CHANGES IN BACTERIAL FLORA DURING STORAGE OF SELECTED AQUACULTURE FEED AND FEED INGREDIENTS

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DEDICATED TO

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Certified that the thesis entitled **"CHANGES IN BACTERIAL FLORA DURING STORAGE OF SELECTED AQUACULTURE FEED AND FEED INGREDIENTS"** is a record of independent bonafide research work carried out by **Shri. Rajanna, M. R.**, during the period of study from September 2000 to August 2002 under our supervision and guidance for the degree of **Master of Fisheries Science (Mariculture)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other title.

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सारांश

दो झीगों के भोजन एवं तीन प्रोटीन समृद्व संघटकों में 60 दिनों के छोटे समय के संग्रहण के दौरान जीवाण्विक बदलाव संघटकों का अध्ययन किया गया. भोजन 'एक', व्यापारिक झींगा 'वर्धक, खुराक तथा भोजन 'दो', सी.एम.एफ.आर.आइ. कोचीन द्वारा तैयार खर्च प्रभावी खुराक था. प्रोटीन समृद्ध भोजन संघटक मछली भोजन, मूंगफली की खली व सोयाबीन आटा इस्तेमाल किये गये. मुहरबंद नमूने समान परिस्थितियों में कमरे के तापमान पर रखे गये तथा जीवाणुओं की आबादी का शून्य, तीस एवं साठ दिनों पर विश्लेषण किया गया. भोजन तथा भोजन संघटकों की पूर्ण तश्तवरी गिनती प्रतिवेदित अनिम्नीकृत सीमा के अन्दर थी. ग्राम घनात्मक गोलिकाऐं (४२.८-७३%) सभी पाँच नमूनों में प्रमुख थीं. संग्रहण के दौरान इसमें थोड़ा कमी आई जबकि ग्राम ऋणात्मक छड़ों में थोड़ी वृद्वि का प्रदर्शन हुआ. भोजन एवं भोजन संघटकों से नियुक्त पाँच सौ नमूने आगे के चरित्र चित्रण एवं पहचान के लिये इस्तेमाल किये गये. पहचाने गये वंशों में माइक्रोकोकस, आर्थ्रोबैक्टर व बैसिलय जातियां प्रमुख थी. संकेतक जीवाणु जैसे विब्रियो जाति की गणना के अलावा, भोजन व संघटकों में मल स्ट्रेप्टोकोकाई, इश्चरिचिया कोलाई एवं स्टेफाइलोकोकस आरियस का अध्ययन किया गया. भोजन 'दो' विब्रियो जाति को छोड़कर उपरोक्त सभी संकेतक वर्गों से विहीन था, जबकि भोजन 'एक' में उपरोक्त सभी वर्ग भिन्न संख्या में प्रदर्शित हुये. छोटे समय के संग्रहण (साठ दिनों तक) का भोजन व भोजन संघटकों के जीवाणु भार पर अधिक प्रभाव नहीं पड़ा.

ABSTRACT

The bacteriological changes in two shrimp feeds and three protein rich ingredients on short-term storage for 60 d was studied. Feed I was a commercial shrimp 'grower' diet and Feed II was a cost effective diet for shrimp formulated at CMFRI, Kochi. The protein-rich feed ingredients used were fishmeal, groundnut oil cake and soybean flour. Sealed samples were stored under identical conditions at room temperature and were analysed for bacterial population at day 0, day 30 and day 60. The total plate count of feeds and feed ingredients was well within the reported non-degrading limit. Gram-positive cocci (42.8- 73%) were dominant in all the five samples. During storage gram-positive cocci showed slight reduction, while, gram-negative rods showed marginal increase. Five hundred isolates from feed and feed ingredients were used for further characterization and identification. The major genera identified were *Micrococcus* spp., *Arthrobacter* spp. and *Bacillus* spp. Besides enumeration of indicator bacteria such as *Vibrio* spp., faecal *streptococci*, *Escherichia coli* and *Staphylococcus aureus* were quantified in the feeds and feed ingredients. Feed II was devoid of all the above indicator forms, except *Vibrio* spp., while, Feed I showed all the above groups in varying numbers. Short-term storage (up to 60 d) did not have much effect on bacterial load in feeds and feed ingredients.

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INTRODUCTION

1. INTRODUCTION

Capture fisheries yielded a steady increase in tonnage for human consumption from 1960s to the late 1980s, but production has remained relatively constant at approximately 60 million metric tons (mmt) since then (FAO, 1995). Aquaculture production has doubled since 1990 (Aiken and Sinclair, 1995), and this remarkable trend will have to continue to meet expected future seafood demand. Aiken and Sinclair (1995) predict that by the year 2025, there will be a shortfall of 55 mmt between seafood harvest of 60 mmt and demand for seafood of 115mmt. Csavas (1994) calculated that aquaculture production would have to increase by 3.5 times over current levels to meet the expected shortfall. It is in this context, the technical advances achieved in aquaculture practices came up as a boom to aquaculture industry. Shrimp farming witnessed spectacular growth during the 1980s. But, majority of shrimp farming countries in Asia had suffered heavy production losses due to outbreak of diseases, starting with the collapse of Taiwanese shrimp industry in 1988. India too became a victim of virulent viral diseases since 1994 onwards. It is believed that unscientific and unscrupulous uses of resources have led to such a collapse. Thus, the only solution for the present problem encountered by entrepreneurs all over the country is to adopt sound management at all steps of aquaculture.

Nutritional cost in shrimp culture amounts to about 40 to 60% of the operational cost. It is by now well established that success of shrimp farming depends upon scientific and sound management at all facets of aquaculture. This is especially important as the focus is getting shifted from extensive to modified-extensive and semi-intensive culture systems. The shrimp feed industry has to shift its focus to cost effective and eco-friendly formulations. As in the case of any other manufacturing enterprise, a number of management interventions are important in aquafeed industry also. Ingredient quality is of prime importance in feed manufacture. Besides, feed should supply all the essential nutrients at required level to sustain the best growth and feed conversion. Feed derived waste also should be minimum, since it can cause deleterious changes in water quality and lead to eutrophication of the

system, which in turn would enable proliferation of pathogenic microorganisms or create a stressed environment, resulting in poor growth of the cultured organisms.

Prepared aquafeeds are perishable products, which require utmost care during storage and supply. Proper storage of feed ingredients and feed is important in maintaining the quality. Feed processors attempt to formulate and manufacture aquafeeds aimed at extending their shelf life. During storage, in many ways feed quality can be diminished, including physical wastage, chemical deterioration and microbial degradation.

Since feed is biological in nature with nutrients, it is equally capable of supporting the growth of contaminating bacteria. Two types of bacterial food borne diseases are recognized; intoxication and infection. Feed borne bacterial intoxication is caused by the ingestion of food containing bacterial toxin resulting from bacterial growth in the feed. On the other hand, food borne infection is caused by ingestion of food containing viable bacteria, which then grow and establish in the host resulting in diseases. Even though certain bacteria are ubiquitous in nature, others enter the system through contaminated feed. This principle is applicable to aquafeed as well as to human food (seafood items). Jansen (1970) has reported that fish may be more important vector of human diseases than has been realized. Uncontrolled and improper feed management could upset the natural flora in the cultural systems, leading to heavy loss. Even though shelf life of aquafeed is not well established, it is being presumed that a well-processed feed could be stored for six months by following proper storage measures, without any nutrient loss. Small farmers store left over feed for subsequent crops in case of a crop failure or when it is bought in excess. Does this practice have any implication on the bacterial flora in the feed? What are the sources of certain bacterial contaminants in feed as well as environment? Present investigation is being carried out in this line for a valuable conclusion. There is hardly any work being conducted on these aspects of aquafeed and thus it will be of use to farmers as well as nutritionists in designing proper feed management practices.

The main objectives of this work are:

1. To study the bacterial profile during short-term storage of feed and feed ingredients.

2. To isolate and identify bacterial strains from the feed and feed ingredients.

2. REVIEW OF LITERATURE

2.1. Feed and Feed Management in Aquaculture

Like any other organism, shrimps also need nutrients that will provide for growth, maintaining life and resistance to diseases (Pascual, 1983). Extensive studies have been carried out on shrimp nutrition during the past two decades as may be seen from the reviews of New (1976, 1987), Pascual (1983), Akiyama and Dominy (1989) Paulraj (1990). In the natural environment, shrimp derive nutrients from a variety of food sources, both of plant and animal origin, like algae, diatoms, small crustaceans, molluscs, polychaetes and fishes that are found at the bottom of the aquatic systems (Gopalkrishnan, 1952; Pascual, 1983; Baily-Brock and Moss, 1992). They also consume significant amounts of detritus aggregates, which serve as important carriers of attached microorganisms that constitute important components in shrimp diet. There are, however, considerable differences of opinion on the food value of bacteria, which are ingested by shrimp along with detritus (Chong and Sesekumar, 1981). Certain bacteria like Purple Sulphur Bacteria (PSB) and *Rhodobacter capsulatus* have been employed in the artificial feed of fish larvae (Bhosle, 1997).

Parallel to intensification of shrimp farming industry, nutritionally balanced and high quality compounded diets were developed and commercialized (New, 1976,1987,1990; Shigueno, 1984; Akiyama and Dominy, 1989; Tacon, 1993; Ali, 1997). Besides commercial aquafeeds, many farmers manufacture their own feeds to reduce the cost of farming to considerable extent.

Selection of proper ingredients is decisive to ensure nutritionally- rich, cost-effective and environment-friendly shrimp feeds. Many have reviewed the conventional feed ingredients and noted fish meal, squid meal, shrimp meal, other crustacean meals, soybean meal, cereals and yeast as the ones extensively used in the manufacture of commercial shrimp feeds in Asian countries (Raveen and Walker

(1980), New (1987), Tacon, (1993), Akiyama (1991), Paulraj (1993, 1998) and FDS (1994).

The best feed produced in the world proves to be worthless if it is not properly managed. Feed management is a sequential process comprising feed selection, handling and storage, feeding regimes and adjustments to feeding rates (New, 1990; De- la-Cruz, 1989; Tacon, 1993; Cruz, 1994; Akiyama and Chwang, 1995; Jory, 1995). Poor storage and handling of feeds results in product deterioration, reduced feed attractability and palatability, nutritional deficiencies and disease out breaks (Jory, 1996). The effects of ambient temperatures on the chemical characteristics or nutritional value of shrimp feeds during storage was reported by De la Cruz *et al.* (1989). Divakaran *et al.* (1994) have demonstrated effects of long term storage.

2.1.2. Quantitative and Qualitative Occurrence of Bacteria In Feed

Studies on fish feeds have shown that commercial feeds do contain a mixed microflora including bacterial species potentially harmful to man and fish (Trust, 1975). Animal feed is a recognized vector in the transmission of pathogens to live stock (Mc Capes *et al.*, 1989; Hinton and Mead, 1992). The ingredients used in feed formulation partly determine the nature of its microflora (Trust and Wood, 1973).

Fish feeds support growth of a wide variety of bacteria (Trust and Money, 1972). Feeds are known to be pathogenic to fish when the moisture content exceeds 25% (Jain, 1998). The enumeration of aerobic hetereotrophic bacterial load of feed (cow-dung and grass) used at Aluu, Nigeria, indicated that, it had higher count of $5.6 \pm 0.4 \times 10^6$ cfu/g compared to that of sediment $8.4 \pm 1.6 \times 10^5$ cfu/ g and water $4.7 \pm 1.0 \times 10^3$ cfu/ml (Opokwasili and Alapiki, 1990). Similar values for 9 indigenous and 16 imported shrimp feed have been reported from India (Kalaimani *et al.*, 1998) and for tilapia feed from Nigeria (Ogbodeminu *et al.*, 1991).

However, the viable microbial counts in the range of 3.7×10^2 to 1.4×10^4 /g of diet (Yoshimizhu, 1980) and total heterotrophic of 2.2×10^5/g diet (Sera and Kimata, 1971) have been reported. On the contrary Ringo and Strom (1994) recorded the total viable counts as low as 3.7×10^2 /g of roe diet and 6.7×10^2 /g of commercial

feed. Trust and wood (1973) reported 10^3 to 10^4 aerobic bacteria per gram of fish diet. There are even instances where the diet for aquarium fishes was found to be sterile (Trust and Money, 1972). Bissetts, *et al.* (1998), studied the characteristics of microbes of a formulated abalone (*Haliotis laevigata*) diet. Bacterial numbers on unimmersed diet and diet immersed in sterile seawater for 2 and 4 days were negligible. Bacteria proliferated after 2 days immersion in seawater with abalone (1.2 × 10^5 cells/mm^2) and without abalone (5.7 × 10^4 cells/mm^2). Murago *et al.* (1987) have reported the total bacterial counts of 2.1 × 10^7 cfu/g for live diets (rotifer and brine shrimps) and 1.2 × 10^4cfu/g for artificial diets. The same author also reported differences in the bacterial counts of these diets on two different media. The mean bacterial counts were 2.1 × 10^7 cfu/g wet weight and 2.2×10^6 cfu/g wet weight for live diets and 1.2 ×10^4 cfu/g and 8.4 × 10 cfu/g for artificial diets respectively in Zobell agar and Bromothymol blue agar.

Trust (1971) examined 47 samples of fish diets belonging to nine different production batches from two commercial hatcheries. The mesophilic, psychrophilic and thermophilic bacterial counts were found to be in the range of 10^3 to10^7/g., <10^2 to 10^5 and 10^3 to 10^5g respectively. Many of the mesophilic bacteria (10^3 to10^6/g) were able to grow on the media, which contained 3% (w/v) fish diets. Aerobic and anaerobic spore forming bacteria (<10^2 to 10^4/g) were also present in the feeds analysed and the faecal *Streptococci* count ranged between 10^2 and 10^3 /g feed.

Trust and Money (1972) have studied bacterial populations of 25 commercial diets formulated and offered to aquarium fishes. The total count of aerobic mesophilic bacteria in 24 samples ranged from 9.0 ×10^2 to 1.3 ×10^6 /g of diet and Enterobacteriaceae were detected in 23 of the feeds analyzed. Their Most Probable Number estimates ranged from 40 to 1100/ 10 gram of diet with a mean of 1023/ 10g. Viable heterotrophic bateria ranged from 10^6 to 10^8 cfu/ ml of *Artemia* naupli homogenate (Lopez-Torres and Lizarraga-Partida, 2001). The same authors also isolated bacteria on TCBS medium with hatched *Artemia* cysts of commercial brands. The TCBS population has shown an inverse correlation (r^2= 0.5775,a= 0.05) with cyst age, values comprising between 10^1 and 10^7 cfu/ml. The standard two-day-old *A.franciscana* had 24000 ± 10700 cfu (mean plus or minus STD) per animal with

presumptive *Vibrio* spp. and haemolytic bacteria constituting 58% and 10% of the total respectively (Olsen, *et al.*, 2000).

Greater variations in the dominant bacterial flora associated with the feeds have been reported (Ogbondeminu *et al.*, 1991). The predominant bacterial genera associated with feed (rice bran) were *Pseudomonas* with the incidence of 25% along with 25% gram positive bacteria, represented by *Streptococcus faecalis* (12%) and *Micrococcus* (15%) and *Staphylococcus* spp (10%). They could identify 80% of feed microflora in culture water as well. Yoshimizhu (1980) reported diminance of *Pseudomonas*, *Coryneforms* and *Micrococcus* with the percentage incidence ranging from 13-20, 44-79 and 4-50 respectively for the diet, which was used to feed the fry and fingerlings of salmons. Ringo and Strom (1994) reported dominance of *Pseudomonas* spp. (31.6%) in capelin roe diet and Enterobacteriaceae (26.9%) in a commercial diet. Dominance of gram-positive bacteria has been noticed in the isolates of feed (cow dung and grass) with *Bacillus* to the extent of 56.25%. Other bacterial species' such as *Streptococcus*, *Staphylococcus*, *Aeromonas* and *Escherichia* spp. constituted 6.25% each (Opokwasili and Alapiki, 1990). However, Hamid *et al.* (1978) reported the presence of 100% *Bacillus* in mullet diets. Among the bacteria isolated from live diets (brine shrimp and Rotifer), *Pseudomonas* was the most predominant bacterial genus, constituting 48,3% and *Vibrio, Cytophaga*, and *Moraxella* constituting 11.4%, 10.2% and 8.1% respectively (Muroga *et al.*, 1987). The same authors reported that gram-positive bacteria (48.4%) dominated in artificial commercial diets.

On TCBS medium, Lopez-Torres and Lizarraga-Partida (2000) isolated bacteria associated with hatched *Artemia* cyst of commercial brands. Of the 617 isolates, 94% were gram positive and only 6% were gram-negative, but were oxidase negative.

2.1.3. Bacterial Diseases of Shrimp and Seafood-borne Human Health Problems

Increasing occurrence of shrimp diseases in aquaculture systems and public health risks have been a serious problem in shrimp farming countries of the world (Jory, 1996; Karunasagar *et al.*, 1998). Though a number of bacterial genera have been found to constitute the normal microflora of the cultured and farmed animals, members of only 10 genera, namely *Aeromonas, Alcaligenes, Alteromonas, Flavobacterium, Flexibacter, Leucothrix, Moraxella, Mycobacterium, Pseudomonas* and *Vibrio* have so far been reported to be pathogenic in penaeid shrimp (Fulks and Main, 1992; Shariff and Subhasinghe, 1992; Lightner, 1993; Otta *et al.*, 1998). Among the genera, *Vibrio* are numerically the most abundant and commonly met with bacterial pathogens in cultured shrimp (Karunasagar and Indrani, 1995; Otta *et al.*, 1998)

Human health hazards associated with consumption of fish and shellfish contaminated with bacterial and pathogenic microorganisms are well recognized (Shewan, 1961; Liston, 1980; Austin and Austin, 1987; Inglis *et al.*, 1993a&b). Many outbreaks of human disease have been associated with marine fishery products (Reilly, 1998), especially those from wild stocks, and similar problems can result from aquaculture due to poor management (GESAMP, 1991). A number of pathogenic or potentially pathogenic bacteria such a *V.parahaemolyticus*, *V.vulnificus*, *V.cholerae* and *Salmonella* sp., which are often implicated with food poisoning or outbreaks like gastroenteritis, typhoid, cholera *etc.* in human beings, have been encountered in significant levels in cultured shrimps and their environment (Fonseka, 1990; Karunasagar *et al.*, 1991; Reilly and Twiddy, 1992; Nayyarahamad *et al.*, 1995; Surendran *et al.*, 1995). Consumption of raw or partly cooked shrimp from affected areas is likely to cause diseases due to pathogens or toxins (Shuval, 1986). Consumption of raw shrimp was identified as the main mode of transmission in the explosive outbreak of cholara witnessed in Philippines in 1961 (GESAMP, 1991) and in many other countries in subsequent years (Reilly, 1998). Karunasagar *et al.* (1990) reported that *V.parahaemolyticus* is the commonest among the potentially pathogenic *Vibrio* spp. encountered in Indian seafood. Though the ingestion of a small number of *V.parahaemolyticus* is apparently harmless, in as little

as two hours they can multiply resulting in a population capable of causing food poisoning. Food poisoning due to *V. parahaemolyticus* has been reported from many parts of the world (Aoki and Chun, 1967; Quadri and Zuberi, 1977).

As reviewed above, many have worked on various aspects of aquafeed, but a lacuna exists in the shelf -life studies of aquafeed, especially on the bacteriological changes during storage of shrimp feeds in tropical countries. An attempt has been made in the present investigation to study the changes in bacterial flora during short-term storage of widely used shrimp feed and feed ingredients.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The change in the bacterial population during short-term (0- 60d) storage of two shrimp feeds and three protein rich feed ingredients was studied. Of the two feeds, Feed I was a commercial grower shrimp feed collected from a farmer and Feed II, a cost effective formulated diet from Central Marine Fisheries Research Institute. The three feed ingredients were fishmeal, groundnut oil cake and soybean flour.

One kg each of feed and feed ingredients was used for the present study. The feed ingredients were purchased from the local market. Dry fish (anchovy) and GNOC were dried in hot hair oven at 60°C overnight and ground in a domestic grinder to make fine particles of <1 mm size. Soybean flour was used as such since the particle size was ≤1 mm.

The feed and feed ingredients were stored at room temperature aseptically in sealed black polythene bags to avoid any aerial contamination. Sampling was done on day 0, day 30 and day 60.

3.1. Quantitative Analysis of Bacteria in Feeds and Feed Ingredients

3.1.1. Quantitative analysis

The quantitative analysis includes Total Plate Count, *Staphylococcus aureus* count, *Faecal streptococci* count, *Escherichia coli* count, *Vibrio* spp. Count.

3.1.1.1. Preparation of sample

Ten grams of the sample (feed and feed ingredients) was aseptically weighed into a sterile mortar and ground with pestle (sterilized by burning with alcohol and flaming). Ninety ml saline was added into mortar and mixed uniformly by

agitating and allowed for 15 min. This becomes 10 times dilution. Using a sterile pipette, 1 ml of the supernatant was aseptically transferred into 9 ml of normal saline. This becomes 10^2 dilutions. Similarly, further dilutions were made whenever required for the inoculation.

3.1.1.2. Total plate counts (TPC)

One ml each of the appropriate dilutions was pipetted to sterile petridishes taken in duplicate for each dilution. About 15-18 ml molten and cooled (45^0C) Zobell marine agar was poured to each plate, mixed well with the inoculum and allowed to set for 30 min. The plates were incubated at 37^0 C for 48 h in an inverted position. After the incubation, the individual colonies were counted. The average count of the duplicates was calculated. The TPC per gram of the sample was calculated as follows:

TPC/g of the sample = (Average count × dilution factor)/ Weight of the sample (g)

3.1.1.3. *Staphylococcus aureus* count

Appropriate dilutions (0.1ml each) were pipetted to pre-set and dried Baired Parker agar plates, taken in duplicate for each dilution. The inoculum was spread well over the surface using the sterile bent glass rod. The plates were incubated at 37^0 C in an inverted position for 48 hr. Black colonies with thin white margin and a zone of clearance around colonies were counted as *Staphylococcus aureus*. The average count of the duplicates was calculated. The *Staphylococcus aureus* count per g sample was *calculated* using the relation.

Staphylococcus aureus count/g sample = (Average count × 10 × dilution factor)/ sample weight

3.1.1.4. *Escherichia coli* count

Appropriate dilutions (0.1 ml each) were pipetted to pre-set and dried Tergitol –7 taken in duplicate for each dilution. The inoculums were spread well over the surface using a sterile bent glass rod. The plates were incubated at 37^0 C for 18 – 24 hr in an inverted position.

Non mucoids not raised, line yellow colonies with or without occasional rets brown at the centre and yellow zones around were counted as *Escherichia coli* .The average of the duplicates was calculated. *E.coli* per gram sample was calculated using the relation:

Escherichia coli count per gram of the sample =Average count ×10 × dilution factor)/ sample weight.

However, to confirm them as *E.coli* the following procedure was also followed.

3.1.1.4a. Streak on Eosine- Methylene Blue (EMB) agar

EMB agar was melted and cooled to 50°C poured into petri dishes and allowed to set. The set plates were dried and cooled to room temperature. Typical yellow colonies from T –7plates were picked with a sterile platinum loop and streaked onto EMB agar plates, by streak dilution method, incubated at 37^0C for 18-24 hr. Well-isolated colonies, 2-3mm diameter with a greenish metallic sheen by reflected and dark purple centre by transmitted light were picked up and sub cultured on to Zobell agar slants and incubated at 37^0C for 18- 24 hr.

3.1.1.5. *Faecal streptococci* count

One ml each of the appropriate dilutions was pipetted to appropriately marked sterile petridishes taken in duplicate for each dilution. About 15-18 ml of molten Kenner Faecal Streptococci Agar medium cooled to 45^0C was poured on to each plate. The medium was mixed well with the inoculum and allowed to set for 30 min. The plates were incubated at 37^0C for 48 hr. All surface and subsurface light

pink to deep red colour colonies were counted as *Faecal streptococci*. The average of the duplicates was counted. *Faecal streptococci* count per gram sample was calculated as follows:

Faecal streptococci count /gram sample = (Average count × dilution factor)/sample weight.

3.1.1.6. *Vibrio* spp. count

Thiosulphate Citrate Bile Salt Sucrose (TCBS) Agar was melted and cooled to 45- 50^{0}C and poured to petridishes and dried. 0.1ml sample of appropriate dilution was inoculated on to preset TCBS plates. Inoculum was spread well over the surface using a sterile bent glass rod. Plates were incubated at 37^{0}C for 48 hr in an inverted position.

Yellow flat smooth colonies with opaque centres and transparent peripheries, 2-3 mm diameter were taken as *Vibrio* spp. and were transferred to Kligler Iron Agar and incubated at 37^{0}C for 18 hr. *V.cholerae* will give acid butt in alkaline slant.

Vibrio spp.count/gram of sample= (Average count×dilution factor ×10)/sample weight weight.

3.1.2. Media used

The media and chemicals used for microbial study were the supply of Hi Media Laboratories Ltd., Mumbai.

The list of media/ broth, staining solution and test reagents used are given below.

3.1.2.1. Alkaline peptone water

Peptone	:	10 g
Sodium chloride	:	5 g
DW	:	1000 ml

3.1.2.2. Baired Parker Medium

Basal medium

Tryptone	:	10 g
Beef extract	:	5 g
Yeast extract	:	1g
Sodium pyruvate	:	12 g
Glycine	:	12 g
Lithium chloride	:	5 g
Distilled water	:	1000 ml

Heated with agitation to obtain complete solution, cooled to 50°-60°C and adjusted to pH 7.0 ± 0.2. Dispensed in 100ml quantities and sterilised at 15lbs for 15 min.

To the melted basal medium of temperature 45-50°C following solutions were added asceptically.

1 % filter sterilized solution of potassium tellurite	:	1 ml
Egg yolk emulsion	:	5 ml

Mixed well and poured immediately in 15-ml quantities into petriplates.

3.1.2.3. Ec broth

Tryptone	:	2 g
Lactose	:	5 g
Bile salt	:	15 g
$K_2H\ PO_4$	:	4 g
$KH_2\ PO_4$	:	15 g
NaCl	:	5g
Distilled water	:	100 ml

Dissolved, adjusted to pH 6.9 ± 0.1 and dispensed in 5 ml quantities in test tubes (with inverted Durhanm's tube). Sterilised at 10 lbs for 20 min.

3.1.2.4. EMB agar

Peptone	:	10 g
Lactose	:	10 g
K_2HPO_4	:	2 g
Eosine Y	:	0.4 g
Methylene blue	:	0.065 g
Agar	:	15 g

pH 7.1±0.1

Heated to dissolve the medium completely and sterilised in autoclave at 15 lbs.

3.1.2.5. Kenner Faecal *Streptococcus* Agar

Peptone	:	10 g
Yeast extract	:	10 g
Sodium chloride	:	5 g
Sodium glycerophosphate	:	10 g
Maltose	:	20 g
Lactose	:	1 g
Sodium azide	:	4 g
Bromocresol purple	:	0.015 g
Agar	:	15 g
DW	:	1000 ml

pH 7.2

Heated with agitation till all ingredients get dissolved and cooled to 45-50°C and pH is adjusted to 7.2. Dispensed in 100 ml quantities and sterilised in autoclave at 15 lbs for 15 min. Before pouring into plates, to melted and cooled to (45-50°c) medium, 1ml of 1% TTC solution (dissolved 1g triphenyl tetrazolium chloride in 100ml sterilized distilled water by steaming for 1 hr) was added.

3.1.2.6. Kligler Iron agar

Peptone	:	20 g
Yeast extract	:	3 g
Beef extract	:	3 g
NaCl	:	5 g
Lactose	:	10 g
Glucose	:	1 g
Ferric citrate	:	0 g
Sodium thiosulphate	:	0.3 g
Phenol red	:	0.05 g
Agar	:	15 g
DW	:	1000 ml

pH 7.4±0.2

Dissolved and dispensed in test tubes (15ml), plugged and sterilized at 121°C, 15 lbs for 15 min. Kept In slanting position, so as to get about of 2.5 cm dept

3.1.2.7. Potassium tellurite

1% potassium tellurite provided by Hi Media Laboratories Ltd., Mumbai, was sterilized by membrane filter technique and was taken in sterilised conical flask kept in refrigerator at 5-8°C. Whenever, it was required it was taken and used.

3.1.2.8. Tergitol-7 agar medium

Peptone	:	10 g
Yeast extract	:	6g
Beef extract	:	5 g
Lactose	:	20 g
Tergitol-7	:	0.1 g
Bromothymol blue	:	0.05 g

Agar	15 g
DW	1000 ml
pH	7.2±0.2

Heated to dissolve medium completely, cooled to 50°C and pH was adjusted to 7.2 ± 0.2. Dispensed in 100 ml quantities and sterilised in autoclave at 15 lbs for 15 min. Before pouring into sterile Petri plates 0.4 ml of 15 2, 3, 5 TTC previously sterilised by steaming, was added.

3.1.2.9. Thiosulphate Citrate Bile Salts Sucrose Agar (TCBS)

Peptone	:	10 g
Yeast extract	:	5 g
Sodium thiosulphate	:	10 g
Sodium citrate	:	10 g
Sodium cholate	:	3 g
Oxgall	:	5 g
Sucrose	:	20 g
Sodium chloride	:	10 g
Ferric citrate	:	1 g
Bromothymol blue	:	0.04 g
Agar	:	15 g

Boiled to dissolve the medium completely. Not autoclaved. Cooled to 50°C and poured into plates.

3.1.2.10. Tryptone broth

Trypone	:	1.0 g
NaCl	:	0.5 g
DW	:	100 ml

pH 7.1±0.1

Dispensed in 5 ml quantities in test tubes and sterilised at 15 lbs for 15 min.

3.1.2.11. Zobell marine agar

Commercially available Zobell marine Broth was taken in appropriate quantities(40.25grams per litre distilled water), into which 2% agar was added. Heated to dissolve the medium completely and sterilized at 15 lbs for 15 min.

3.1.2.11a. Zobell Slant preparation

Zobell marine broth in appropriate quantity was taken and into which Agar was added in appropriate proportion and heated to dissolve medium completely. Medium was transferred to test tubes and kept in slanting position and allowed till it got solidified.

3.1.3. Staining Solutions

3.1.3.1. Gram's crystal violet

Crystal violet	:	2 g
Ethyl alcohol	:	20 ml
Ammonium oxalate	:	0.8 g
DW	:	80 ml
Gram's Iodine		
Potassium iodide	:	2 g
Iodine crystal	:	1 g
Distilled water	:	300 ml

3.1.3.2. Gram's saffranine

Saffranine	:	1 g
Ethyl alcohol	:	40 ml
Distilled water	:	360ml

Commercilly available Grams staining kit was used (HIMEDIA).

3.1.4. Indicator Solutions

3.1.4.1. Andrade's indicator (for sugar fermentation test)

Acid Fuschsin	:	0.5 g
Distilled water	:	100 ml
1N NaOH	:	16ml

3.1.4.2. Bromothymol blue indicator

Sodium hydroxide	:	40 mg
Bromothymol blue	:	250 mg
DW	:	100 ml

40 mg NaOH was dissolved in 100ml distilled water. Bromothymol blue was added and dissolved.

3.1.4.3. Methyl red indicator

Methyl red	:	50 mg
Ethyl alcohol	:	150 ml
Distilled water	:	100 ml

Methyl red was dissolved in alcohol and was diluted with distilled water.

Test: To a tube of 48-h old bacterial culture in MRVP medium, 0.25ml of MR indicator was added. Development of red colour was taken as positive.

3.1.4.4. Kovac's Indole reagent

P-dimethyl amino benzaldehyde : 5 g
N- butyl alcohol or amyl alcohol : 75 ml

Dissloved and kept in amber coloured bottle overnight before test.

Test: To a tube of 48h old tryptone broth culture, 0.2ml of indole reagent was added, shaken and allowed to stand. Development of pink or red colour at the top indicated a positive test for indole.

3.1.4.5. Vogue's Parker test reagents

Solution A

α-Naphthol : 0.25 g
Ethyl alcohol : 5 ml

Solution B

KOH : 2 g
Distilled water : 5 ml

Test: In a small test tube, 1 ml of 48h old bacterial culture grown in MRVP medium was taken and 0.6 ml solution A and 0.2 ml solution B was added, shaken well, small crystals of creatine was added, shaken and allowed to stand for 4 hours. Eosin pink colour indicated positive VP test.

3.1.5. Prepartion of Sterile Egg-yolk (50%) for Baired Parker Agar

Five chicken eggs were washed free of dirt with vim and water, wiped, dried and kept immersed in rectified spirit in a 1L beaker for 2 hours. The alcohol was drained into a bottle, eggs were taken out (one at a time), small opening was made at one end and all the egg white was poured out carefully. The egg yolk was transferred into a sterile conical flask, each egg provided around 15 ml of yolk. Equal volume of sterile normal saline was added, agitated well and allowed to stand. 5 ml each of the yolk saline was pipetted into sterile test tubes plugged with sterile cotton and kept in refrigerator (5 to 8° C).

3.2. Qualitative Analysis of Bacteria in Feeds and Feed Ingredients

3.2.1. Isolation and identification of colonies

3.2.1.1. Isolation

About 20-30 cultures from each feed giving preferably a count of 30- 60 well separated colonies on Zobell marine agar were randomly selected and transferred into peptone water tubes. The tubes were incubated at 37°C for 18-24 hr. The broth cultures were purified on Zobell marine agar by streak dilution method.

3.2.1.2. Streak dilution method for purification of cultures

Pre set and dried Zobell plates were prepared. A loopful of the culture grown in peptone water broth was streaked across the first quarter on the surface of Zobell plates. The loop was sterilized, continued as above to the third quarter and similarly to the fourth quarter. The plates were incubated at 37°C for 18-24 hr. A well isolated colony was picked to a zobell slant, labeled and incubated at 37°C for

18-24hr. The pure culture thus obtained was maintained at room temperature and used for identification.

3.2.1.3. Identification

The cultures were initially differentiated in to gram positive and gram-negative bacteria. Gram-positive bacteria were further identified upto the genus level by following the identification scheme of Surendran and Gopakumar (1981). The identification scheme includes gram reaction and microscopy, catalase and motility.

3.2.1.4. Gram reaction and microscopy

Preparation of smear: Only young cultures (18-24 h old) were used for staining. On to a dust free microscopic glass slide a speck of young culture was emulsified with a drop of sterile physiological normal saline into middle and spread uniformly. Dried in the air. Heat fixed by passing the slide three to four times through the blue flame of Bunsen burner.

Staining: The slides were placed on a staining bridge. The smear was flooded with gram crystal violet for one minute. Washed with water. Gram iodine was flooded for one minute. Washed with water. Gram's decolouriser was added for 30 seconds. Washed with water. Counter stained with saffranin for one minute. Washed and dried.

Microscopy: Using oil immersion objective, slides were observed under microscope. Cells stained violet, bluish violet or bluish purple were taken as gram positive. Cells stained red were considered gram negative. Shape, size and arrangement of cells were also noted. In case of gram-positive observation of spore formation was done.

3.2.1.5. Motility of bacteria

Hanging drop method was adopted to observe motility of bacteria under microscope using high power objective. (40x, 45x, 50x). Small drop of distilled water was placed on the middle of a cover slip; a speck of young culture from agar slant was emulsified with it. A cavity slide was taken; the margin of the cavity was smeared with paraffin jelly. The slide was inverted on the cover slip in such a way that the cover slip gets attached to the slide and on turning upside down, the culture drop hangs in to the cavity. Observed under a high power objective.

3.2.1.6. Catalase test

A speck of young culture was placed on a clean glass slide and flooded with two drops of 30% of hydrogen peroxide. Evolution of gas from the culture indicates positive test for catalase.

3.2.1.7. Oxidase test

One percent N: N: N: N- Tetramethyl- p- phenylene diamine dihydrochloride was freshly prepared and used for the test. A smear of test culture was prepared and streaked on to a filter paper impregnated with Kovac's cytochrome oxidase reagent using a sterile platinum loop. Development of blue colour indicates positive reaction.

RESULTS

4. RESULTS

4.1 Quantitative Variation of Bacterial Population in Feeds and Feed Ingredients on Short Term Storage

The bacterial loads determined in two shrimp feeds and three protein rich feed ingredients during 60 d storage are shown in Tables 1 to 5. The total plate counts (TPC) of bacteria in feeds and feed ingredients showed variations during the storage period of 60 days.

In the commercial shrimp feed (Feed I), the initial TPC was 2.765×10^3 cfu/g, which increased to 8.8×10^4 cfu/ g in 30 days and then reduced to 2.73×10^4 cfu/g. The number of colony forming units (cfu) of *Vibrio* spp. almost doubled from 3.0 to 6.4×10^1 cfu/g on the 60th day. The faecal *Streptococci* count also showed a marginal increase. The *Escherichia coli* count remained constant throughout *ie.* 3×10^1 cfu/g. *Staphylococcus aureus* appeared only on 30 days of storing (2.2×10^2 cfu/ g), while it was absent in the 60th day sampling (Table 1).

In Feed II, which is a cost effective formulated feed of CMFRI, the TPC showed a decline after 60 days. The total count of *Vibrio* spp., however, increased from 2×10^1 to 4×10^1 cfu/ g in 30 days and remained the same on day 60. Faecal *Streptococci, E. coli* and *S. aureus* were absent in this feed throughout the period of observation (Table 2).

In all the three feed ingredients there was variation in bacterial count during storage. The total plate count of fishmeal which was 5.12×10^3 cfu/g on day 0 showed a decline to 2.7×10^3 cfu/g in 60 days. The counts of *Vibrio spp., E. coli* (2×10^1 cfu/g) and *S. aureus* (3×10^2 cfu/gm) did not show any significant variation along with *F. streptococci* (6.5×10^2 cfu/gm and 5.1×10^2 cfu/gm). *F. Streptococci* and *S. aureus* were absent in fishmeal on day 60 (Table 3).

The total plate count of groundnut oil cake showed an increase from 3.3 $\times 10^3$ cfu/g to 5.1 $\times 10^3$ cfu/g in 30 days and then a reduction to 2.5 $\times 10^3$ cfu/g. *Vibrio*

spp. count remained almost the same (3×10^1 cfu/g) with an increase in 30 days (1.2×10^2 cfu/gm). *Faecal streptococci* and *E.coli* count showed little or no variation. *S.aureus* though occurred in day 0, in the remaining samplings it was absent (Table 4).

Total plate count of soybean flour showed a decline from 3.15×10^3 cfu/g to 2.75×10^3 cfu/g on day 60. *Vibrio spp.* did not show much quantitative variation and remained at 2×10^1 cfu/g. *Faecal streptococci* and *E.coli* were absent in the soyflour. *S.aureus* count remained a 3.5×10^2 cfu/gm in 30 d sample (Table 5).

4.2. Qualitative Analysis of Bacterial Genera in Feeds and Feed Ingredients

For qualitative study of the bacterial isolates, Zobell Marine Agar was used. For this purpose 500 isolates were maintained. The tests done for characterization include gram staining, motility, catalase, oxidase, nitrate reduction.

Micrococci were gram positive, aerobic cocci. The cells non-motile, and were showing oxidative respiration. *Arthrobacter* were gram positive, aerobic, asperogenus, coccoid bacteria, which were non-fermentative and nitrate reducing forms. *Bacillus spp.* were facultatively fermentative rods, gram positive, motile and showed catalase positive and were nitrate reducing.

Vibrio spp., Faecal Streptococci, E.coli and *Staphylococcus aureus* were plated in their respective selective media. *Vibrio spp.* from TCBS media were gram-negative rods, oxidase and catalase positive with round entire, glossy and yellow or green colonies. *Faecal streptococci* plated on Kenner Faecal Agar medium was gram positive, facultatively anaerobic, non-motile, catalase and oxidase negative cocci. *E. coli* plated on Tergitol-7agar was gram-negative, motile, and oxidative. The positive indole test was used as confirmative test. *Staphylococcus aureus* plated on Baired Parker Agar medium is cocci form, gram positive, facultatively anaerobic, and non-motile showing catalase test positive.

The percentage frequencies of occurrence of respective bacterial colonies in feed and feed ingredients are shown in Figures 1-5. The commonest genera was *Micrococcus spp.* in all the samples. Other genera were *Arthrobacter spp* and *Bacillus spp.* The occurrence of these genera did not show much variation on storage. Gram-negative rods increased from 13.6% to 23.8% in 60 days in Feed I (Fig.1).

In Feed II, the occurrence of *Micrococcus* spp. reduced from 73% to 53.27% in 60 days, while, the percentage of *Arthrobacter* (11.5% to 14.7%), *Bacillus spp.* (4.6%- 10.74%) and gram-negative rods (10.6 - 10.74%), increased during storage (Fig. 2).

In all the three feed ingredient samples, *Micrococcus* was the dominant group. In fishmeal, *Micrococcus* spp. decreased from 73% to 65.17% in 60 days. *Arthrobacter* (6- 7.74%) and *Bacillus spp.* (2.0 - 5.74%) showed slight increase, whereas, gram negative rods showed slight increase in 30 days (22%) and then a reduction on the 60th day (19.04 - 17%) (Fig.3). In groundnut oil cake, *Micrococcus* reduced from 73% to 53.33%, while, *Arthtrobacter* spp., *Bacillus spp.* and gram negative rods were 6-13.3%, 5.3- 13.33%, 15.7 to 20% respectively on the 60th day (Fig.4).

In soybean flour *Micrococcus* spp. was less than 50% (42- 48%) and the percentage occurrence increased on storage (42.8 - 51.72) while, *Arthrobacter* occurrence increased from 17.8 to 28.5% in 30days and then reduced to 23.9% on the 60th day. Gram-negative rods showed a gradual reduction in percentage occurrence (28.5 to 24.3). *Bacillus* also showed an increasing trend in 30 d (28.5%) and then reduced to 20% (Fig. 5).

Table 1. Variation of bacterial populations in Feed I

Days	TPC	*Vibrio* spp	*F. streptococci*	*E. coli*	*S. aureus*
0	2.765×10^3	3×10^1	3.6×10^1	3×10^1	NIL
30	8.8×10^4	5.3×10^1	2.9×10^1	3×10^1	2.2×10^2
60	2.73×10^4	6.4×10^1	4.5×10^1	3×10^1	NIL

Table 2. Variation of bacterial populations in Feed II

Days	TPC	*Vibrio* spp	*F. streptococci*	*E. coli*	*S. aureus*
0	5.75×10^3	2×10^1	NIL	NIL	NIL
30	5.5×10^4	4×10^1	NIL	NIL	NIL
60	3.65×10^4	4×10^1	NIL	NIL	NIL

Table 3. Variation of bacterial populations in Fishmeal

Days	TPC	*Vibrio* spp	*F. streptococci*	*E. coli*	*S. aureus*
0	5.12×10^3	2×10^1	6.5×10^2	2×10^1	NIL
30	3.87×10^3	3×10^1	5.1×10^2	2×10^1	2.2 x102
60	2.7×10^3	2×10^1	NIL	2×10^1	NIL

Table 4. Variation of bacterial populations in Groundnut oil cake

Days	TPC	*Vibrio* spp	*F. streptococci*	*E. coli*	*S. aureus*
0	3.3×10^3	3×10^1	6×10^1	3×10^1	2.3×10^2
30	5.1×10^3	1.2×10^2	5×10^1	3×10^1	NIL
60	2.51×10^3	3×10^1	4.9×10^1	3×10^1	NIL

Table 5.Variation of bacterial population in Soybean flour

Days	TPC	*Vibrio* spp	*F. streptococci*	*E. coli*	*S. aureus*
0	3.15×10^3	2×10^1	NIL	NIL	3.5×10^2
30	2.075×10^3	10×10^1	NIL	NIL	3.1×10^2
60	2.75×10^3	2×10^1	NIL	NIL	NIL

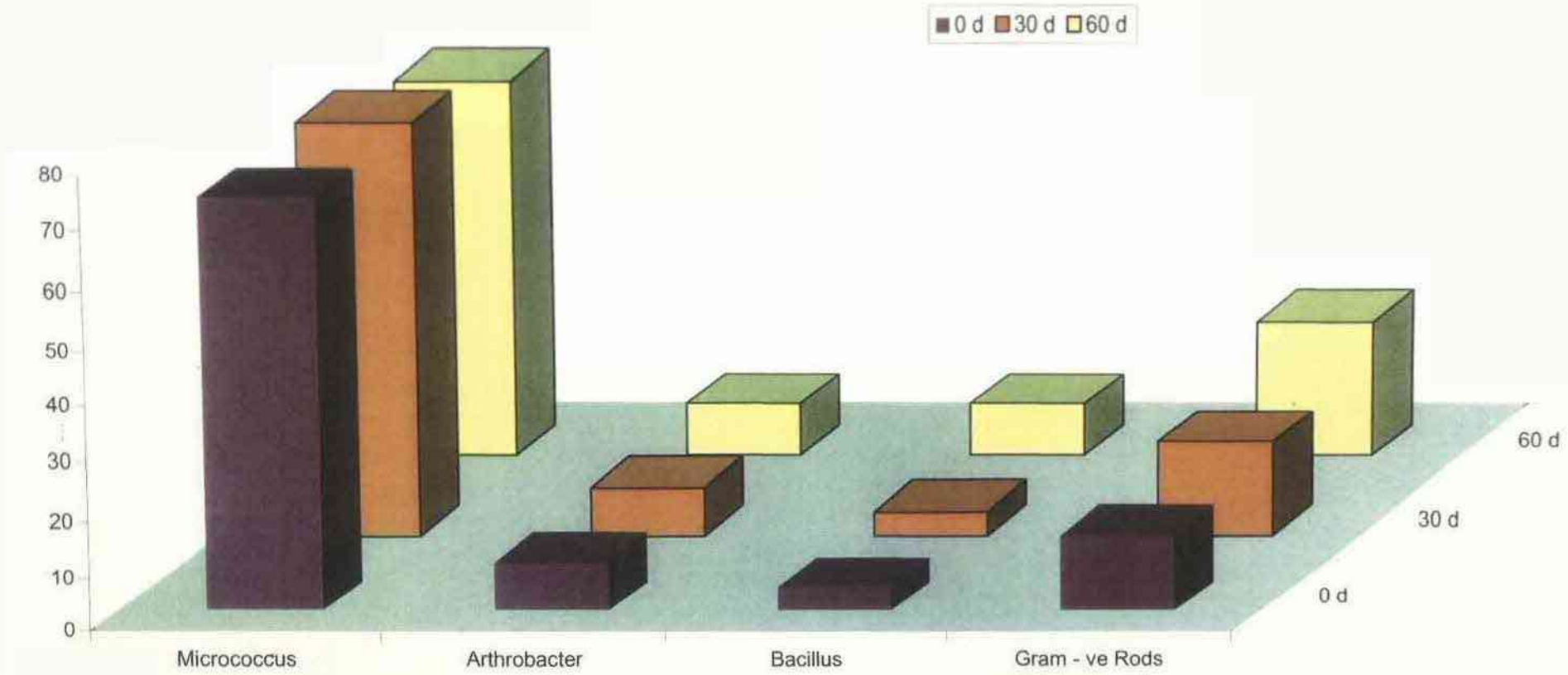


Figure 1. Percentage occurrence of different bacterial genera in Feed I

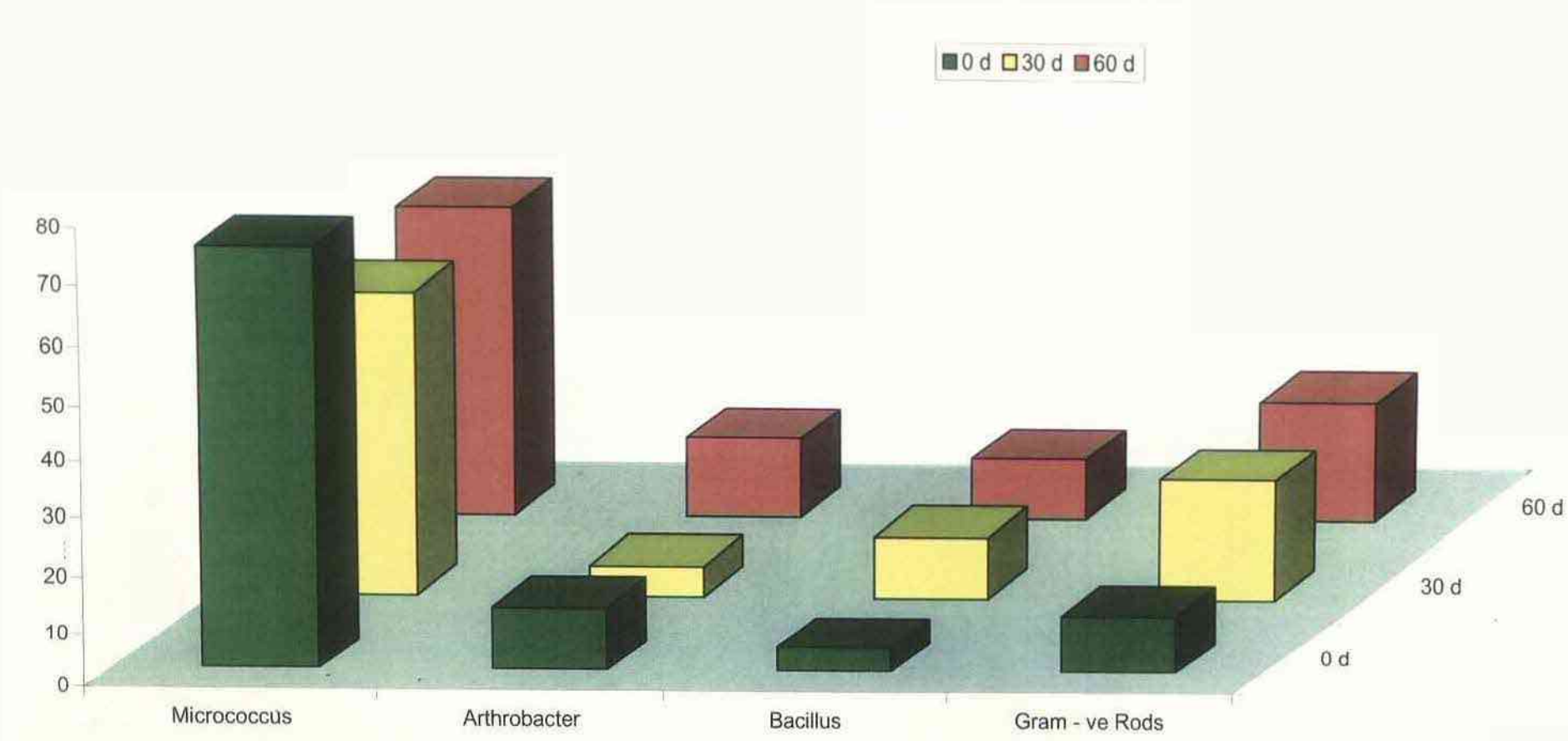


Figure 2. Percentage occurrence of different bacterial genera in Feed II

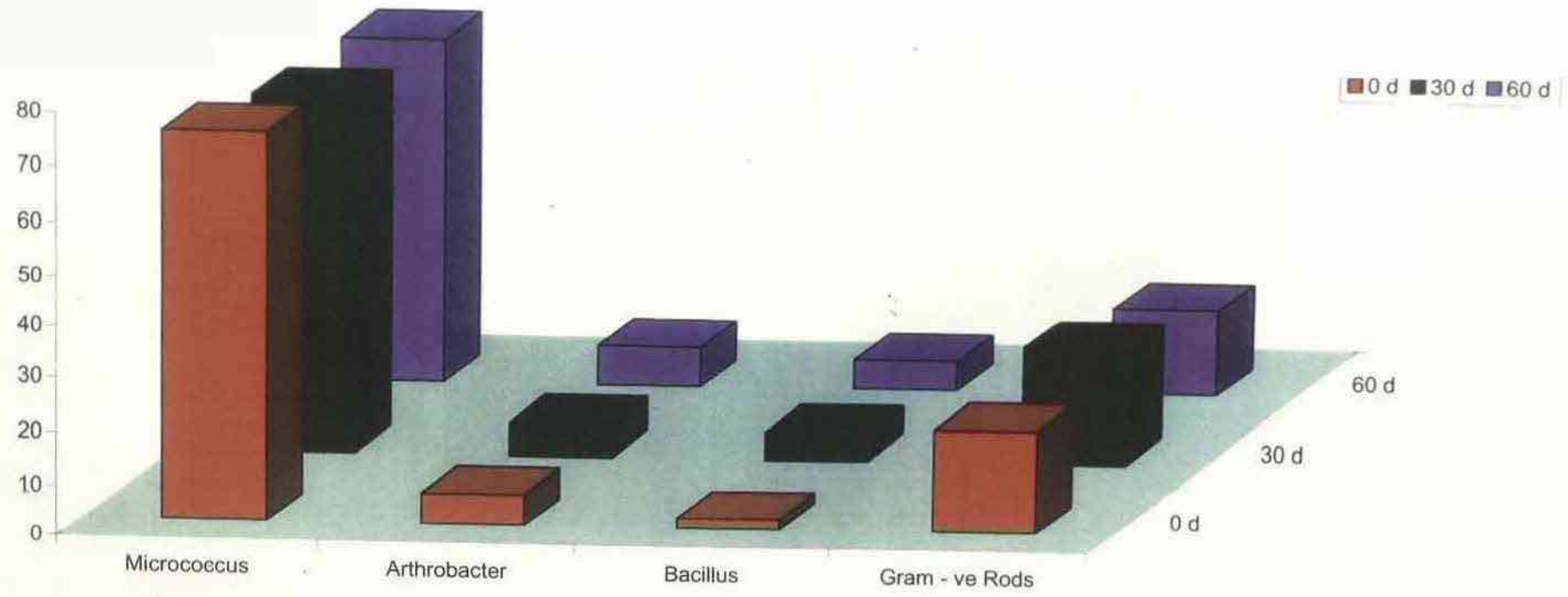


Figure 3. Percentage occurrence of different bacterial genera in Fish Meal

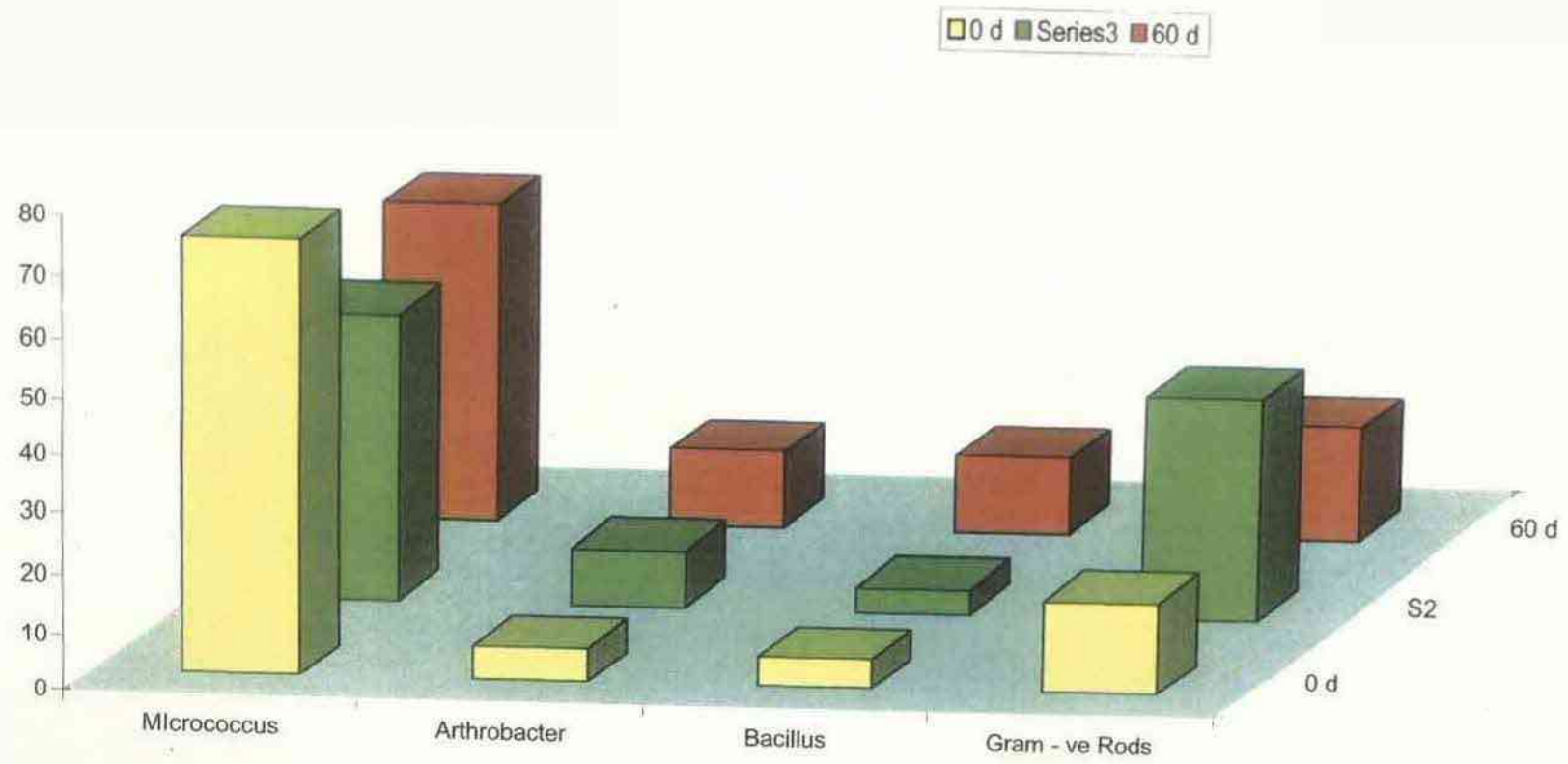


Figure 4 Percentage composition of different bacterial genera in ground nut oil cake

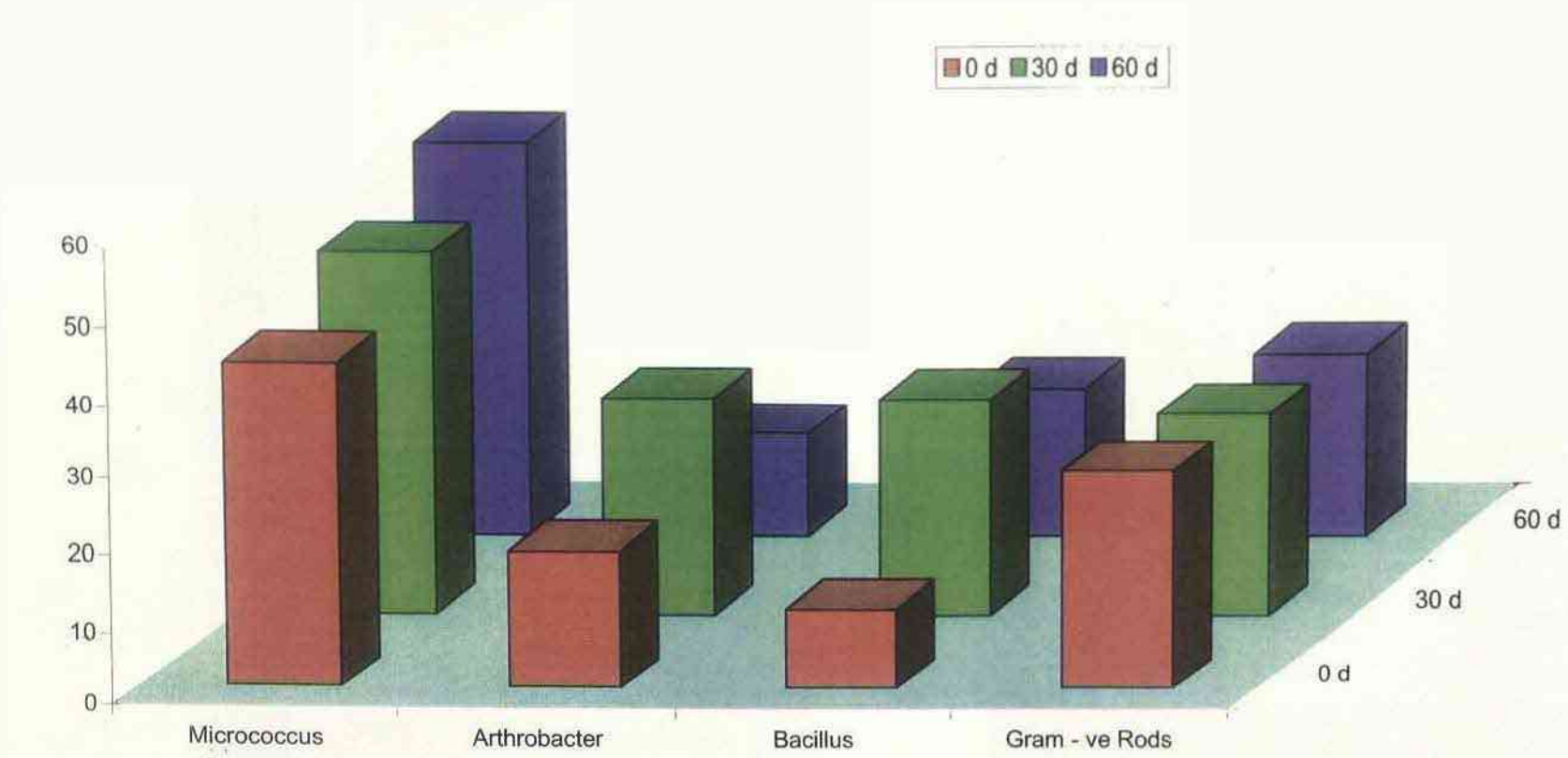
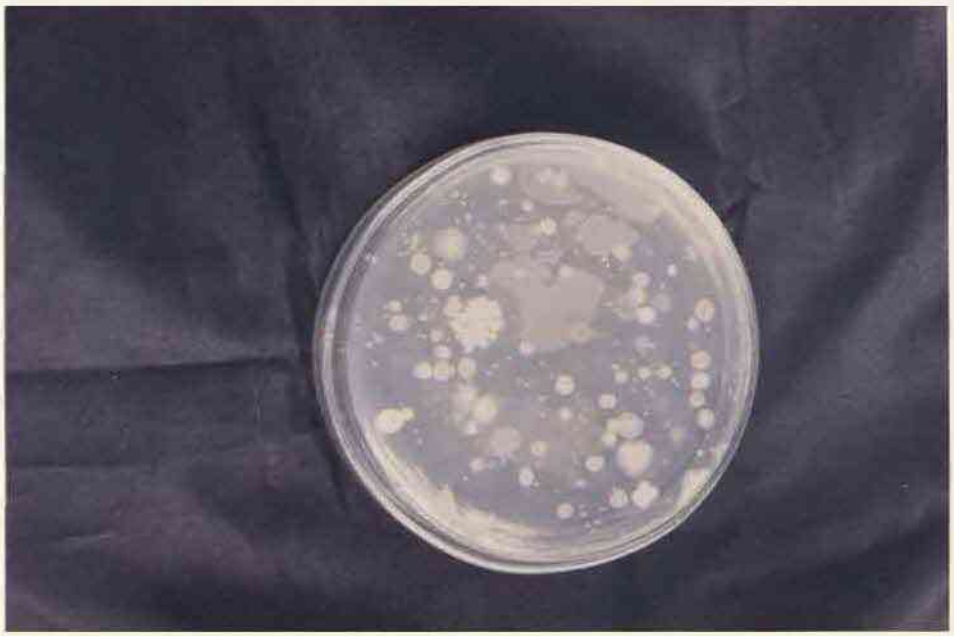


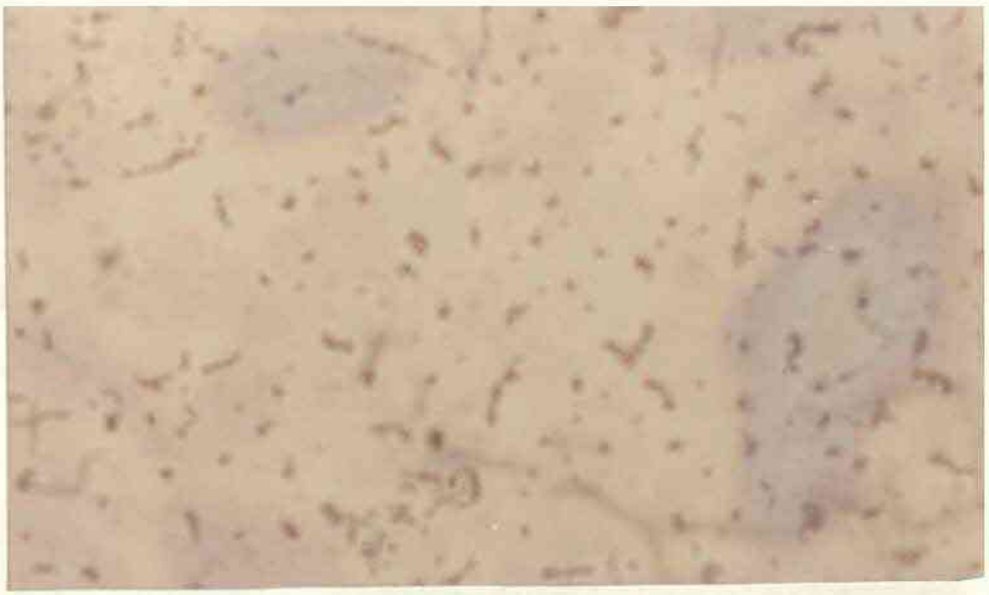
Figure 5. Percentage occurrence of different bacterial genera in Soybean flour

1. Total Plate Count in Zobel marine agar medium in Fish meal

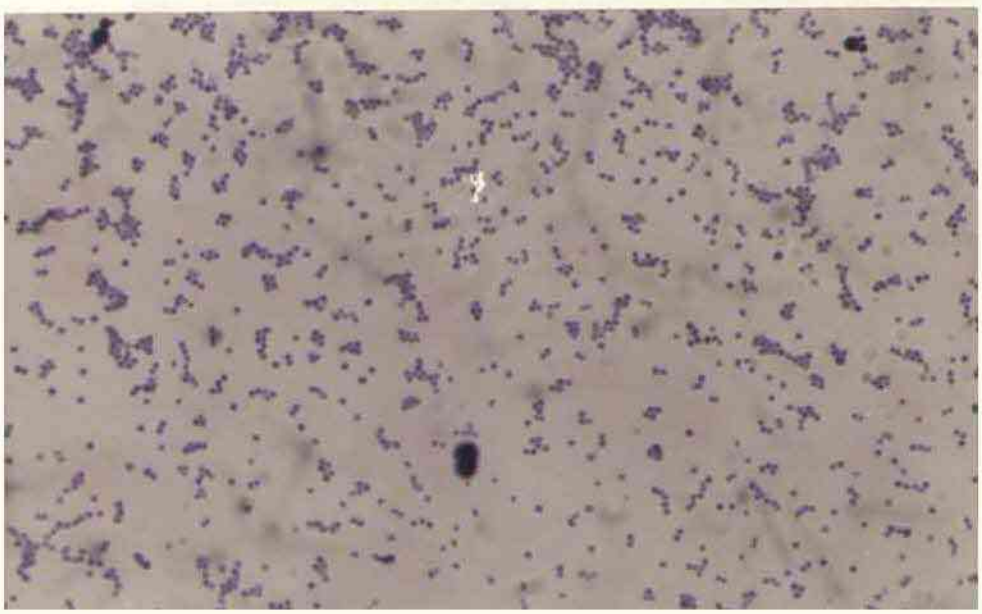


2. Total Plate Count in Zobel marine agar medium in Ground nut oil cake

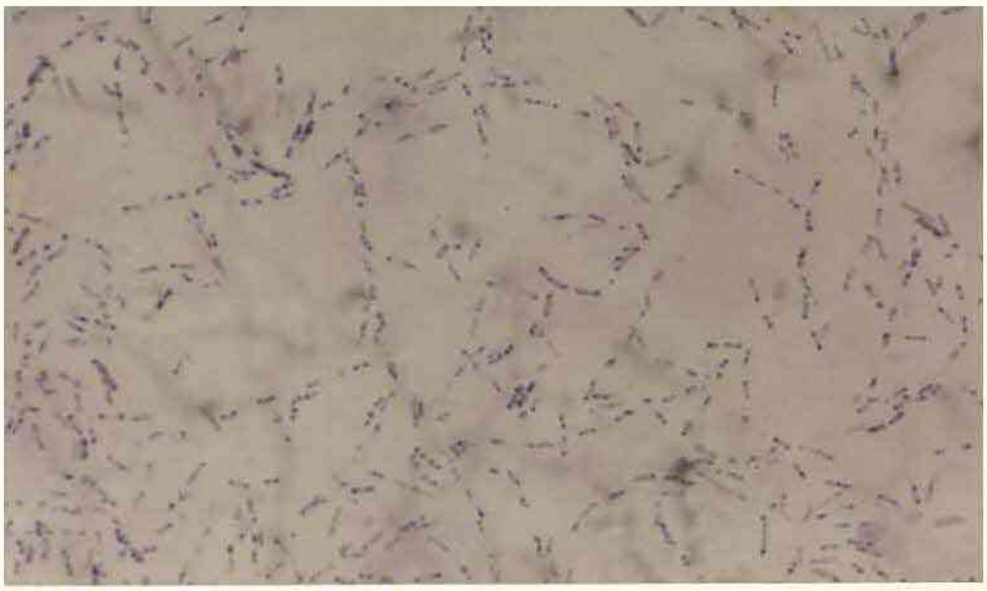
3. *Staphylococcus aureus* gram positive cocci in soybean flour



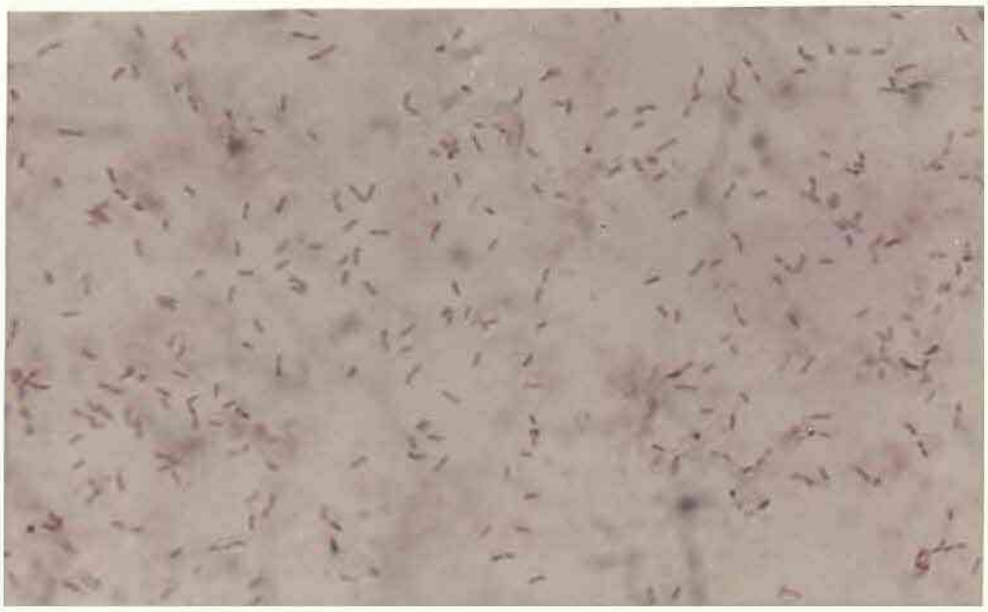
4. *Staphylococcus aureus* gram positive cocci in Feed I



5. *Bacillus spp* in Fish meal



6. Gram negative rods in Fish meal



DISCUSSION

5. DISCUSSION

In addition to enumerating the bacterial load of two shrimp feeds and three feed ingredients, the present study also attempted to evaluate the ability of these samples to support bacterial growth on short-term storage. The rationale for the choice of these bacteria for testing in samples has been based on their role in sea food- borne epidemics, in feed decomposition and causing diseases in cultured aquatic organisms. Also, feeds and feed ingredients being sources of nutrients required for bacterial growth and contain a significant inoculum in it. Bacteria being able to produce extracellular enzymes could affect the quality of a diet or play a role in pathogenicity to fish or man, as were spore-forming bacteria that resist pasteurization.

5.1. Qualitative Study of Bacteria Present in Feeds and Feed Ingredients

5.1.1. Total Plate Count

When bacterial count is taken into account it was observed that either of the two shrimp feeds or the feed ingredients were sterile in the present study. TPC of Feed I was in the range of 2.7×10^3 cfu/g to 8.8×10^4 cfu/g and that of Feed II was in the range of 5.75×10^3 cfu/g to 5.5×10^4 cfu/g. Sera and Kimata (1971) observed 2.2×10^5 cfu/g TPC in fish diet. The mesophilic bacterial counts in fish diets were reported as 10^3 to 10^7 cfu/g by Trust (1971) and Trust and Wood (1973). Muroga *et al.* (1994), have reported difference in counts when Zobell agar and Bromothymol blue medium were used. They could obtain 1.2×10^3 cfu/g in artificial fish diet using Zobell agar medium. The present observations also show a similar trend within the range as in the above studies when Zobell agar was used.

For feed ingredients, TPC of fish meal was between 2.7×10^3 cfu/g to 5.12×10^3 cfu/g, of ground nut oil cake 3.3×10^3 cfu/g to 5.1×10^3 cfu/g and soybean flour 2.075×10^3 cfu/g to 3.15×10^3 cfu/g. There are no studies pertaining to change

of bacterial flora in feed ingredients on storage. Thus, the present observations form a baseline in the area.

Feeds are considered unsatisfactory when they contain a large population of bacteria, even if they are not pathogenic or have not altered the material. The TPC in the range of 10^6 to 10^8 /g feed results in decomposition of feed (Trust, 1971). In the present study, TPC of feeds as well as feed ingredients were well within this range and there was no drastic variation in the count during 60 d storage period.

Vibrio spp, *Faecal streptococci*, *Escherichia coli* and *Staphylococcus aureus etc* are indicators of contamination as well as potential pathogens. The feed containing these bacteria would form an inoculum in the culture systems either through the uneaten feed or through faeces. The bacterial population would result in oxygen depletion and increase in ammonia level, thus affecting animal health. The presence of indicators or pathogens or any other bacteria beyond a limit in feed is, therefore,dangerous to the culture systems (Trust, 1971).

5.1.2. *Vibrio* spp.

Vibrio spp. count in Feed I was between 3 x 10 cfu/g and 6.2 x 10 cfu/g. during 60 d of storage. In Feed II, it ranged from 2 x 10 cfu/g to 4 x10 cfu/g. Reilly and Twiddy (1992) in their attempt to identify the source of *V.cholerae* in shrimp feeds have concluded that commercial shrimp feed is a potential source of the pathogen. *Vibrio parahaemolyticus* is another potentially pathogenic species reported to be dangerous to cultured shrimp and seafood (Karunasagar *et al.* 1990). Muroga *et al.* (1987) reported 10% *Vibrio* spp. from live diets, Artemia and rotifers. In fishmeal, *Vibrio* spp. count was between 2-3 x 10 cfu/g, while, in groundnut oil cake it was between 3 x 10 cfu/g and 1.2 x 10^2 cfu/g and in soybean flour between 10- 20 cfu/g.

5.1.3. *Faecal streptococci*

Faecal streptococci was present in the Feed I, a commercial shrimp feed, in the range of 2.9 -4.5 x 10 cfu/g, while it was absent in the Feed II, which was formulated at the Central Marine Fisheries Research Institute. In fishmeal, the count ranged from 5.1 x 10^2 to 6.5 x 10^2 cfu/g and in groundnut oil cake it ranged from 4.9 x 10 to 6 x 10 cfu/g,whereas in soybean flour it was absent. Storage didn't have much effect in the count of *Faecal streptococci.*

5.1.4. *Escherichia coli*

Feed I has shown *E.coli* count in the range of 3 x 10 cfu/g throughout the period of observation, whereas, Feed II had no *E.coli* in the sample. Trust and Money (1972) observed that Enterobacteriaceae in feeds could indicate a potential for contamination with enteric pathogens.

5.1.5. *Staphylococcus aureus*

S. aureus count in Feed I was found to be in the range of 0 to 2.2 x 10^2 cfu/g, whereas it was nil throughout the storage period in Feed II. It was observed that *S. aureus* appeared in 30 days of storing, while, it was absent in the 60th day sampling. Inhibition of *Salmonella enteritidis* isolated from fishmeal on 60-d storing is reported (Pelagic *et al.*, 1998). Probably similar may be the case here. The processing method employed during feed preparation, especially heat treatment, seems to destroy the bacteria. That might be the reason for the absence of *Faecal streptococci, E.coli* and *S. aureus* in Feed II, which was formulated at the Nutrition laboratory of the Central Marine Fisheries Research Institute. Feed I, a commercial shrimp feed, had occasional to regular contamination with the indicator bacteria. This may be attributed to unhygienic handling by the farmer after opening of the feedbag or storage. Ramalinga (2000) has also reported presence of *faecal streptococci* in aquafeeds.

For human consumption permitted *E.coli* count is <10/g, Faecal coliforms nil, and *Faecal streptococci* <20/g (Trust, 1971). Such restriction should be imparted for aqua feed also, because fish have been shown to be vectors of bacterial

disease to humans (Jansen, 1970). It is, therefore, important that such bacteria are not present in the diets of shrimp being raised for human consumption.

When consideration is given for feed preparation and handling under hygienic conditions and proper heat treatment, the number of bacteria present in the feed ingredients could be reduced as seen in the case of Feed II in the present study. The possible source of contamination of Feed I would be during handling and storage by the farmer.

5.2. Qualitative Study of Bacteria Present in Feeds and Feed Ingredients

For qualitative study of bacteria present in feeds and feed ingredients, 500 isolates were maintained in Zobell Marine Agar slants. Only gram-positive strains were identified upto genus level, whereas gram-negative isolates were taken as such. As shown in Figs. 1-5, gram-positive cocci were the dominant group in both the feeds as well as the ingredients. *Micrococcus* was followed by *Arthrobacter* spp. and *Bacillus* spp. A similar observation was made by Muroga *et al.* (1994) in commercial fish diets. Hamid *et al.* (1978) reported only *Bacillus* sp. from grey mullet diet. In the present study, the percentage occurrence of gram negative bacteria in both the feeds gradually increased on storage. While in feed ingredients, there was no noticeable trend observed. There was gradual increase in percentage composition of *Arthrobacter* spp. in Feed I, II, fishmeal, and groundnut oil cake. In soy flour, *Arthrobacter* spp and *Bacillus* spp were higher in 30 d storage period.

Ringo and Strom (1994) have reported predominance of Enterobacteriaceae in commercial feeds and *Flavobacterium* spp in capelin roe diet. According to Surendran and Gopakumar (1981), the gram-positive cocci, usually found in association with fish and fishery products or aquatic environments are either of three genera, *viz.*, *Staphylococcus*, *Streptococcus* or *Micrococcus*. Since *Staphylococcus* and *Streptococci* were absent in feed II, it can be inferred that Micrococcaceae observed in Feed II belonged to the genus *Micrococcus*.

Many factors like nature of microflora of the ingredients used in feed formulation, type of heat treatment that the feed has undergone and the final

moisture content would have an influence on the final bacterial load of diet (Trust and Wood, 1973; Blank *et al.*, 1996)

Christian (1980) has observed a shift from gram negative to gram-positive flora in proteinaceous foods when the water activity dropped from 0.98 to 0.96. As a result of this, the major bacterial flora of such feed could be gram positives. In the present study, in both the feeds gram positive bacteria were dominant throughout the period of study, though it gradually reduced, yet, overall, remained as the dominant group. Sugita *et al.* (1986, 1987) studied the microflora in pellet diets and found them to be composed of *Bacillus* spp. (4.8×10^3/g) and *Clostridium* (6×10^2/g). In the present study, it was observed that *Bacillus* spp. formed 4.5-10% in feeds and 2.0-20% in feed ingredients. It is also a well-established fact that feed consumed by cultured aquatic organisms does have a definite influence on the gut microbial flora (Del-Rio-Rodriguez *et al.*, 1997). Ogbondeminu *et al* (1991) have reported that 80% of the feed microflora were identified in the water and of the bacterial population in feed 25% was *Pseudomonas* spp., *Staphylococcus* spp., 10%, *Streptococcus* as 12%, *Micrococcus* 15% and *Aeromonas* 8%.

The fact that *Staphylococcus* spp., *Streptococcus* spp (*faecalis*) etc are facultative pathogens or agents of food poisoning is of importance. Although the presence of these bacteria is not often associated with fish diseases or enteric diseases in human beings, the health implications, on the introduction of these organisms into natural water via the unconsumed feed and faeces in shrimp culture waste water cannot be ignored.

Feed quality, generally perceived as the responsibility of the feed manufacturer, is affected by factors outside the plant such as handling, storage, and use. Thus, the maintenance of feed quality becomes partly the responsibility of the farmer too. Every shrimp farmer must be familiar with the nature and occurrence of major feed quality problems and be able to prevent and control them by practicing proper storage measures.

Based on the results of the present study, it is concluded that the bacterial microflora of selected feeds and feed ingredients reflects their handling,

storage and processing temperature. The commensal bacteria included a diversity of potential pathogens and indicators. It is suggested that feed manufacturers and farmers should be more aware of the bacterial load of fish/ shrimp diets, and a minimum standard might need to be imposed in the processing methods. Strict hygiene and sanitary procedures in aqua feed production and handling would be of immense help in avoiding problems during later stages of culture operations. Further studies in this line could help in imposing proper standards for feed ingredients as well as feeds used in aquaculture.

SUMMARY

6. SUMMARY

1. Two fish feeds, one a commercial shrimp 'grower' feed (Feed I) and another a cost effective shrimp feed formulated at CMFRI, Kochi (Feed II) and three protein rich feed ingredients (fish meal, ground nut oil cake and soybean flour) were stored at room temperature and were analysed quantitatively and qualitatively for their bacteriological profile, on 0, 30 and 60 days.

2. TPC of both the feeds were in the range of 2.7×10^3 to 8.8×10^4 cfu/g, whereas, TPC of fish meal was 2.7 to 5.12 x 10^3 cfu/g, groundnut oil cake 3.3 to 5.1 x 10^3 cfu/g and soybean flour 2.075 to 3.15 x 10^3 cfu/g during 60 d storage period.

3. Vibrio spp., count in both the feeds was in the range of 2×10^1 to 6.2×10^1 cfu/g. Fish meal showed *Vibrio* spp., count in the order 2-3 $\times 10^1$ cfu/g, ground nut oil cake had *Vibrio spp.* count in the range 3×10^1 cfu/g. to 1.2×10^2 cfu/g and soybean flour in the range of 10×10^1 2.0×10^1

4. Faecal *streptococci* count was in the range of 2.9×10^1 to 4.5×10^1 cfu/g in Feed I, while, it was absent in Feed II. In fishmeal, it ranged from 5.1×10^2 to 6.5×10^2 cfu/g in groundnut oil cake it was found to be between 4.9×10^1 to 6×10^1,while it was absent in soybean flour during the storage period.

5. *Esherichia coli* count was 3×10^1 cfu/g in the Feed I, whereas, it was absent in Feed II. Fishmeal has shown *E.coli* count of 2.0×10^1 cfu/g, groundnut oil cake 3×10^1 cfu/g and soy bean flour nil during storage.

6. *Staphylococcus aureus* count ranged from 0 to 2.2×10^2 cfu/g in Feed I, whereas, it was absent in Feed II. Fishmeal, groundnut oil cake and soybean flour had *Staphylococcus aureus* within 10^2 cfu/g during the 60 d storage period.

7. Qualitatively, in both the feeds, *Micrococcus* was predominant, followed by *Arthrobacter* and *Bacillus*, with marginal decrease in *Micrococcus* and proportionate increase in *Bacillus* in 60 d.

8. In fishmeal, *Micrococcus* spp. (65-73%) was predominant, followed by *Arthrobacter* spp., (6-7.4%) and *Bacillus* spp., (5.3-5.74%). Gram-negative bacteria ranged from 15.7% to 22.0%.

9. In groundnut oil cake *Micrococcus* spp., (48 to 73%) was dominant, followed by *Arthrobater* spp (6-13.3%) and *Bacillus* spp., (2-13.3%). Gram-negative bacteria varied between 19.04 – 37.8%.

10. In soybean flour, *Micrococcus* sp was dominant in the range of 42.8- 51.72%, followed by *Arthrobacter* spp. showing variation between13.79- 28.55% and *Bacillus* spp. in the range of 10.7 - 28.5%. Gram-negative bacteria showed slight reduction during storage (28.57- 24.13%).

11. Overall, short-term storage of 60 days had no drastic effect on the bacterial flora of feeds and feed ingredients. Yet, the effect on long-term storage cannot be overruled, especially of qualitative changes. So, there is a wide scope in this area for further studies.

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7. REFERENCES

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