amaranth meal and *Carnobacterium maltaromaticum* bacteria (ACF 4 °C) stored at a temperature of 4 °C, and (**F**) feed supplemented with 20% amaranth meal and *Carnobacterium maltaromaticum* bacteria (ACF 20 °C) stored at a temperature of 20 °C during a 98 day experiment. The mean values, standard deviations, standard errors, and confidence interval of three replicates of microbial counts are shown in Table S2 (Supplementary Materials).

The counts of nearly all microorganisms (excluding ASBF) increased by around 100% in CF 20 °C samples after 14, 28, and 42 days. In CF 20 °C samples, TVC 28 °C and yeast counts peaked on day 28 at 92×10^7 and 12×10^4 CFU·g^{-1}, respectively. The highest counts of potentially pathogenic bacteria (Hem 37 °C, *Staphylococcus* sp., *Clostridium* sp.) were noted after 42 days of feed storage. The maximum counts of Hem 37 °C, *Staphylococcus* sp., *Clostridium* sp., and molds were determined at 2.8×10^6, 3.0×10^2, 45, and 2.5×10^3 CFU·g^{-1}, respectively. A minor decrease in microbial counts was noted in successive weeks of the experiment. However, on day 98, the counts of all evaluated microorganisms in CF 20 °C samples were several-fold to several hundred-fold higher than those in CF 4 °C samples (Figure 2B).

In feed samples supplemented with 20% amaranth meal stored at a temperature of 4 °C (AF 4 °C), the counts of all analyzed microbial groups decreased by several orders of magnitude after 14 days of the experiment. On day 28, Hem 37 °C (20 CFU·g^{-1}) was the only potentially pathogenic microorganism in the studied samples. Toward the end of the experiment, AF 4 °C samples were colonized only by TVC 28 °C (500 CFU·g^{-1}) and ASFB (5 CFU·g^{-1}) (Figure 2C).

In AF 20 °C samples, the decrease in the counts of potentially pathogenic Hem 37 °C bacteria was considerably lower than that in AF 4°C samples. On day 28, Hem 37 °C counts in AF 20 °C samples were determined at 1.0×10^4 CFU·g^{-1}, and they were 500-fold higher than those in AF 4 °C samples on the same day. The counts of TVC 28 °C, ASBF, and yeasts were also several-fold to several dozen-fold higher in AF 20 °C samples than in AF 4 °C samples on the same days (Figure 2D). Hem 37 °C, *Staphylococcus* sp., and *Clostridium* sp. survived for longer periods of time in AF 20 °C than in AF 4 °C. Hem 37 °C, *Staphylococcus* sp., and *Clostridium* sp. were eliminated from AF 20 °C samples only after 56 days, and from AF 4 °C—already after 14 or 28 days of the experiment (Figure 2C,D).

Feed samples supplemented with 20% amaranth meal and a highly active environmental strain of *C. maltaromaticum* probiotic bacteria (ACF 4 °C, ACF 20 °C) were characterized by the lowest counts (Figure 2E,F) and the lowest survival rate of all analyzed microbial groups, regardless of storage temperature (Table S3). Potentially pathogenic *Staphylococcus* sp., *Clostridium* sp., and Hem 37 °C bacteria were not detected in ACF 4 °C and ACF 20 °C samples already after 7 days. In the first two weeks of the experiment, TVC 28 °C counts decreased around 1000 fold, ASFB counts decreased more than 100-fold, and yeast counts decreased several fold in ACF 4 °C and ACF 20 °C samples relative to the initial values. On day 98, ACF 4 °C samples were colonized only by TVC 28 °C and ASBF at 10 and 5 CFU·g^{-1}, respectively (Figure 2E). TVC 28 °C and ASFB counts were higher in ACF 20 °C at 120 and 20 CFU·g^{-1}, respectively (Figure 2F). Additionally, Spearman's test revealed significant ($p \leq 0.05$) negative correlations between *C. maltaromaticum* counts and almost all microbial populations (except for *Clostridium* sp. and molds) in ACF, regardless of storage temperature (Table 2).

The differences in the quantitative and qualitative composition of bacterial and fungal microbiota in the analyzed types of fish feeds (CF, AF, and ACF) stored at different temperatures (4 °C and 20 °C) and for different periods of time were confirmed by the statistical analysis (Table 3). The Kruskal−Wallis test revealed significant ($p \leq 0.05$) differences in the counts of all analyzed microorganisms between the evaluated feeds (CF, AF, and ACF) and in ASFB and yeast counts in feed samples stored for different periods of time. Significant ($p \leq 0.05$) differences were also observed in *Staphylococcus* sp., *Clostridium* sp., and mold counts in feed samples stored at different temperatures, and in TVC 28 °C, Hem 37 °C, *Staphylococcus* sp., and *Clostridium* sp. counts in feed samples stored for different periods of time.

Table 2. The values of correlation coefficients between microbial counts in feed supplemented with 20% amaranth meal and *C. maltaromaticum* probiotic bacteria (ACF) stored at 4 and 20 °C. The correlations between microbial counts in ACF 4 °C (N = 14) and ACF 20 °C (N = 14) samples were analyzed with Spearman's test.

	Microorganisms									
	C. maltaromaticum		TVC 28°C [1]		Hem 37 °C [2]		*Staphylococcus* sp.		Yeasts	
	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C
TVC 28 °C [1]	−0.904 *	−0.535 *								
Hem 37 °C [2]	−0.763 *	−0.713 *	0.723 *	0.763 *						
Staphylococcus sp.	−0.677 *	−0.580 *	0.511	0.577	0.715 *	0.755 *				
Yeasts	−0.763 *	−0.578 *	0.763 *	0.975 *	0.997	0.782 *	0.755 *	0.591		
ASFB [3]	−0.894 *	−0.535 *	0.957	0.999	0.723 *	0.763 *	0.577	0.577	0.763 *	0.975 *

[1]—mesophilic bacteria (TVC 28 °C); [2]—hemolytic mesophilic bacteria (Hem 37 °C); [3]—aerobic spore-forming bacteria (ASFB); *—statistically significant correlations in Spearman's test ($p \leq 0.05$).

Table 3. The quantitative composition of microorganisms in control feed (CF), feed supplemented with 20% amaranth meal (AF), and feed supplemented with 20% amaranth meal and *Carnobacterium maltaromaticum* bacteria (ACF) stored at different temperatures (4 and 20 °C) for 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, and 98 days, validated in the Kruskal−Wallis test (N = 84).

Microorganisms	Differences (*p*) between		
	Feed Type	Storage Temperature	Storage Time
TVC 28 °C [1]	0.0000 *	0.2194	0.0242 *
Hem 37 °C [2]	0.0000 *	0.3058	0.0476 *
Staphylococcus sp.	0.0000 *	0.0456 *	0.0493 *
Yeasts	0.0050 *	0.0550	0.0011 *
ASFB [3]	0.0005 *	0.2785	0.0005 *
Clostridium sp.	0.0001 *	0.0238 *	0.0483 *
Molds	0.0003 *	0.0048 *	0.9979

[1]—mesophilic bacteria (TVC 28 °C); [2]—hemolytic mesophilic bacteria (Hem 37 °C); [3]—aerobic spore-forming bacteria (ASFB); *—statistically significant differences; one-way ANOVA, $p \leq 0.05$.

4. Discussion

The analyses of the quantitative and qualitative composition of microbiota in fish feed samples revealed significant differences ($p \leq 0.05$) across the examined types of feed (CF, AF, ACF), feed storage temperatures, and feed storage times. Control feed (CF) was characterized by the highest counts, highest survival rates, and longest survival times of all analyzed microbial groups, which indicates that feed ingredients promote the growth of both specific feed microorganisms and potentially pathogenic microorganisms [37,53,54]. Similar results were reported by Petreska [4] and Gołaś et al. [55] who analyzed the counts of heterotrophic mesophilic bacteria and selected potentially pathogenic bacteria, yeasts, and molds in commercial feeds administered to intensively reared *Silurus glanis* L.

In our study, the counts of all specific feed microbiota and potentially pathogenic microorganisms (Hem 37 °C, *Staphylococcus* sp., *Clostridium* sp.) in feed supplemented with 20% amaranth meal (AF 4 °C, AF 20 °C) decreased by 1 to 4 orders of magnitude relative to those determined in CF 4 °C and CF 20 °C. The survival times of potentially pathogenic bacteria (Hem 37 °C, *Staphylococcus* sp., *Clostridium* sp.) were also significantly shorter in AF 4 °C and AF 20 °C than in CF 4 °C and CF 20 °C. The obtained results and the presence of significant differences ($p \leq 0.05$) in the counts of all analyzed microbial groups between CF and AF samples indicate that feed supplementation with 20% amaranth meal inhibits the growth of bacterial and fungal microbiota regardless of storage temperature or duration (Table 3). The antibacterial and antifungal properties of amaranth meal are also confirmed by the decrease in the counts of the remaining microbial groups (TVC 28 °C, ASFB, yeasts, molds) in

AF 4 °C and AF 20 °C samples in successive weeks of the experiment. The above could be attributed to the fact that amaranth meal contains lignins whose antioxidant, antibacterial, antiviral, and fungistatic properties contribute to the maintaining of the adequate microbiological quality of feed [31,56–59]. The addition of amaranth meal stabilizes natural microbiota in animal feeds, enhances the nutritional value of feeds, and improves performance.

Research studies have confirmed the beneficial influence of amaranth-supplemented feeds on the health status and body weight gains of rats [60], intensively farmed pigs [61,62], chickens [63], calves, lambs, sheep, and ruminants [56]. Studies investigating the effect of amaranth-supplemented feeds on fish in different farming systems also demonstrated that amaranth meal stimulated the immune system of fish [64], their growth performance, and the enzymatic activity of their gut microbiota [5,65]. The results of the present study indicate that amaranth meal can be effectively used to improve the quality and microbiological safety of fish feeds.

The counts of all studied microorganisms (TVC 28 °C, Hem 37 °C, ASFB, *Staphylococcus* sp., *Clostridium* sp., yeasts, and molds) were lowest in ACF 4° and ACF 20° relative to AF and CF stored at the corresponding temperatures. The counts, percentage viability, and survival times of the evaluated microbial groups were considerably lower in ACF 4° and ACF 20° than in AF 4° and AF 20° (Figure 2C–F; Table S3), which indicates that amaranth meal and *C. maltaromaticum* probiotic bacteria exert antibacterial and antifungal effects on natural microbiota and potentially pathogenic microorganisms in compound feed. The synergistic effects of the tested feed additives could be attributed to the symbiotic relationship between amaranth meal and the evaluated probiotic bacteria, and their ability to inhibit the growth and development of various microbial groups and genera. An in vitro study [57,66] revealed that amaranth is a source of bioactive compounds that suppress the proliferation of many microorganisms, including *Staphylococcus aureus*, *Bacillus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Proteus mirabillis*, *Klebsiella pneumoniae*, and *Candida albicans*. Amaranth meal also promotes the development of many species of probiotic bacteria, such as *Lactobacillus plantarum*, L. *paralimentarius*, L. *helveticus*, L. *sakei*, *Pediococcus pentosaceus*, L. *paralimentarius*, *Enterococcus mundtii*, *E. hermanniensis*, *E. durans*, *Enterococcus* sp., and *Leuconostoc mesenteroides*, whose metabolic activity enhances the nutritional value and health benefits of food products [67–69]. An in vitro study conducted by Gullón et al. [70] demonstrated that amaranth was characterized by a high prebiotic potential and promoted the growth of probiotic microflora isolated from the human digestive tract. By inhibiting the growth and development of naturally occurring microorganisms and pathogenic microbiota in foodstuffs and feedstuffs [5,66] probiotic bacteria and amaranth contribute to improving fish welfare and performance in various aquaculture systems [71–73].

The lowest counts of all evaluated microbial groups and genera and the shortest microbial survival times were noted in ACF samples regardless of storage temperature and storage time, which indicates that amaranth meal and *C. maltaromaticum* probiotic bacteria exert synergistic effects on the quantitative and qualitative composition of feed microbiota. Feed supplementation with 20% amaranth meal and *C. maltaromaticum* (ACF) bacteria completely inhibited the growth of most analyzed microorganisms (excluding ASFB and TVC 28 °C) in feeds stored at 4 °C and 20 °C for 7 days. The results of our in vitro study were validated statistically, which suggests that the novel tested additives contribute to the microbiological stability of fish feeds regardless of storage conditions and storage time.

5. Conclusions

The results of the present study, which investigated the supplementation of compound fish feeds with innovative additives, amaranth meal, and a highly active environmental strain of probiotic bacteria, *C. maltaromaticum*, indicate that the tested additives exert synergistic effects and contribute to the microbiological stability of fish feeds regardless of the temperature and time of storage. The evaluated components decreased the counts, percentage viability, and survival times of various groups and genera of microorganisms that occur naturally in feeds, which suggests that they can minimize feed losses resulting from the growth and metabolic activity of autochthonous

and allochthonous microbiota in feeds that are stored for excessive periods of time and/or at inadequate temperature. Excessive microbial growth lowers the nutritional value of feed, and decreases nutrient digestibility and assimilability, which may negatively affect fish performance in aquaculture. The addition of 20% amaranth meal and a highly active environmental strain of probiotic bacteria, *C. maltaromaticum,* to fish feed inhibited the growth of potentially pathogenic microbiota (Hem 37°, *Staphylococcus* sp., and *Clostridium* sp.) in vitro, which is important for the growth rate and welfare of fish. Due to their novel synergistic health-promoting properties, amaranth meal and environmental *C. maltaromaticum* bacteria could be recognized as the key ingredients of organic, environmentally friendly fish feeds, which guarantee the high quality of fish intended for human consumption.

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