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Effect of two carbon sources in microbial abundance in a Biofloc culture system with *Oreochromis niloticus* (Linnaeus, 1758)

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Abstract
The goal of this research was to compare the microbial community production in a culture system Biofloc in tilapia using molasses and molasses + rice powder as carbon sources. With molasses it were found 18 heterotrophic bacteria species, 11 opportunistic pathogen and seven probiotic bacteria. With molasses and rice powder it were found 17 bacteria species, six pathogen, nine degradative and two probiotic bacteria's. The obtained results in this study suggested that BFT technology could be a new strategy to control pathogens differently as with conventional culture mediums, since experiments show that while microbial flocs development proceeds, the bacterial degradative heterotrophic and potential probiotics displace the opportunistic pathogen bacteria that communally causes infection process in cultured fish and crustaceans, like genus: *Aeromonas*, *Pseudomonas*, *Vibrio*, *Enterobacter*, *Klebsiella* among others, because of a competitive exclusion by heterotrophic and probiotic bacteria with respect to another bacterial groups since discharge a variety of exoenzymes and polymers that created an hostile bacteria environment, especially pathogens. Biofloc production is an alternative technique system which eliminates the use of chemical substances and antibiotics that negatively impact on aquaculture production and their environment.

Keywords: *Litopenaeus vannamei*, Shrimp farming, Nellore District, Andhra Pradesh

Introduction
The microbial community studies in aquaculture production has taken today great relevance, especially because it has been recognized their role to remove contaminants, nutrient recycling and in some cases the potential pathogen control [7, 19]. The aquaculture producers must be benefit to power handling the microbial communities in their production units' ponds and reduce the environmental impact generated by aquaculture process [15]. Regarding to this, has been developed new culture systems like Biofloc technique, that consist to develop microbial bio flocs from external carbon source which allow an aggregate of microbial bacteria, microalgae and protozoans that together with detritus and dead organic matter, allow the formation of microbial protein that can be used as primarily food source for cultured species with a significate saving of commercial food used in this industry [10]. Likewise, in this Biofloc system, it helps to minimize or avoid water change ponds with a substantial improvement in water quality discharge [3], because bacteria and other microorganisms acts as efficient biochemical systems to degraded and metabolize organic waste compounds [12]. Despite many benefits that have been achieved in fish and crustaceans cultivation in Biofloc, this biotechnology is still developing and has many questions to answer, especially regarding to the development of microbial communities with different carbon and species cultivation sources [20]. An excellent approach to characterize trophic status of production systems was to specify the principal microbial composition of Biofloc systems [8]. That is why the goal of this research was to compare microbial community production in a Tilapia Biofloc culture system using molasses and molasses + rice powder as carbon sources.

Materials and methods
The experimental work was made in Live Food Production Laboratory from Universidad Autonoma Metropolitana-Xochimilco.

Experimental design and culture conditions

The biofloc production was made in six fiber glass cylindrical beakers of 1,000 L capacity, with a central air diffuser to assure particle continuous movements and resuspension. At each cylinder beaker it was introduced with 60 juvenile tilapias with a mean length of 5.0±0.95 cm and a mean weight of 4.2±1.08 g. Daily it was supplied commercial food (Alimentos del Pedregal®, Toluca, Estado de México, México) with 45% protein and a particle size of 0.6-0.8 mm (10% of their total body weight) and adjusted every 15 days. To assure microbial community development at culture system, a C/N =20:1 relation was maintained [3], by a controlled supply of external carbon source. Three cylindrical beakers had molasses as external carbon source and the other three cylinders had molasses and rice powder as external carbon source, for which carbon source needs were calculated as recommended by Emerenciano [9].

Water quality and sedimentation solids samples

Water quality evaluation was made twice per week. Water temperature (°C) and pH were determined with a potentiometer PHEP 4 Hanna (HI 98127), for dissolved oxygen (DO, ppm) it was used a portable and waterproof oximeter with microprocessor (HI9146). Total ammoniac nitrogen was analyzed (TAN, mgL^{-1}), and nitrites (NO_2^-, mgL^1), nitrates (NO_3^- mgL^{-1}) and phosphate (PO_4^{++}, mgL^{-1}) analyzed with spectrophotometry with Hanna auto analyzer Aquaculture Photometer (HI83203), according standard HANNA methods (HANNA Company, 2003).

The sediment solids quantity produced in Biofloc system was measured with an Inhofe cone, avoiding pouring the sample on walls cone to the mark of one liter, for later sedimentation during 15-20 minutes. Finally, the total sediment solids was registered every third day like mL L^{-1} during 25 days. Later every week it was taken until experiment finished [3, 14].

Microbial communities' identification

Counting and characterization of microbial communities was made by APHA (1) proposed method. Every week a sample was taken of 2 grams of flocs from each cylindrical beaker. These samples was supplied with sterile saline solution (90 mL), later make dilutions (1:10) and put in (0.1 mL) in MSR (Man-Rogosa-Sharpe), BHI (Brain-Herat-Infusion), TCBS (Thiosulphate-Citrate-Bile salts) and TSA (Tryptone Soya Agar) agar plates with three replicates each one, incubated at 27 °C. The colonies forming units (UFC mL^{-1}) were counted after 24 hours and their colony morphology was characterized and later purified by sowing.

DNA isolation

The molecular microbial identification was made for gen 16S of rRNA detection, extracting DNA using the genomic DNA extraction kit (Wizard Genomic DNA Purification Kit (PROMEGA ™)) fulfilling with manufacture specifications. To determine the purity and integrity of specific genomic DNA an electrophoresis in agarose gel at 1% was made and observed at UV light in 302-366 range wave length in a BioRad photo documentation [22, 13].

PCR (Polymerase Chain Reaction)

After DNA isolation from bacterial strains 16S gen RNAr amplifications were made using worldwide primers 8 for. (5'-AGACTTTGATCATGGCTCAG-3') and 1492 rev. (5'-TACGGCTACCTTGTTACGACTT-3'). The amplification reaction was made with elements mentioned at Table 1.

Table 1: PCR pool components.

Reagent	Pool
Water MQ	Variable
Buffer solution 10X	1X
$MgCl_2$	1.5 mM
dNTP´s	10 mM from each dNTP
8 for	10 pmol µL
1492 rev	10 pmol µL
Taq polymerase	5 U µL 1 U µL
DNA sample	0.25 µg reaction

The pool reagents were placed in PCR microtubules and reactions were made in Thermal cycler's equipment (Amplitron II Thermolyne Barnstead Internacional), with following conditions: Pre incubation at 94 °C during five minutes; denaturation at 94 °C, during 38 seconds; hybridization at 52 °C, during 40 seconds; and extension at 72 °C, during seven minutes. Thirty cycles were made, finished in 4 °C step [13].

Detection of 16 S DNAR gen by electrophoresis of PCR products

The PCR amplified product was subjected to electrophoresis in 1% agarose gel (TAE 1X), using 1 µL of molecular marker of 100 bp to assure the size of resulting web from each sample [22, 13].

PCR products purification

To remove primers wastes, nucleotides and polymerases, the samples were purified by QIAquick PCR Purification Kit (Qiagen), following manufacture specifications.

Sequencing and identification of isolated strains

The purification products were send to sequencing Macrogen DNA service and obtained sequences were interpreted by Chromas and Blast programs. To finish the bacterial identification, obtained information were compared with worldwide sequencing base (GENEBANK) to obtain phylogenetic relationships.

Once obtained the bacterial identification a bibliographic search was made to found the metabolic and ecological characteristics of identified bacterial strains, the bacterial strains were separated in three groups: 1) opportunistic pathogen bacteria of fish and crustaceans; 2) degradative bacteria group, corresponding heterotrophic bacteria which can break down cellulose, lignin, cheratina; and 3) heterotrophic bacteria which can break down nitrogen compounds and other natural molecules difficult to transform [17].

Results

Physical-chemical parameters

The physical and chemical parameters were maintained stable during all experiment for both treatments. Water temperature was maintained at 22-24 °C range, pH at 8.26-8-46 range, and dissolved oxygen at 2.90-4.07 ppm. Nitrites (NO_2^-) were maintained at 0.31-0.91 mgL^{-1} range; nitrates (NO_3^-) between 12.85 – 27.31 mgL^{-1} range and ammonium (NH_4) in 0.01-0.10 mgL^{-1} range.

Microbial community's treatment with molasses

Table 2 show the bacterial abundance values (UFC mL^{-1}) in Biofloc system with molasses like carbon source. It was observed during 10 culture weeks that degradative heterotrophic bacteria were most abundant with 18 species (50%); eleven species were recognized like opportunistic

pathogenic bacteria (30.55%), and only seven species like probiotic bacteria (19.44%).
With respect to recognized bacteria as opportunistic pathogen of aquatic organisms, the genera *Aeromonas* was predominant. However, in most cases it dies in last two culture weeks. Instead *Vibrio fluvialis* and *Erwinia* spp. were observed in seven culture weeks of the 10 culture weeks. The bacteria group which present biggest number of UFC mL^{-1} at first culture weeks was *Pasteurella neumotropica* with 198 at first week, but density was decreased from the six week onwards. The probiotic strains with better culture presence were: *Saccharomyces* sp. and *Rhodotourula* sp. However, *Bacillus cereus* was the bacterial strain with highest UFC mL^{-1} with 416 at the 10 culture week.

Table 2: Bacterial abundance (UFC mL-1) present in Biofloc culture system with molasses in *Oreochromis niloticus* experimental culture

Bacteria group	1	2	3	Weeks 4	5	6	7	8	9	10
Pathogen										
Aeromona hydrophila	98	97	0	0	0	95	84	76	0	0
Aeromona salmonicidae	0	0	23	0	0	0	0	0	0	0
Aeromona sobria	0	0	0	0	0	23	40	25	0	0
Aeromona veronii	0	0	0	0	0	0	35	22	15	21
Enterobacter sakasakii	0	260	0	0	5	0	0	0	0	0
Vibrio fluvialis	103	120	0	0	0	125	119	127	72	73
Pasteurella neumotropica	198	0	38	68	0	54	0	0	0	0
Brucella spp.	0	129	0	69	13	17	0	0	0	0
Moraxella spp.	121	0	0	0	0	0	0	0	0	0
Citrobacter freundii	0	0	154	185	0	145	139	0	0	165
Erwinia spp.	250	0	0	0	5	10	17	12	19	24
Degradative										
Pseudomona cepacea	0	0	112	266	376	120	130	0	132	184
Pseudomona luteola	0	157	43	140	310	160	171	152	198	213
Pseudomona fluorescens	0	95	195	114	360	378	369	386	382	394
Enterobacter cloacae	56	156	166	212	415	424	426	468	420	415
Enterobacter sp.	0	0	0	0	0	0	139	0	0	0
Bordetella spp.	0	150	142	238	400	426	0	0	0	0
Actinobacter baumannii	0	98	44	274	245	254	268	325	289	396
Sphingomonas paucimobilis	0	59	130	236	460	430	401	0	0	0
Ochrobactum anthropi	0	0	135	65	410	0	0	0	0	0
Pseudomona aeruginosa	0	78	78	173	110	100	103	0	0	0
Burkholderia cepacia	0	69	69	132	250	241	236	278	301	321
Burkholderia gladiolis	0	0	124	190	450	410	323	318	24	49
Pantoea spp.	0	43	43	223	75	65	37	19	24	36
Prolinoborus fasciculus	0	0	0	0	0	210	350	356	320	346
Probiotics										
Bacillus simplex	0	0	0	0	0	320	329	350	301	324
Lactococcus sp	0	0	0	0	0	176	229	241	216	204
Lactococcus lactis	0	0	0	0	0	230	227	227	296	368
Cedacea lapagei	0	67	67	0	90	0	0	0	0	0
Cedacea davisae	0	84	84	0	54	21	37	0	0	0
Rhodoturula sp	0	0	94	235	74	113	148	168	55	25
Saccharomyces sp	0	48	183	377	34	59	156	246	256	251
Pseudomona onyzihabitans	0	0	259	310	165	0	0	0	0	0
Bacillus cereus	0	0	0	0	0	320	348	379	410	416
Lactobacillus sp	0	0	0	0	0	210	150	265	147	126

Table 3 show the mean values of abundance (UFC mL^{-1}) grouped in pathogen, degradative and probiotic bacteria, as well as variance analysis (ANOVA) observed per bacteria groups during 10 culture weeks (Fig.1). Although the pathogen bacterial group was present during all 10 culture weeks, their best abundance were presented at two first culture weeks. The degenerative bacterial group were most active at third culture week and probiotic bacteria group dominated from third culture week until experiment finished with a mean abundance value of 100-150 UFC mL^{-1}.

Table 3: Abundance (UFC mL^{-1}) per bacterial group in Biofloc culture system with molasses in *Oreochromis niloticus* experimental culture.

Bacterial group	1	2	3	4	Weeks 5	6	7	8	9	10
Pathogen	70	101	43	64	2	43	39	24	10	26
	±9	±10	±6	±8	±1	±5	±5	±4	±2	±5
Degradative	3	28	145	40	12	25	14	12	9	5
	±1	±5	±12	±5	±2	±5	±4	±4	±2	±1
Probiotic	-	33	98	184	60	103	120	151	124	117
		±4	±9	±18	±6	±12	±12	±15	±15	±16

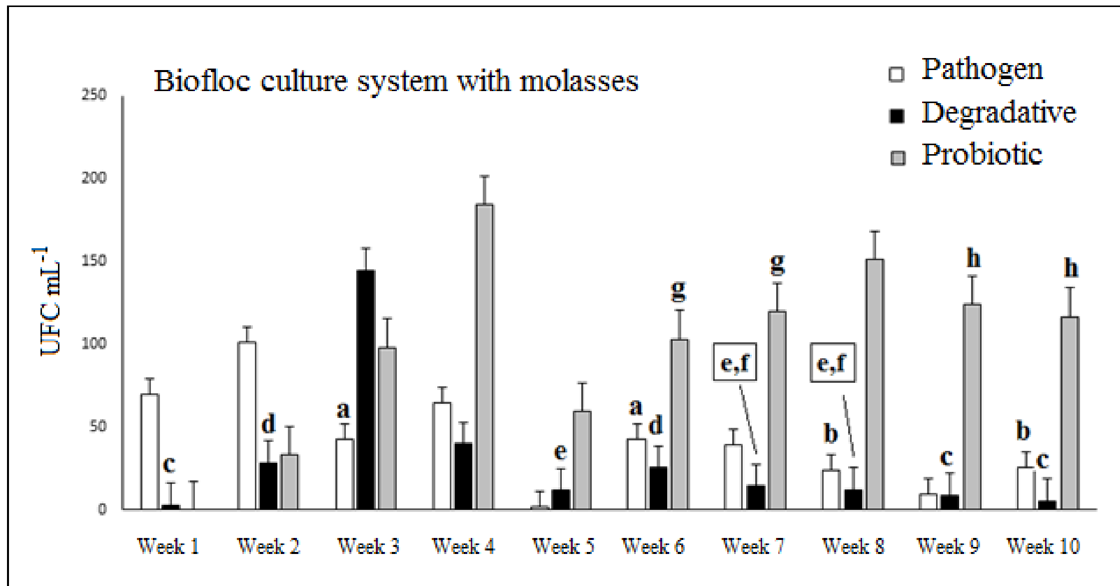

Fig 1: ANOVA analysis per bacterial group with Biofloc experiment with molasses in *Oreochromis niloticus* culture experiment.

Same letters show no significant differences (P>0.05). Columns with no letters shown significant differences (P<0.05).

The weight of significance (P<0.05) founded in this experimental treatment showed that interaction between culture weeks and bacterial group has 40.06% of significance; the week variable only have 36.64% and bacterial group variable (pathogen, degradative and probiotic) with only 21.86% of significance.

Microbial communities with molasses + rice powder

Table 4 show the abundance information (UFC mL^{-1}) of 17 bacterial groups founded in this Biofloc system, which carbon source was molasses plus rice powder. During 10 culture weeks it presented six pathogen bacteria (35.29%), nine degradative bacteria (52. 94%) and two probiotic bacteria (11.76%).

With respect to founded pathogenic bacterial group, the strains with most abundance (UFC mL^{-1}) was *Aeromonas sobria* with 230 at five culture week and *Vibrio fluvialis* with same quantity but at six culture week.

Table 4: Bacterial abundance (UFC mL-1) present in Biofloc culture system with molasses + rice powder in *Oreochromis niloticus* experimental culture.

Bacterial group	1	2	3	4	Weeks 5	6	7	8	9	10
Pathogen										
Aeromonas hydrophila	-	-	230	130	110	50	20	-	-	-
Aeromonas salmonicida	-	-	130	90	50	100	40	-	-	-
Aeromonas sobria	-	-	100	170	230	140	20	-	-	-
Vibrio fluvialis	-	-	80	90	150	230	10	-	-	-
Vibrio vulmificus	-	-	50	110	110	90	20	-	-	-
Enterobacter sakazakii	-	-	30	120	70	60	10	-	-	-
Degradative										
Nitrospira	-	-	-	140	350	450	670	770	1100	1080
Sphingomonas paucimobilis	-	-	-	420	390	560	550	560	460	650
Psudomonas luteola	-	-	-	380	420	460	580	650	750	450
Pseudomonas mendocina	-	-	-	270	340	450	230	170	340	300
Microthrix sp	-	-	-	340	450	560	650	340	230	250
Nitrobacter sp	-	-	-	20	150	190	250	380	540	500
Micrococcus sp	-	-	-	450	410	520	480	350	490	450
Alcaligenes sp	-	-	-	120	150	240	340	250	150	190
Probióticos										
Bacillus subtilis	-	-	-	450	360	340	470	650	860	800
Bacillus sp	-	-	-	560	450	430	480	660	790	760

Table 5 show mean values of abundance (UFC mL^{-1}) grouped in pathogen, degradative and probiotic bacteria, as well as variance analysis (ANOVA) observed for bacterial group during 10 culture weeks. (Figure 2).The pathogen bacterial group although was present in all experimental weeks, their best presence was at the two first culture weeks. The degradative bacterial group was present principally at the third culture week and probiotic bacterial group starting from the third culture week until the end of experiment with an abundance range of 100-150 UFC mL^{-1}.

Table 5: Abundance (UFC mL-1) per bacterial group in Biofloc culture system with molasses + rice powder in *Oreochromis niloticus* experimental culture.

Bacterial group	1	2	3	4	Weeks 5	6	7	8	9	10
Pathogen	-	-	103	118	120	112	20	-	-	-
			±7	±3	±7	±7	±1			
Degradative	-	-	-	268	333	429	469	434	508	484
				±16	±12	±14	±18	±21	±30	±28
Probiotics	-	-	-	505	405	385	475	655	825	780
				±8	±6	±6	±7	±7	±5	±3

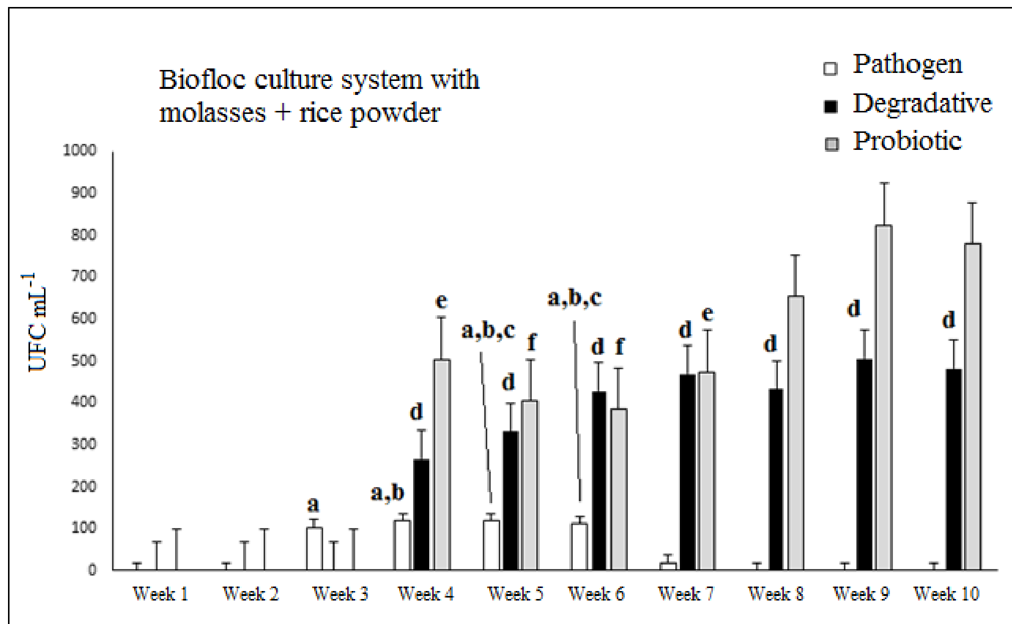

Fig. 2: ANOVA analysis per bacterial group with Biofloc experiment with molasses + rice powder in *Oreochromis niloticus* culture experiment.

Same letters show no significant differences (P>0.05). Columns with no letters shown significant differences (*P*<0.05).

Table 6 show bacterial abundance mean values (UFC mL^{-1}) grouped in pathogen, degradative and probiotic bacteria, as well as variance analysis (ANOVA) observed by bacterial group during all 10 culture weeks (Fig.2). The UFC mL^{-1} values are bigger that those obtained with Biofloc only with molasses but also less diversity was observed.

Table 6: Significance values per culture week between experimental treatments (Biofloc with molasses, Biofloc with molasses + rice powder.

Week	Bacteria's founded in Biofloc system with two experimental treatments						
	Pathogen		Degradative		Probiotics		Significance (*P*>0.05)
	Molasses	Molasses + rice powder	Molasses	Molasses + rice powder	Molasses	Molasses + rice powder	
1			X		X		P = 0.161
		X		X			P = 1.000
				X		X	P = 1.000
		X				X	P = 1.000
2				X	X		P = 1.000
				X		X	P = 1.000
		X		X			P = 1.000
		X			X		P = 1.000
3		X	X				P = 0.749
			X		X		P = 0.997
				X		X	P = 1.000
		X			X		P = 0.946
4	X		X				P = 0.999
			X		X		P = 0.335
	X				X		P = 0.178
5	X		X				P = 0.989
			X		X		P = 0.989
	X				X		P = 0.815
6			X		X		P = 0.944
	X	X					P = 0.998

7		X	X				P = 0.999
			X		X		P = 0.994
		X			X		P = 0.948
8	X			X			P = 0.218
		X		X			P = 0.980
				X	X		P = 0.998
	X	X					P = 1.000
9	X		X				P = 0.362
		X	X				P = 0.997
			X		X		P = 0.997
							P = 0.152
		X	X				P = 1.000
			X		X		P = 1.000
	X		X				P = 1.000
		X			X		P = 1.000

The weight of significance ($P<0.05$) founded in this treatment show that variable bacterial group has the highest value with 35.22%; the culture week variable with 31.79% and the interaction of these two variables with only 29.33%.

Variance analysis between treatments

These analysis showed significant differences between treatments ($P<0.001$) for each considered culture weeks.

Discussion

The results obtained in this study show that regardless the carbon source the microbial communities which develop in this zero water exchange systems makes an efficient debugging of nitrogen compounds potentially toxic for fish, that were maintained stable during all experimental period, like normal parameters for culture of tilapia (nitrites: 0.31-0.91 mgL^{-1}; nitrates between 12.85 – 27.31 mgL^{-1} and ammonium between 0.01-0.10 mgL^{-1}). These results are in consistent according to reported by others authors [15], who reported a decrease of nitrites, nitrates and ammonium in ornamental fish water culture in Biofloc in comparison with conventional culture systems. Also is mentioned that this type of culture systems (Biofloc), microbial transformation was quick and efficient because nitrogen waste compounds were transformed and used in three steps: first by matter oxidation by chemical autotrophic bacteria; second by assimilation by heterotrophic bacteria; and third, for their compounds disposition to micro fauna like ciliates, rotifers and nematodes, which exploit fully these nitrogen compounds [21].

With respect to bacterial abundance and diversity it is noted in molasses treatment 36 bacterial strains, 11 bacteria heterotrophic, six opportunistic pathogen bacteria and seven potential probiotic, while in molasses + rice powder treatment were identified 17 species, of which nine are degradative heterotrophic bacteria, followed for six pathogenic strains and only two probiotic species. While there are not enough studies that shown bacterial species present in this culture system, it is well known that heterotrophic bacteria are predominant in flocs and its grow will depend on starch, cellulose, fructose, sucrose content and other constituent elements of external carbon source, which could be exploited by bacteria as indicated by other authors [22, 2, 6, 10]. The obtained results in this study suggested that BFT technology could be a new strategy to control pathogens differently as with conventional culture mediums, since experiments shows that while microbial focs development proceeds, the bacterial degradative heterotrophic and potential probiotics displace the opportunistic pathogen bacteria that communally causes infection process in cultured fish and crustaceans, like genders: *Aeromonas*, *Pseudomonas*, *Vibrio*, *Enterobacter, Klebsiella* among others, because of a competitive exclusion by heterotrophic and probiotic bacteria with respect to another bacterial groups since discharge a variety of exoenzymes and polymers that created an hostile bacteria environment, especially pathogens [15, 25], so Biofloc production is an alternative technique system which eliminates the use of chemical substances and antibiotics that negatively impact on aquaculture production and their culture environment.

Another important aspect in recent studies [5, 12] which mentioned that Biofloc culture systems can promote the probiotic bacteria development, because in faeces part of intestinal micro biota is discharge and can be mixed with nutrient rich culture medium that allows their grow and therefore their use for cultured species as it happened in this study to identify probiotic bacteria for both treatments. Meanwhile, there was a variation in type and quantity with relation external carbon source, since molasses treatment were found seven genders represented by: *Cedacea lapagei*, *Cedecea davisae*, *Rhodotourula sp*, *Saccharomyce*s sp, *Pseudomona oryzihabitans, Bacillus cereus* and *Lactobacillus* sp. For molasses + rice powder treatment only two strains were isolated: *Bacillus subtilis* and *Bacillus* sp. This is because molasses is a source rich of saccharose, glucose, fructose and dextrose which are not assimilated for cultured bacteria as mentioned by other study [24], in comparison with rice powder which content starch, amylopectin and cellulose, these polymers were subunits of different conformation of chemical compounds and they need diverse enzymatic systems and it is more difficult and slow to degrade Barrera [4].

It was therefore the BTF technology has some advantages above conventional culture systems. It can be noted a better removal of contaminant nitrogen compounds, the growth increase of heterotrophic bacteria, which together probiotic bacteria, inhibit the development of potential pathogen bacteria in aquaculture and recognized the benefit effect of external carbon source used to obtain the type and quantity of bacteria that development in these culture production systems. While the results are important, it must be made a further microbial variation research starting from carbon sources to stablish better options to culture fishes and crustaceans.

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