



Different levels of probiotics affect growth, survival and body composition of Nile tilapia (*Oreochromis niloticus*) cultured in low input ponds



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ABSTRACT

A 7-month experiment was carried out to determine the effects of different levels of probiotics (baker's yeast (*Saccharomyces cerevisiae*) and *Bacillus subtilis*) on Nile tilapia (*Oreochromis niloticus*) reared in low input ponds. Monosex male fingerlings (40 g) were randomly distributed into 28, 1.25 m³ net cages at 50 fish m⁻³ and fed twice daily at 3% body weight on seven isonitrogenous (28% crude protein) diets supplemented with either *Saccharomyces cerevisiae* (1×10^{10} CFU g⁻¹) or *Bacillus subtilis* (1×10^9 CFU g⁻¹) at different levels: Diet 0 (control); Diet 1–3 were supplemented with *S. cerevisiae* at 2 g kg⁻¹ (Diet 1); 4 g kg⁻¹ (Diet 2) and 6 g kg⁻¹ (Diet 3) whereas Diet 4–6 were supplemented with *B. subtilis* at 5 g kg⁻¹ (Diet 4); 10 g kg⁻¹ (Diet 5) and 15 g kg⁻¹ (Diet 6). Higher final weight (255.31 ± 3.19), Specific Growth rates (SGR) (0.77 ± 0.01) and better Feed Conversion Ratio (FCR) (1.61 ± 0.02) were recorded in fish fed on Diet 2 followed by fish fed on Diet 5. Fish fed on probiotic-supplemented diets had significantly better growth, nutrient utilization and FCR compared to fish fed on the control diet ($P < 0.05$). Probiotic supplementation significantly affected the body composition of the fish ($P < 0.05$). *S. cerevisiae* led to significantly high protein (86.06%) at 4 g kg⁻¹ (Diet 2) ($P < 0.05$) while *B. subtilis* led to significantly higher protein (89.40%) at 5 g kg⁻¹ (Diet 4) ($P < 0.05$). Crude lipid and ash content were significantly lower in the fish fed on probiotic supplemented diets ($P < 0.05$) compared to the control. This study has demonstrated that the application of probiotics in diets of Nile tilapia in low input ponds promotes growth and enhances body composition. The two probiotics have got different effects depending on the level of application. *S. cerevisiae* exhibited the best performance at 4 g kg⁻¹ while *B. subtilis* had the best performance at 10 g kg⁻¹.

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Introduction

Nile tilapia (*Oreochromis niloticus*) is the second most farmed finfish species globally after carps [23]. Its vast culture is attributed to its ability to feed on various feeds, rapid growth, tolerance to a wide range of culture conditions, popularity with consumers, ease of breeding in captivity and wide availability to farmers [15,47,48]. In Kenya, Nile tilapia is either cultured in monoculture or polyculture with African catfish (*Clarias gariepinus*) in semi-intensive earthen ponds which are fertilized with livestock manure alongside supplementary feeding to reduce the cost of feeds [21,22]. These practices have led to poor growth rates and low yields [43]. Low input ponds are manured fertilized ponds with little or no supplementary feeding and have been in existence for ages in Asia and many parts of the world [15,19]. Livestock manures have been used to provide nutrients in form of nitrogen (N) and phosphorus (P) in low input ponds [36,58]. Of the nutrients applied in low input ponds, only 5–15% is converted to harvestable products with the remaining nutrients making the environment susceptible to invasion by disease causing microorganisms leading to poor growth and losses [4,38,50]. In the past decade, probiotics have received a lot of attention as a strategy towards enhancing performance and health management in aquaculture [51,54].

Probiotics are live microbes that when administered in sufficient amounts improves digestion, growth and enhances fish welfare [1,11]. They enhance growth by stimulating fish appetite and production of vitamins, fatty acids and additional digestive enzymes thereby breaking down indigestible feed components and improving digestion [3,33,34,60]. Previous studies have reported improvement in growth and feed utilization of different fish species including Nile tilapia [34], Indian major carp (rohu, *Labeo rohita*) [55] and rainbow trout (*Oncorhynchus mykiss*) [10]. The commonly used probiotics in aquaculture belongs to microalgae (*Tetraselmis*), yeast (*Debaryomyces*, *Phaffia* and *Saccharomyces*); gram positive (*Bacillus*, *Lactococcus*, *Micrococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Weissella*) and gram negative bacteria (*Aeromonas*, *Alteromonas*, *Photobacterium*, *Pseudomonas* and *Vibrio*) [17,25,31,40,41,44,59,64].

Saccharomyces cerevisiae and *Bacillus subtilis* are two of the commonly used probiotics in aquaculture to enhance growth and improve feed utilization due to their low cost, viability, ability to survive in the digestive tract of fish and the ability to colonize the gut of the fish [1,2,6,8,10,16,27,29–32,35,57]. Though probiotics are widely used in the livestock industry in Kenya, their use in aquaculture appears to occur inadvertently [43]. This may be related to lack of knowledge on their importance, efficacy and application levels. To improve the overall nutrient utilization by fish and growth of fish in low input ponds, the use of dietary probiotics is essential to keep fish healthy and promote growth [35,59,65]. This study was undertaken to assess the effect of different levels of *S. cerevisiae* and *B. subtilis* on growth, feed utilization, survival and body composition of Nile tilapia cultured in low input ponds.

Materials and methods

Experimental design

The experiment was carried out at Kenya Marine and Fisheries Research Institute, Sagana, Kenya. All male monosex *O. niloticus* fingerlings produced through hormonal sex reversal according to Phelps and Popma, [45] were used in this study. Fingerlings of an average weight of 40 g were acclimatized on a control diet at 3% body weight for 10 days and randomly allocated to seven treatments in four replicates. The experimental units consisted of 1.25 m³ net cages (1.0 × 1.0 × 1.25 m); placed in four earthen ponds of 150 m² surface area. Each pond was holding seven cages representing replicates of the seven treatments. The fingerlings were stocked at 50 fish m⁻³ according to Yi et al. [63] and Chakraborty et al. [14]. Ponds were dried and treated using agricultural lime (CaCO₃) at 100 g m⁻² [46]. Pond fertilization was done 2 weeks before stocking using dry chicken manure at 50 g of dry matter m⁻² and thereafter on a weekly basis to stimulate natural productivity following Charo-Karisa, [15].

Diet preparation

Dry ingredients (Table 1) were used to formulate seven isonitrogenous (28% crude protein) diet. Prepared diets were supplemented with dietary probiotic *Saccharomyces cerevisiae* (1×10^{10} CFU g⁻¹) FURAHA® (Agro Chemical and Food Company Limited, Kenya) at 3 concentrations; Diet 1 (2 g kg⁻¹), Diet 2 (4 g kg⁻¹) and Diet 3 (6 g kg⁻¹) and *Bacillus subtilis* (1×10^9 CFU g⁻¹) ULTRALACT® (Gee Dee Enterprises, India) at 3 concentrations; Diet 4 (5 g kg⁻¹), Diet 5 (10 g kg⁻¹) and Diet 6 (15 g kg⁻¹) according to Abdel-Tawwab et al. [1] and Hai [27]. The control diet (Diet 0) was not supplemented with any probiotic. The basal diets were blended with respective proportion of probiotics and an additional 100 mL of water per 1 kg diet. The diets were pelleted using an electric meat mincer (2–3 mm die), dried at room temperature, packed in plastic bags and refrigerated at 4 °C to maintain microbial viability as described by Allameh et al. [6]. Diets were repeatedly prepared every 2 weeks during the experiment [49]. Fish were hand-fed daily at 3% of the total biomass, twice daily at 1000 h and 1500 h for a period of 7 months. Feed adjustments were done for each cage every month after sampling.

Diet and fish body composition analysis

Experimental diets and fish samples were analyzed for proximate composition. A random sample of ten fish was taken at the time of stocking to serve as an initial carcass sample and three fish from each cage at harvest for the final carcass

Table 1
Ingredients and proximate composition of the experimental diets.

Ingredients (g kg ⁻¹)	Diet						
	Diet 0 (control)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Fish meal	190	190	190	190	190	190	190
Wheat bran	390	390	390	390	390	390	390
Wheat pollard	160	160	160	160	160	160	160
Maize germ	120	120	120	120	120	120	120
Cotton seed cake	120	120	120	120	120	120	120
Soybean oil	20	20	20	20	20	20	20
<i>S. cerevisiae</i>	0	2	4	6	0	0	0
<i>B. subtilis</i>	0	0	0	0	5	10	15
Proximate composition (% of dry matter)							
Dry matter	88.9	89.2	88.3	87.9	88.4	87.6	88.7
Crude protein	29.4	29.9	30.2	29.7	29.3	29.9	29.4
Crude lipids	3.4	4.1	3.4	3.1	3.8	3.5	4.1
Crude fiber	5.7	6	6.2	4.7	5.1	5.8	6.1
Moisture	8.2	9.2	9.2	9.1	8.4	8.6	8.7
Ash	8.9	8.9	7.5	9.2	10.4	10.9	11.3

* *Saccharomyces cerevisiae* treatments: Diet 1 (2 g kg⁻¹); Diet 2 (4 g kg⁻¹) and Diet 3 (6 g kg⁻¹).

** *Bacillus subtilis* treatments: Diet 4 (5 g kg⁻¹); Diet 5 (10 g kg⁻¹) and Diet 6 (15 g kg⁻¹).

composition analyses. Analysis for crude protein, lipid, moisture and ash were carried out using standard methods of the Association of Official Analytical Chemists [7]. Table 1 shows the proximate compositions of the experimental diets.

Sampling

Once a month, 30 fish were caught randomly from each cage and measured individually for total length and weight. The fish were weighed with a digital balance (0.01 g) and total length was measured using a measuring board (0.10 cm) as described by Caspers, [13]. Fish growth performance and feed utilization were calculated using standard formulae: specific growth rate SGR (%) = $100 (\ln W_t - \ln W_0) / t$ where: $-\ln$ = Natural logarithm, W_0 = initial weight (g), W_t = final weight (g) and t = period in days; Weight gain (WG) = final weight (g) - initial weight (g) and feed conversion ratio (FCR) = Feed intake (g)/Weight gain (g). Logarithmic regression formula, $W = aL^b$ was used to calculate the length-weight relationship (LWR) whereas condition factor (K) was calculated by the formula, $K = 100(W/L^3)$; where W = weight (g) and L = total length (cm), a and b are the regression slope and intercept (regression coefficient), respectively, according to Froese [24]. Survival of the fish was calculated as; survival (%) = number of fish at harvest/number of fish stocked) \times 100.

Water quality parameters were analyzed weekly using standard methods by Boyd and Tucker [12] and in situ measurements were done using a multi-parameter water quality meter model number H19828 (Hanna Instruments Ltd., Chicago, USA). The mean values for water quality parameters during the experiment were as follows: water temperature (25.40 °C), dissolved oxygen (4.76 mg L⁻¹), pH (8.06), total ammonium nitrogen (0.01 mg L⁻¹), nitrates-nitrogen (0.03 mg L⁻¹) and phosphates (0.02 mg L⁻¹). Water quality parameters were within the recommended levels for tilapia culture [12].

Data analysis

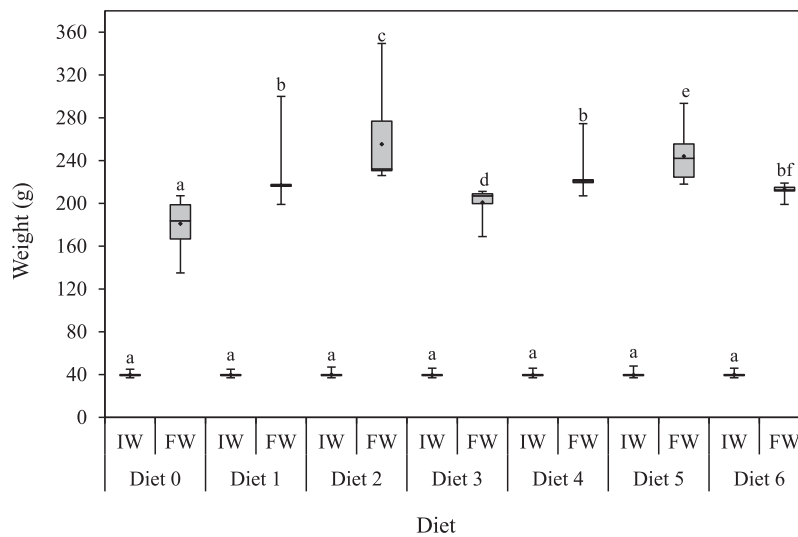
Data are expressed as mean \pm SE. All data were analyzed using one-way ANOVA at $P < 0.05$ for significance differences among groups. Differences between means were further analyzed using Duncan Multiple Range Test (DMRT) at $P < 0.05$. Percent survival data were arcsine-transformed before statistical analysis. Analyses were carried out using Statistical Product and Service Solutions (SPSS version 20).

Results

Fish growth performance was improved by feeding probiotic supplemented diets in comparison to the control (Table 2). Fish fed on probiotic supplemented diets had higher final weight compared to the control (Fig. 1). Final weight and SGR were highest in fish fed on Diet 2 followed by fish fed on Diet 5. FCR was more than 1.00 in all the treatments (Table 2). The lowest FCR was recorded in fish fed on Diet 2 while the highest FCR was in fish fed on the control diet (Table 2). Fish growth was significantly affected by the levels of probiotics in the diets ($P < 0.05$). Fish fed on the control diet had significantly lower growth, SGR and weight gain ($P < 0.05$) compared to other groups. Fish fed on Diet 2 had significantly higher growth performance followed by the fish fed on Diet 5 ($P < 0.05$). The SGR was significantly higher in fish fed on Diet 2 and Diet 5 followed by fish fed on Diet 4 ($P < 0.05$). Condition factor (K) was affected by probiotic supplementation, with fish fed on Diet 2 and 5 having significantly higher condition factor compared to the control and Diet 6 ($P < 0.05$). There were no significant differences ($P > 0.05$) in condition factor among fish fed on Diet 2, 3, 4, and 5. Results of LWRs indicated that the values of b during the culture period were not significantly different and were ≥ 3 with an R^2 value of

Table 2Growth performance parameters of *O. niloticus* fed on diets supplemented with different probiotic levels in low input ponds.

Parameter	Diet 0 (control)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Initial length (cm)	13.24±0.08 ^a	13.20±0.04 ^a	13.26±0.04 ^a	13.35±0.04 ^a	13.26±0.04 ^a	13.28±0.04 ^a	13.22±0.04 ^a
Initial weight (g)	39.90±0.10 ^a	39.75±0.14 ^a	39.99±0.15 ^a	39.65±0.14 ^a	39.63±0.15 ^a	39.57±0.14 ^a	39.90±0.15 ^a
Final length (cm)	22.18±0.08 ^a	23.46±0.06 ^a	23.88±0.05 ^a	23.10±0.05 ^a	23.31±0.07 ^a	23.93±0.06 ^a	23.29±0.07 ^a
Final weight (g)	180.96±1.74 ^a	216.93±1.74 ^b	255.31±3.19 ^c	200.84±1.08 ^d	220.62 ±1.59 ^b	243.99±1.89 ^e	212.93±1.33 ^{bf}
SGR (% day ⁻¹)	0.63±0.01 ^a	0.71±0.01 ^b	0.77±0.01 ^c	0.67±0.01 ^d	0.72±0.01 ^b	0.76±0.01 ^c	0.70 ±0.01 ^b
Weight gain (g)	140.92±1.76 ^a	177.18 ±0.75 ^b	215.32±3.22 ^c	161.29±1.08 ^d	204.17±1.00 ^b	179.07±1.02 ^{be}	173.03±0.40 ^f
FCR	2.03±0.03 ^a	1.87±0.01 ^{ab}	1.61±0.02 ^c	1.95±0.02 ^d	1.85±0.01 ^b	1.67±0.02 ^e	1.73±0.01 ^f
Condition factor (K)	1.74±0.01 ^a	1.78±0.01 ^b	1.83±0.01 ^c	1.82±0.01 ^c	1.80±0.02 ^{bc}	1.83±0.01 ^c	1.75±0.02 ^a
Survival (%)	77.00±1.00 ^a	83.50±0.50 ^a	89.50±0.56 ^b	81.50±5.25 ^{ab}	87.50±2.06 ^b	88.50±1.50 ^b	80.00±6.00 ^a

* *Saccharomyces cerevisiae* treatments: 2 g kg⁻¹ (Diet 1); 4 g kg⁻¹ (Diet 2) and 6 g kg⁻¹ (Diet 3).** *Bacillus subtilis* treatments: 5 g kg⁻¹ (Diet 4); 10 g kg⁻¹ (Diet 5); and 15 g kg⁻¹ (Diet 6).*** Means within the same row with different superscript letters are significantly different at $P < 0.05$ where $a < b < c > d < e < f$ (one-way ANOVA, Duncan Multiple Range Test (DMRT). For every diet, $n = 30$).**Fig. 1.** A box plot showing the initial (IW) and final weight (FW) of *O. niloticus* fed on diets supplemented with different levels of probiotics in low input ponds. The box plot indicates the lower and upper quartiles (bottom and top box lines), the median (horizontal line in the box), the minimum and maximum values (top and bottom whiskers) and the means (black circles inside the box). Different letters on top of the box denote significant differences at $P < 0.05$; where $a < b < c > d < e > f$ (one-way ANOVA, Duncan Multiple Range Test (DMRT). For every diet, $n = 30$).**Table 3**Body composition of *O. niloticus* fed on diets supplemented with different levels of probiotics in low input ponds.

Parameter (% dry matter)	Initial	Diet 0 (control)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Protein	69.95±0.00	83.33±0.28 ^a	83.73±0.36 ^a	86.06±0.18 ^b	85.81±0.05 ^b	89.40±0.16 ^c	86.63±0.09 ^b	78.94±1.68 ^d
Lipids	18.90±0.00	22.16±0.00 ^a	12.85±0.06 ^b	13.13±0.47 ^b	12.54±0.21 ^b	11.81±0.01 ^c	12.56±0.03 ^b	12.06±0.05 ^{ac}
Moisture	74.00±0.00	74.18±0.01 ^a	73.28±0.20 ^a	71.20±0.14 ^b	72.53±0.18 ^a	71.29±0.05 ^b	70.80±0.01 ^b	76.55±0.86 ^c
Ash	24.33±0.33	16.78±0.33 ^a	14.67±0.34 ^b	11.31±0.93 ^c	13.22±0.30 ^b	10.53 ±0.12 ^c	12.47±1.65 ^{bc}	11.52±1.53 ^c

* *Saccharomyces cerevisiae* treatments: 2 g kg⁻¹ (Diet 1); 4 g kg⁻¹ (Diet 2) and 6 g kg⁻¹ (Diet 3).** *Bacillus subtilis* treatments: 5 g kg⁻¹ (Diet 4); 10 g kg⁻¹ (Diet 5); and 15 g kg⁻¹ (Diet 6).*** Means within the same row with different superscript letters are significantly different at $P < 0.05$; where $a > b < c > c$ (one-way ANOVA, Duncan Multiple Range Test (DMRT).**** Initial values were excluded in the comparisons between treatments. For every diet, $n = 12$.

0.95, 0.97, 0.96, 0.96, 0.97, 0.97 and 0.98; regression slopes of 2.86, 2.84, 3.02, 2.93, 2.98, 3.05 and 2.99 for Diets 0,1,2,3,4,5,6, respectively. The experimental diets significantly affected the survival of fish ($P < 0.05$). The highest survival was recorded in fish fed on Diet 2 while the lowest was in fish fed on the control. Generally, fish fed on *S. cerevisiae* supplemented diets had better performance and survival compared to fish fed on *B. subtilis* supplemented diets.

Results for body composition analyses of experimental fish are presented in Table 3. Different probiotic levels significantly increased protein content but reduced lipid content when compared to the control group in fish fed on *S. cerevisiae* supplemented diets ($P < 0.05$). Similarly, *B. subtilis* supplemented diets led to significantly higher protein content in comparison to the control ($P < 0.05$). Nonetheless, lipid content was significantly lower in fish fed on the control diet compared to fish

fed on Diet 4 ($P < 0.05$). Ash content was significantly higher in fish fed on Diet 5 ($P < 0.05$) and no significant difference was recorded between fish fed on Diet 4 and Diet 6 ($P > 0.05$). The initial values for crude protein and lipid were lower than the levels after feeding experimental feeds, while ash content was higher before the experiment.

Discussion

Diet supplementation with probiotics (*S. cerevisiae* and *B. subtilis*) in the current study resulted to better growth and feed utilization, but indicated a non-linear relationship between the level of probiotic supplementation and the growth performance of the fish (Table 2). The highest growth performance exhibited in fish fed on probiotic supplemented diets in the present study could be attributed to improved nutrient digestibility and availability to fish. According to Merrifield et al. [39] and Welker and Lim [61], probiotics have been reported to improve digestion of feed by producing digestive enzymes or alterations of the gut environment, translating to better growth. Several studies have demonstrated that *S. cerevisiae* and *B. subtilis* affects growth of several fish species including Nile tilapia [1,29], common carp (*Cyprinus carpio*) [62] and rainbow trout (*Oncorhynchus mykiss*) [5].

Enhanced growth observed in fish fed on *S. cerevisiae* at 4 g kg^{-1} concurs with the results of Diab et al. [18] who recorded high average body weight of Nile tilapia fed on diets containing dried yeast at 1–5%. Hassaan et al. [28] also reported increased final weight and improved FCR of *O. niloticus* with increasing yeast levels from 0% to 0.5% or 1.0%. Better FCR in fish fed on probiotic supplemented diets could be attributed to enhanced levels of gastrointestinal bacteria involved in the decomposition of nutrients thereby providing additional enzymes, vitamins and amino acids to the fish [52,56]. Lara-Flores et al. [35] reported that live yeast supplementation in diets of Nile tilapia improved feed and protein digestibility which could have led to improved feed utilization and efficiency in the present study. The higher growth reported in fish fed on diets with *B. subtilis* in the present study is in agreement with El-Haroun et al. [20] who reported an increase in the daily growth rate by 33% and lower FCR by 43% reported in *O. niloticus* fed on Biogen® (a commercial product that contains *B. subtilis*).

The non-linear relationship between the level of *S. cerevisiae* and *B. subtilis* supplementation and the growth performance of the fish in this study is in agreement with the findings of Goda et al. [26] who reported reduced growth in fish fed on high levels of baker's yeast. Rainbow trout (*O. mykiss*) had significantly lower growth when fed on commercial probiotic containing *S. cerevisiae* and *S. elipsoedae* at 1.5% and above [5]. Additionally, Bagheri et al. [10] established that higher levels of a combination of *B. subtilis* and *B. licheniformis* led to lower growth of rainbow trout fry. Reduced growth at higher levels of probiotic dosage could be an indicator of depressed nutrient utilization attributed to variations in experimental conditions and duration of probiotic administration. This suggests that higher probiotic levels do not necessarily result in improved growth and fish fed on intermediary levels of probiotics may have acquired better health conditions compared to the other treatments hence increase in growth.

Improvement of feed digestibility (although not specifically measured in the present study) and nutrient utilization associated with enhanced presence of gut microbes could have led to the observed higher growth rates since gut microbes are known to produce additional amino acids that are beneficial to the fish especially when there is nutrient deficit fish [41,42,61,65]. Moreover, probiotics may have led to improved gut microvilli morphology and digestive enzymes which enhances nutrients absorption [65]. The LWR during the culture period indicates that fish fed on Diet 2 and Diet 5 had isometric growth which is the ideal growth recommended by Froese [24]. The improved survival of the fish fed on probiotic supplemented diets could be an indication of better health conditions and this concurs with the findings of Welker and Lim [61] and Hai [27] who established that *S. cerevisiae* and *B. subtilis* contains peptide antibiotics, including subtilin and bacitracin which improves immunity hence higher survival.

Body composition analysis in the current study shows that fish fed on probiotics supplemented diets had significantly higher protein content and lower lipid content compared to the control (Table 3). The increase in protein content could have resulted from increased nutrient deposition. This is in agreement with El-Haroun et al. [20] and Bagheri et al. [10] who reported an increase in the level of protein and reduction in crude lipid content in *O. niloticus* and *O. mykiss* fed on probiotic supplemented diets. According to Abdel-Tawwab et al. [1], *S. cerevisiae* plays a key role in enhancing food intake resulting to improvement of fish body composition. Therefore, the higher carcass protein content in this study could be attributed to more proteins secreted by the probiotics in the gut of Nile tilapia and effective conversion of ingested food into structural protein building more muscle [34,37,53]. On the contrary, other studies have documented that probiotic treatments have no significant effect on protein, lipid or ash content [29,39]. The crude lipids were lower in the fish fed on probiotic supplemented diets compared to the control. This represents fish with more protein and less fat which is desirable in aquaculture [9,29]. The quantities of the probiotics used during the study implies that only little amounts are required for improved growth and can lead to more yield and economic benefits. Furthermore, probiotics may also lead to a better fish culture environment by stimulation growth of non toxic algae in fish ponds hence improved fish welfare.

This study has demonstrated that supplementation of baker's yeast (*Saccharomyces cerevisiae*) at 4 g kg^{-1} and *Bacillus subtilis* at 10 g kg^{-1} of feed led to improved growth performance of *O. niloticus* in low input ponds indicated by better final weight, weight gain, SGR and FCR. From these results, we conclude that dietary probiotics increase the growth of *O. niloticus* reared in low input ponds but higher levels of inclusion may lead to depressed growth. Therefore, feed manufacturers/farmers should include baker's yeast (*S. cerevisiae*) at 4 g kg^{-1} and *B. subtilis* at 10 g kg^{-1} of feed to supplementary feeds used by majority of farmers in low input ponds to promote growth of Nile tilapia. Further studies should focus on

evaluating the efficacy of these probiotics on immunity and intestinal morphology of the Nile tilapia cultured in low input ponds and other culturable fish species.

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Competing interests

None.

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