SENSORY AND MICROBIAL ANALYSIS OF *Chirrinus mirgala* (Hamilton, 1822) AT THE MARKETS OF KATHMANDU VALLEY

MABILA KHADKA
T.U. Registration No: 5-2-37-429-2008
T.U. Examination Roll: 26
Batch: 2070/2072

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Submitted to:
Central Department of Zoology
Institute of Science and Technology
Tribhuvan University
Kirtipur, Kathmandu
Nepal

January, 2017

DECLARATION

I hereby declare that the work presented in this thesis has been done by myself, and has not been submitted elsewhere for the award of any degree. All sources of information have been specifically acknowledged by reference to the authors or institutions.

Date 1 January 2017 ……

Mabila Khadka

TRIBHUVAN UNIVERSITY
01-4331896
CENTRAL DEPARTMENT OF ZOOLOGY
Kirtipur, Kathmandu, Nepal.
Institute of Science and Technology
Ref.No.:

RECOMMENDATION

This is to recommend that the thesis entitled **"Sensory and Microbial Analysis Of *Chirrinus mirgala* At The Markets Of Kathmandu Valley"** has been carried out by Ms. Mabila Khadka for the partial fulfillment of Master's Degree of Science in Zoology with special paper Fish And Fisheries. This is her original work and has been carried out under my supervision. To the best of my knowledge, this thesis work has not been submitted for any other degree in any institutions.

Date: 1 January 2017

……..

Prof. Dr. Kumar Sapkota

Professor

Central Department of Zoology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

TRIBHUVAN UNIVERSITY
01-4331896
CENTRAL DEPARTMENT OF ZOOLOGY
Kirtipur, Kathmandu, Nepal.
Institute of Science and Technology
Ref.No.:

LETTER OF APPROVAL

On the recommendation of supervisor "Prof. Dr. Kumar Sapkota" this thesis submitted by Ms. Mabila Khadka entitled **"Sensory and Microbial Analysis Of *Chirrinus mirgala* At The Markets Of Kathmandu Valley''** is approved for the examination and submitted to the Tribhuvan University in partial fulfillment of the requirements for Master's Degree of Science in Zoology with special paper Fish And Fisheries.

Date: 1 January 2017

…….
Prof. Dr. Ranjana Gupta
Head of Department
Central Department of Zoology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

TRIBHUVAN UNIVERSITY
01-4331896
CENTRAL DEPARTMENT OF ZOOLOGY
Kirtipur, Kathmandu, Nepal.
Institute of Science and Technology
Ref.No.:

CERTIFICATE OF ACCEPTANCE

This thesis work submitted by Ms. Mabila Khadka entitled **"Sensory and Microbial Analysis Of *Chirrinus mirgala* At The Markets Of Kathmandu Valley''** has been accepted as a partial fulfillment for the requirements of Master's Degree of Science in Zoology with special paper Fish And Fisheries.

EVALUATION COMMITTEE

……..	……
Supervisor	Head of Department
Prof. Dr. Kumar Sapkota	Prof. Dr. Ranjana Gupta
Professor	Head of Department
Central Department of Zoology	Central Department of Zoology
Tribhuvan University	Tribhuvan University
Kirtipur, Kathmandu, Nepal	Kirtipur, Kathmandu, Nepal

……..	……..
Internal Examiner	External Examiner

Date of Examination: 22 January 2017

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LIST OF ABBREVIATIONS

Abbreviated form	Details of abbreviations
ADP	Adenosine Di phosphate
AMP	Adenosine Mono phosphate
ATP	Adenosine Tri phosphate
Cfu	Colony Forming Unit
EFSA	European Food Safety Authority
FAO	Fisheries And Aquaculture Organization
FDA	Food and Drug Administration
H_2S	Hydrogen Sulfide
ICMSF	International Commission for Microbiological Specification of Food
P^H	Percentage of Hydrogen
TPCA	Total Plate Count Agar
QI	Quality Index
QIM	Quality Index Method
SIM	Sulphur reduction, Indole production and Motility Test
SSA	Salmonella Shigella Agar
SSO	Specific Spoilage Organism
TMA	Trimethyl Amine
TMAO	Trimethyl Amine Oxide
TSI	Triple Sugar Iron Test
TVB-N	Total Volatile Basic Nitrogen
TVC	Total Viable Count
WHO	World Health Organization

SENSORY AND MICROBIAL ANALYSIS OF *Chirrinus mirgala* (Hamilton, 1822) AT THE MARKETS OF KATHMANDU VALLEY

Abstract

Fish meat is an excellent source of long chain omega 3 polyunsaturated fats, riboflavin, pantothenic acid, selenium and vitamin D and is low in fat and sodium. Meat is highly perishable food as it is an ideal culture medium for growth of many organisms because of the high moisture, high percentage of nitrogenous compounds of various degree of complexity, plentiful supply of minerals, accessory growth factors and some fermentable carbohydrates (glycogen) of a favorable pH for most of the enteric microorganisms.

The development of various spoilage indicators in fish and fish products is due to a combination of microbiological, chemical, enzymatic and physical phenomena. This research objects the sensory and microbial Analysis of *Chirrinus mirgala* at the markets of Kathmandu Valley. Two hundred twenty five samples collected randomly from 5 stations in the time period of March 2016 to June 2016 were examined for sensory and microbial analysis. Sensory analysis was performed by the Quality Index Method. For microbial analysis, enumeration of total bacterial load in gills, muscle and intestine along with examination for the presence of *Salmonella* spp. in samples was performed. Standard microbiological techniques were used for the preparation of media, isolation and identification of the microbes. Pour plate technique was employed for enumeration of total bacterial load.

The Quality Index for the samples was in increasing order for Kalimati, Balkhu, Lagankhel, Khicchapokhari and Bhaktapur with the value of 6.3, 7.0, 7.7, 8.7 and 11 respectively. The microbial load in the edible portions (muscle and head with gills) of *Chirrinus mirgala* had the lowest value 1.51 $X10^7$ for the samples from Khicchhapokhari and highest microbial load was found in the samples of Bhaktapur with the value 3.33 $X10^{9.}$ Microbial loading was found to be the highest in intestine and the least in muscles. *Salmonella* spp. was found in the intestine of samples but not in the muscle and gills.

Though Sensory analysis results of samples inferred that the fishes are not evidently spoiled to reject for the consumption and *Salmonella* spp. was isolated only from the inconsumable body parts, the high microbial loading recommends further examination of fish especially for the presence of pathogens, during handling, storage and up to the very point of consumption is needed for the protection and maintenance of community health by keeping food borne diseases to a minimum.

Key Words: Sensory analysis, Microbial loading, *Chrrinus mirgala*

1. INTRODUCTION

1.1 Background of the study

According to the Food Act, 2023 (1967), "food" means any unprocessed, semi-processed, processed or produced food or drinking substance which the human being generally consumes and drinks, and includes any spp., food additives, color or flavor to be used in any food or drinking substance. Likewise, "adulterated foodstuffs" means the foodstuffs under any of the following conditions:- (1) foodstuffs rotten so as to be harmful to health, or prepared or kept in a filthy or toxic condition, (2) foodstuffs wholly or partially made of any sick or disease carrying animal or bird or of harmful plant so as to be unfit for human consumption, (3) foodstuffs that may be harmful to health as any food additive, colour, preservative, internally or externally mixed chemical compound or pesticide exceeds the ceiling as prescribed.

According to Thompson *et al.* (2012) adulterated food is impure, unsafe, or unwholesome food. Incidents of food contamination have occurred because of poor harvesting or storage of grain, use of banned veterinary products, industrial discharges, human error and deliberate adulteration and fraud.

The rate at which the world is being modernized and globalized is sky rocketing. The 21st century world is a small village in which the exchange of food materials between different parts of the world is so common. With the increase in the rate of food exchange, the cases of food adulteration is also increasing alarmingly. Due to the well-developed infrastructures and the sense of awareness of Consumers towards their health, the cases of food adulteration in Developed Countries is less common.

But, food chains are generally long in developing countries like Nepal as compared to those in developed countries due to poor infrastructure which makes the food more vulnerable to be contaminated with harmful agents (microorganism and chemicals). In addition, infrastructures related to technical regulation, conformity assessment and safety of food are still in developing phase which requires more focus and investment for better functioning. Similarly, Inspections and regulation of food related business are challenging and difficult due to scattered and large number of primary producers, traders and retailers (Bajagai, 2012).

Among the various food items, meat and meat products are considered as an ideal culture medium for growth of many organisms because of the high moisture, high percentage of nitrogenous compounds of various degree of complexity, plentiful supply of minerals, accessory growth factors and some fermentable carbohydrates (glycogen) of a favorable pH for most of the enteric microorganisms (Frazier and Westhoff, 1978). Meat is every edible part of any slaughtered animal, whether the same is in its natural state or has been subjected to freezing, chilling, salting, canning or other preservative processes (OYSGN, 1978). Nowadays, the consumption of meat and meat products is increasing with increasing population, economic growth, urbanization, modernization and industrialization. Meat and meat products are regarded as high nutritive value food. A

typical meat contains about 20% protein, 70% water, 5% lipid, and 5% other substances example carbohydrate, salts, vitamin, etc. (Nakai and Modler, 2000). Meat of various faunas has been brought into use by human beings very early from the Stone Age. On a global scale, fish and fish products are the most important source of protein and it is estimated that more than 30% of fish for human consumption comes from aquaculture (Hastein*et al.*, 2006). At present context, due to the raised consumer awareness, fish meat is preferred among various meat items in Nepal. Fish meat is an excellent source of long chain omega 3 polyunsaturated fats, riboflavin, pantothenic acid, selenium and vitamin D. It is also low in fat and sodium. Meat and meat products are also an excellent source of high quality animal protein, vitamins especially B complex, and certain minerals, especially iron (Gracey*et al*., 1986). Generally, fish are good sources of vitamins B12 and B6. It is also good source of fluorine and iodine which are needed development of strong teeth and the prevention of goiter in man (Andrew, 2001).

The increasing demand of fish meat cannot be fulfilled by the fish production of Nepal alone. Hence, Nepal has to rely on the fish meat imported from various parts of world, especially India. Food products (including meat) are contaminated with soil, air and waterborne micro-organisms during harvesting, processing, distribution and preparation. Extremely high numbers of micro-organisms are found in meat animals intestinal tracts, and some of these find their way to the carcass surfaces during slaughter. Some apparently healthy animals may harbour various micro-organisms in the liver, kidneys, lymph nodes and spleen. These micro-organisms and those from contamination through slaughtering can migrate to the skeletal muscles via the circulatory system (Marriot, 1994).

Fishes are highly perishable, and prone to vast variations in quality due to differences in spp., environmental habitats, feeding habits (Yagoub, 2009). In addition, they can also function as carriers of several microbial and other health hazards (Yagoub, 2009). Microbial contaminant are rather common than any other form of contaminant as food animal itself harbor them. Microbial status of fresh meat depend on animal rearing, transportation, slaughtering and cutting and packing, besides hygiene and processing conditions of the slaughter plant. The natural surface flora of fish meat is usually derived from the water bodies in which they are reared and the food materials they are fed. The same microorganisms are found in their intestinal or respiratory tracts. Bacteria present in the muscle fibers and other parts may be due to slaughtering practices or infection of the animal prior to slaughter, such as *Brucella, Salmonella, Streptococcus,* and *Mycobacterium tuberculosis,* also certain anaerobic bacteria may be present. *Achromobacter* and *Pseudomonas* are predominant in meat held at low temperatures. Also the presence of *Bacilli, Staphylococci,* and *Lactobacilli* may contribute to surface slime (Biswas, 2011). Bacterial diseases in fish are a serious threat to aquaculture systems that cause severe damage and mortality in Egypt (Noor El Deen*et al.*, 2010). Enterobacteraceae in fish are considered as an indicator to sewage pollution and has been reported as opportunistic pathogen in fish (Rajasekaran, 2008). The pathogenic strains of Enterobacteraceae may cause diarrhea in fish (Shender *et al.*, 2009). The source of water for abattoir activities is very paramount to meat hygiene as water is needed in maintaining cleanliness of the abattoir environment and for making the raw fish meat appears fresh.

Meat contamination in abattoirs and meat stalls could result from contaminated water, unhygienic practices like poor handling, use of contaminated tables to display meat meant for sale and the use of contaminated knives in cutting operations. Contamination of meat and meat products occur when raw meat is exposed or makes contact with pathogenic microbes such as which are ubiquitous in nature (WHO, 1982).

According to Cahill (1990), the microbiological diversity of fresh fish muscle depends on the fishing grounds and environmental factors around it. Williams and Wilkins (1948) suggested that the type of micro-organisms that are found associated with particular fish depends on its habitat. Kyenberg (1991), Rodricks (1991) classified the bacterial pathogens associated with fish as indigenous and non-indigenous. The non-indigenous contaminate the fish or the habitat one way or the other and examples include *Escherichia coli, Clostridium botulinum, Shigelladysenteriae, Staphylococcus aureus, Listeria monocytogens andSalmonella*. The indigenous bacterial pathogens are found naturally living in the fish's habitat for example *Vibrio*spp. and *Aeromonas* spp. The bacteria from fish only become pathogens when fish are physiologically unbalanced, nutritionally deficient, or there are other stressors, i.e., poor water quality, overstocking, which allow opportunistic bacterial infections to prevail. Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include *Mycobacteium, Streptococcus* spp., *Vibrio* spp., *Aeromonas*spp., *Salmonella* spp.and others (Lipp and Rose, 1997). The sources of these microbes in meat could be inherent micro flora in normal tissues of animals, air, environment or contamination due to unhygienic slaughtering, handling and processing conditions. The particular isolation of some most pathogenic organisms such as *Salmonella* spp., *E. coli* and potential pathogenic organisms *as Klebsiella* spp., *Citrobacter* spp. *and Proteus* spp., which when isolated from fish and fish products gives an indication about environmental fecal pollution of fish (Wogu and Maduakol, 2010).

Microbial contamination of meat and meat products must not exceed levels which could adversely affect the shelf life of the product if it does it renders the meat unwholesome and hence not fit for human consumption (Fasanmi and Sansi, 2008). Although only a few infectious agents in fish are able to infect humans, some exceptions exist that may result in fatalities. However, the greatest risk to human health is due to the consumption of raw or insufficiently processed fish and fish products (Yagoub, 2009).

Reduction of risk for human illness associated with raw product can be better achieved through controlling points of potential contamination in the field, during harvesting, during processing or distribution, or in retail markets, foodservice facilities, or the home (Scates*et al.*, 2003; FDA 2007).

Food security is a complex issue, which is influenced by a number of factors. Increasing national agricultural production alone cannot improve food security. The Food and Agricultural Organization (FAO) of the United Nations and the World Health Organization (WHO) state that illness due to contaminated food is perhaps the most widespread health problem in the contemporary world and an important cause of reduced economic productivity (Edema *et al.*, 2005). Healthy food leads to the healthy body. In the same manner, healthy body leads to the healthy mind. The combination of healthy body and healthy mind is the most influential and detrimental factor for the development of a country. Hence food safety is the most essential requirement of today's world.

1.2 Fish marketing in Nepal

There are two groups of fish traders involved in fish marketing in Nepal: those from India and those from Nepal. Compared to their Nepalese counterparts, the Indian traders are well established and organized in terms of manpower, resources and working capability. Fish imported from India and fish produced in Nepal is traded in the fish market in Nepal. The fish from India is more consistent in size and supply, whereas the fish from Nepal is superior in quality and freshness. These are some of the factors which determine the fish prices in the market. There is no organized database on imports and exports of fresh fish. The importance of this has been realized and it is being addressed accordingly. Imports of processed fish and fishery products from other countries in the urban affluent market have been documented in value terms. In 2003/2004 Nepalese Rupees 0.7 million worth of salmon, frozen fish and fish in brine were imported.

Fish marketing infrastructures have been developed in most cities in the Terai along with agriculture marketing networks. In Kathmandu,Kalimati wholesale market centre has developed a fish marketing infrastructure that includes chilled, refrigerated and icing facilities. These facilities are used by fish traders at all levels, including middlemen, wholesalers, retailers and vendors on a community and co-operative basis. This model has been successfully operated for several years and is being assessed with a view to wider application in other areas. The system of labeling/certification of product safety of fish and fishery products have not yet been well developed. However, monitoring of this is done at random by the Municipality, Consumers' forum, Department of Food technology and Quality (FAO)

1.3 Objectives

1.3.1 General Objective

The general objective of the study was to analyze the sensory and microbial quality of *Chirrinusmirgala*atmarkets of Katmandu Valley.

1.3.2 Specific Objectives

The specific objectives of the study were:

i. To assess the sensory analysis of the fish meat.
ii. To enumerate the total bacterial load of raw fish meat (*Crrihinusmirgala*).
iii. To examine the occurrence of *Salmonella*spp.
iv. To explore the differences in the quality of fish meat at different study sites.

1.4 Significance of the study

Road, bank of the river and ground floor aside the dusty road are used for selling the fish meat rather than the scientifically designed retail selling stations. Due to the scarcity of clean water in the Kathmandu, butcher use ground water or even sometimes the water from the polluted water bodies for various meat processing processes and to make it appear fresh. There could be possible cross contamination between adjacent raw meat through unclean hands of the handlers and flies. Careless sneezing and coughing with butchers, handling the carcasses and the money with the same unwashed hands could be good sources of contamination of the product (Joshi *et al*., 2003). In addition, fish meat is always kept openly while awaiting the buyers, making it naturally vulnerable to infection with different types of microorganism. Therefore, improper handling and improper hygiene may lead to the contamination of fresh meats and eventually affects the health of the consumers (Koussema*net al*., 2008).Likewise,frequent interrupted power supply is a day to day problem in countries like Nepal. Under these situations, people held the fish meat at ambient temperature hence the microbial load of meat may increase (Kandeepan*et al.,* 2010**).**Similarly, food chains are generally long in developing countries like Nepal as compared to those in developed countries due to poor infrastructure which makes the food more vulnerable to be contaminated with harmful agents (microorganism and chemicals). In addition, infrastructures related to technical regulation, conformity assessment and safety of food are still in developing phase which requires more focus and investment for better functioning. Similarly, Inspections and regulation of food related business are challenging and difficult due to scattered and large number of primary producers, traders and retailers (Bajagai, 2012).

1.5 Limitations

The present study was carried out in the limited time and budget. Quality examination could only be done on the basis of physical and microbiological aspect. Chemical analysis of the samples could not be done.

2. LITERATURE REVIEW

Fish are recognized as being highly perishable, having a relatively short shelf life, which is defined as the length of time from the day of catch that fresh fish can be in the marketplace unspoiled (Regenstein and Regenstein, 1991). Therefore fish requires proper handling and preservation to increase its shelf life and retain its quality and nutritionalattributes. Quality is defined as the aesthetic appearance and freshness or degree of spoilage which the fish has undergone (FAO). Immediately as fish is caught, it loses its natural resistance to attack by microorganisms and also starts to undergo both physical and chemical changes that in return bring changes in appearance, taste, smell and texture.

2.1 Forms of fish spoilage

"Spoilage" can be defined as a change in fish or fish products that renders them less acceptable, unacceptable or unsafe for human consumption (Hayes,1985). Fish undergoing spoilage has one or more of the following signs: slime formation; discolouration; changes in texture; off-odours; off-flavours and gas production. The development of these spoilage indicators in fish and fish products is due to a combination of microbiological, chemical and enzymatic and physical phenomena (Huis in't Veld, 1996).

2.1.1 Microbiological spoilage

Live fish is normally considered to be sterile, but microorganisms are found on all the outer surfaces (skin and gills) and in the alimentary tract of live and newly caught fish (Liston, 1980). When fish dies, its entire body resistance mechanisms breakdown, giving way to microorganisms or the enzymes they secrete to invade or diffuse into the flesh where they react with the complex mixture of natural substances present. During storage a characteristic flora develops, but only a part of this flora, known as the specificspoilage organisms (SSO), contribute to spoilage. The SSO counts reach a minimal spoilage level where the fish is sensorially rejected (Dalgaard, 1993).

Bacteria are able to decompose proteins, other nitrogen containing compounds to ammonia, hydrogen sulphide, which produce an unpleasant and disgusting flavour (Herbert and Shewan, 1975).

Microbiological growth may develop in different ways and depends on intrinsic, extrinsic factors, the variety of processing practice and preservation and implicit parameters (Huisin't Veld, 1996).

2.1.2 Chemical oxidation

Chemical spoilage processes are changes taking place in the lipid fraction of the fish. Living cells in fish have enzymatic protection mechanisms against lipid oxidation by

having an enzyme, glutathione peroxidase, which acts by reducing hydro-peroxides in cellular membranes to corresponding hydroxyl-compounds. This reaction requires a supply of the enzyme in a reduced form and thus the reaction stops when the fish die.
Lipids are oxidized to peroxides, aldehydes, ketones and lower aliphatic acids. The hydro-peroxides are tasteless but can cause brown and yellow discolouration of the fish tissue. The degradation of hydro-peroxides gives rise to the formation of aldehydes and ketones that result in rancid off-flavours. All the chemical by-products eventually reach a level where the fish is rejected (Dalgaard, 1993).
High temperatures are partly responsible for the speed of the oxidation processes. In addition, direct sunlight, wind, heat, light (especially UV-light) and several organic and inorganic substances may also accelerate oxidative processes.

2.1.3 Autolytic spoilage

Initial quality deterioration is caused by the action of autolytic changes in fresh fish flesh, which is later followed by microbiological activity (Gram and Huss, 1996). Since post-mortem pH decreases immediately after death, enzymes are responsible for sensorial changes of fish flesh during the first days of storage (Hultmann and Rustad, 2004; Morkore*et al.*, 2008). Endogenous fish enzymes are highly active during ice storage (Morkore*et al.*, 2008). Protein degradation is one of the factors that lead to the fish muscle softening (Haard and Simpson, 2000).
As fish dies, its enzymatic activity doesn't stop immediately but continues resulting in proteolytic changes that are responsible for early quality loss in fresh fish. The more these enzymes get in contact with the fish's flesh the greater the spoilage. Adenosine triphosphate (ATP) is broken down through a series of products such as adenosine diphosphate (ADP), inosine monophosphate (IMP), inosine and hypoxanthine (HX) as: HX IIMP energy ADPATP.
IMP and HX may be responsible for the sweet and mild tastes in the later stages of shelf life and these products accumulate especially when the respective step is rate- limiting (Regenstein and Regenstein, 1991). From previous studies, these changes precede microbiological spoilage and have been seen to contribute very little to spoilage of chilled fish and fish products (Huss, 1994).

2.2 Analysis methods for quality evaluation

Fish grading and analysis can be done by methods such as sensory evaluation, microbiological and chemical methods that can be rapid or slow but the principle of analysis in both slow and rapid methods is based on measurement of the products resulting from fish post mortem spoilage.
Fung (2002) defines "rapid methods" as miniaturized biochemical kits, antibody, DNA-based tests, which are modifications of conventional tests aimed at making analysis and detection faster, more convenient, sensitive, and more specific than conventional methods.

According to Bourgeois *et al.* (1995) the use of rapid methods in fish quality grading is based on the detection of physical or chemical signals which are indicative of the presence and activity of microorganisms even though they may not have a direct relationship with their possible harmfulness. These signals include: cellular bodies, macromolecules, intracellular bio-molecules, excreted metabolites, physical parameters of the medium and indicators changing colour under microbial metabolic influence.
Various rapid methods can be used to measure these signals/parameters and among others they include electrical, chemical, physiological and immunological changes.

2.2.1 Sensory analysis method

As demonstrated by Meilgaard *et al.* (1991), a sensory method is a scientific discipline that the analyst uses to measure and interpret the characteristics of a product (food) following given described quality parameters (appearance, texture, odour and taste or flavour) on the prepared quality index (QI) scheme with 0-3 demerit score for each quality attribute. With reference to QIM_eurofish (2005), QIM is an objective method used for evaluation of raw fish, based on specific attributes such as the eyes, skin, and gills using a scoring system from 0-3 with a description of each parameter written in a guideline. The scores for all the attributes are added up to give an overall sensory score referred to as a quality index (QI), the lower the score, the fresher the fish. The QI increases linearly with the keeping time of fish in ice.
Sensory methods are commonly used for food grading today in food production including fish or everything else that can be used and it is considered to be more reliable particularly if it is done properly. In accordance with Meilgaard *et al.* (1991) and Alejandra *et al.* (1992), the primary purpose of evaluation is to conduct valid and reliable tests, to provide data upon which informed decisions on the product can be made.
The evaluation of firmness is usually performed through pressing on the skin or the fillet of fish by finger, and this method depends to a large extent upon subjective assessments of the expert panel. At present, the popular improvement of a quality index method of sensory evaluation with different scores showing the degree of firmness of fish texture indicating the freshness quality has been implemented in several European countries (Barbosa and Vaz-Pires, 2004; Cardenas *et al.*, 2007; Sant'Ana*et al.*, 2011; Massa *etal.*, 2012; Cyprian *et al.*, 2013).

2.2.2 Microbiological analysis method

Bacteria naturally exist on the skin, gills, and in the gut but they are not able to cause spoilage because of the natural defensive mechanism in healthy living fish. After slaughter of fish, bacteria find access into the fish flesh not only due to lost sterility, but because of the various mechanical and autolytic changes that rupture gut walls and soften fish flesh, making it easy for bacteria to access fish tissue. The activity of bacteria in the fish, especially the specificspoilage bacteria (SSB), including (*Shewanella, Photobacterium or Pseudomonas*) decomposes various fish components such as TMAO

and produces undesirable odours, flavours and taste in the fish as well as being a health hazard to consumers (Bremner, 2002).

According to Bourgeois and Mafart (1995) the various ways that can be used to determine bacteriological contamination in food/fish include; Total Plate Count (TPC), Most probable number (MPN) and other instrumental methods e.g. (ATP, microscopy, turbidometry, conductance, others). Total Viable Count / Total Plate Count/ Standard Plate Count/ Aerobic Plate Count SPC, APC; all mean the number of bacteria (colony forming units, cfu/g or ml) in a food product under specified standard and uniform conditions of culturing. In general, these methods rely on the estimation of the fraction of the micro flora able to produce colonies in the medium used under specified incubation conditions (Huss *et al.,* 2004). Therefore, the temperature during incubation of the plates has greater influence on the number of colonies developing in the sample thus, in the examination psychrophilic bacteria, pour plating and a 3-4 day incubation period at 25oC is recommended other than at 30 or 37°C (Huss, 1994;Huss, 1995). The enumeration of bacterial counts in food can be done using a variety of medium, thus the classification of these methods for instance; plate count agars (PCA) are commonly used for enumeration of bacteria (Huss, 1995; ICMSF, 1998)

2.2.3 Chemical methods

The evaluations of food using chemical methods are considered to be more objective than sensory methods especially when it is done accurately using appropriate method (Huss, 1995).These methods involve determination of the concentration of a specific chemical(s) in the food under study. Chemical methods of food evaluation are normally used to indirectly predict the level of a sensory attribute, which allows for immediate determination of freshness. To use chemical methods to serve this purpose, well set, quantified and standardized tolerance levels of chemical spoilage indicators need to be established (Huss, 1995). With regard to evaluation of fish quality using chemical methods, the total volatile basic amines (TVB) constitute to the commonly measured chemical indicators. TVB is a general phrase used to include volatile amines such as, trimethylamine (TMA), ammonia (NH3) produced by spoilage bacteria; dimethylamine (DMA) and produced by autolytic enzymes during frozen fish storage (Huss, 1988). The concentration of these chemicals in fish tissues can be determined by steam distillation method (Malle andPoumeyrol, 1989). Conversely the measurement of the amount of hypoxanthine (Hx) in fish is one of the chemical methods of determining fish freshness. Hx is one of the products of nucleotides degradation mediated by bacterial activity (Proteus bacterium) is known to be responsible for bitter, off-flavours of spoil fish (Huss, 1995). Studies have shown that the degradation of nucleotides progresses vary greatly from one fish to the other but often coincidently progresses with the preserved level of spoilage as may be determined by trained analysts thus the development of the formula for fish freshness based on these autolytic changes that entailed. K-value, (freshness) can be determined by calculating the ratio of inosine and hypoxanthine to the sum of ATP and all the other products of ATP degradation multiplied by 100 (Huss, 1995; Connell, 2001; Haard, 2002). The interpretation is that the smaller the K-value the more fresh the product

is and high K-value indicate unacceptable fish product. K-value of 20% has been suggested as a freshness limit and 60% as the rejection point. Nevertheless; its application has not been popular in fish industry due to loss of recognition from the EU system (Huss, 1995). Other methods such as Peroxide Value (PV), Thiobarbituric Acid (TBA), Iodine value (IV) and also constitute to the chemical methods that are used to measure rancidity in fish and fish products

2.2.4 Physical method

It has long been known that the electrical properties of skin and tissue change after death, and this has been expected to provide a means of measuring post mortem changes or degree of spoilage. However, many difficulties have been encountered in developing an instrument: for example, species variation; variation within a batch of fish; different instrument readings when fish are damaged, frozen, filleted, bled or not bled; and a poor correlation between instrument reading and sensory analysis. Most of these problems, it is claimed, are overcome by the GR Torrymeter (Jason and Richards, 1975). However, the instrument is not able to measure quality or freshness of a single fish, although it may find application in grading batches of fish.

Texture is an extremely important property of fish muscle, whether raw or cooked. Fish muscle may become tough as a result of frozen storage or soft and mushy as a result of autolytic degradation. Texture may be monitored organoleptically but there has for many years been a need for the development of a reliable objective rheological test which would accurately reflect the subjective evaluation of a well-trained panel of judges. Gill *et al.,* (1979) developed a method for evaluating the formaldehyde-induced toughening of frozen fish muscle. The method utilized an Instron Model TM equipped with a Kramer shear cell with 4 cutting blades. This method correlated well with data obtained from a trained texture panel. A method for measuring hardness/softness of fish flesh, designated as compressive deformability, has been reported by Johnson *et al.* (1980). An accuratelycut fish sample is compressed by a plunger, and the stress-strain curve recorded. A modulus of deformability is calculated from the recorded graph. The results from such measurements may, however, be difficult to interpret.

Another method, measuring the sheer force of fish flesh, has been investigated by Dunajski (1980). From this work, it has been concluded that a thin-bladed shear force cell of the Kramer type can be applied.

These are but a few of the examples cited in the literature and until recently all involved expensive equipment and destructive sampling. Therefore, Botta (1991) developed a rapid non-destructive method for the measurement of cod fillet texture. It is a small, portable penetrometer which measures both firmness and resilience. Each test takes only 2-3 seconds to complete and results appear to correlate well with subjective texture grades.

2.3 Microorganisms found in fish meat

A study was performed to determine the microbiological status of cooled and frozen fish products collected from retail markets in the Republic of Bulgaria. The highest total viable counts (TVCs) in cooled fish products were established for silver carp, Black sea roach and trout followed by common carp, vacuum packed trout fillets and horse mackerel. The highest *Aeromonas* spp. load was established in cooled trout and vacuum packed trout fillets followed by cooled silver carp, common carp and horse mackerel (Stratev*et al.*,2015).

A study was carried out for detection and identification of Enterobacteriaceaein retailed and farm fish revealed that the most dominants isolated strains were *Citrobacter* spp., *Enteriobacter* spp.,*Klebsiella*spp., *Proteus* spp., *and Serratia*spp.with the highly pathogenic Enterobacteriaceae including *Salmonella* spp. *and E. coli (*Elsherief, 2014*).*

A study was conducted aiming at the isolation of human pathogenic bacteria in gills, intestines, mouth and the skin of apparently healthy fish, Tilapia rendali and *Oreochromicmossambicus*, from the Fletcher dam. Differentiation and characterization of various isolates was based on their growth characteristics on specific culture media (biochemical and gram staining reactions).The following human pathogenic bacteria were isolated *Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Vibrio cholerae, Shigelladysenteriae and Enterococcus faecalis* (Sichewo*et al.*, 2013).

Microbial quality of frozen fishes was studied by Adebayo-Tayo*et al.,* (2012) and found that the Bacterial isolated from frozen fish samples were *Staphylococcusaureus, Esherichia coli, Vibrio* spp., *Salmonella* spp., *and Pseudomonas* spp and *Micrococcus* spp.while fungi isolated were *Aspergillus niger, Penicillium* spp., *Rhizophus stolonifer and Monilia* spp.The bacterial isolates that occurred most frequently in the three different types of frozen fish samples included *Staphylococcus aureus* (20.0%), *Escherichia coli* (20.0%), and *Pseudomonas* spp.(20.0%). Others were *Micrococcus* spp.(15.0%) and *Vibrio* spp.(10.0%). Among the fungal isolates, *Aspergillusniger*was the most predominant (35.0%),followed by *Penicilliums*pp. (30.0%), *Rhizopus stolonifer* (20.0%) and *Monilia* spp.(15.0%) occurred least.

An investigation on the microbiological qualities of some frozen fishes available in a major market in Lagos was conducted by determining the total plate count, isolation, identification and estimation of some bacterial pathogens. Total plate count ranged between 3.6×10^6- 25.60×10^6cfu/gm, total coliforms ranged between 3.01×10^6- 24.66×10^6cfu/gm. Other bacterial pathogens isolated include: *E. coli, Pseudomonas* spp., *Vibrio* spp., and *Staphylococcus* spp. indicating the poor sanitary and hygienic conditions of the markets where these fishes are sold or countries where they are being imported (Babalola*et al.*, 2011).

Enterobacteriaceae were isolated from gills, skin, muscles and the intestine of 83 out of 150 (55%) randomly collected fishes, the most dominants isolates were *E. coli*,

Citrobacter spp., *Enteriobacter* spp. and *Klebsiella* spp. Highly pathogenic *Enterobacteriaceae*including *Salmonella*spp.,*Shigella*spp., *Proteus* spp., *Alklegens*spp., *Pseudomonas* spp. *(*62%*)* were estimated from all parts of collected samples (Yagoub, 2009).
Bacterial micro biota associated with fresh raw shrimp was *Aeromonas, Pseudomonas, Vibrio, Flavobacterium* and *Serratia* (Jeyasekaran *et al.,* 2006).Arannilewa*et al*., (2006) found that the total coliform count range in fish was between 3.0 x 103 - 7.5 x 106 with increasing values, as the duration of storage increases.

The microbial qualityof the tilapia reported by Thampuran*et al.,* (2005) indicated that all tissue samples exceptmuscle tissues were contaminated with fecal coliformwere *Escherichia coli* is the most common contaminantand is often encountered in high numbers.

The presence of *Listeria* spp.and *Salmonella* spp.from the external surface of tilapias were shown by Morales *et al.*, (2004).

Thepresence of eight potentially pathogenic *Vibrio* spp., with overall incidence in the samples as 4.6% for *V.cholerae,* 4.7% for *V. parahaemolyticus,* 6.0% for *V.vulnificus,* 11% for *Vibrio alginolyticus,* 9.9% for *Vibriometschnikovii,* 1.3% for *Vibrio mimicus,* 13% for *Vibriodamsela,* 7.6% for *Vibrio fluvialis,* and 52% for acombined population of all of the above was detected by Hadi*et al.,* (2004).

According to the Center for Food Safety and Applied Nutrition in Washington (2001), most fish related food borne illness are traced to *Salmonella, Staphylococcus* spp.,*Escherichia* spp., *Vibrio parahemolyticus, Clostridiumperfringens, Clostridium botulinum*and *Enteroviruses.*

10% of imported and2.8% of domestic raw seafood was positive for*Salmonella, Enterococcus*spp.and *Aeromonas*spp., fecaland total coliform according to Heinitz*et al.,* (2000).

2.4 Causative factors of spoilage

2.4.1 Time/temperature conditions

The most crucial factors determining the quality of fishery products are time and temperature tolerance. Proliferation of microorganisms requires appropriate high temperatures, while at lower temperatures close to 0ºC, their activity is reduced, thereby extending the shelf life of fish products. Temperature is the single most important factor affecting post-harvest quality of the products. It is often critical to reach the desired short-term storage temperature rapidly to maintain the highest visual quality, flavour, texture, and nutritional content of fresh fish.

The rate of spoilage is dependent upon the holding temperature and is greatly accelerated at higher temperatures, due to increased bacterial action.

2.4.2 Hygiene during handling

Apart from the microorganisms that fishes have at the time of capture, more is added via unhygienic practices and contaminated equipment such as storage facilities. This was demonstrated by studies that compared the quality and storage life of completely aseptically treated fish (aseptic handling), washed fish, iced in clean plastic boxes, with clean ice (clean handling) and with un-washed fish, iced in old, dirty wooden boxes (normal handling). A considerable difference was found in the bacterial contamination of the three batches, the latter heavily contaminated with a reduction in storage life compared with the other samples (Huss *et al.,* 1974).
The design of a fish hold is of great importance as far as hygiene in the hold is concerned. Hold design should enable the purge (drip loss) to be collected easily. The amount of purge was suggested to be higher at 5-7ºC; at which temperature there is greater spoilage since the purge is a very good medium for bacterial growth (Hermansen, 1983).

2.4.3 Rough handling

Rough handling will result in a faster spoilage rate. This is due to the physical damage to the fish, resulting in easy access for enzymes and spoilage bacteria. Physical mishandling in the net, such as very large catches, fishermen stepping on fish or throwing boxes, containers and other items on top of the fish, may cause bruises and rupture of blood vessels. When fish is in rigor mortis (a complicated series of chemical changes that result in stiffening of the fish's muscle shortly after death), rough handling can cause gaping (Huss, 1995) .

2.4.4 Initial bacterial load

The micro flora on tropical fish often carries a slightly higher load of Gram-positives and enteric bacteria but otherwise is similar to the flora on temperate-water fish (Liston, 1980). Basically, bacteria populations on temperate fish are predominantly psychotropic reflecting water temperatures of about 10oC while fish from the tropics have largely mesophilic bacteria (Gram and Huss, 1996).

2.4.5 Methods of capture

The fishing gear and method employed determines the time taken between capture and death. Fish caught in gillnets struggle much to escape, and in so doing, they are bruised by the net which increases exposure to microbial entry and subsequent deterioration. Fish caught by hook and line methods, on the other hand, die relatively quickly and therefore

bruises and stresses are likely to be minimal. Physical mishandling in the net due to long trawling nets and very large catches accelerates spoilage. The large catches in the net are compacted against each other resulting in the fish getting bruised and crushed (especially small sized fish) by the heavy trawl net.

2.4.6 Mode of storage

In bulk-storage, the weight of the pile may crush the fish at the bottom, leading to a loss of weight (yield) as well as other physical damage. It has been reported that when haddock is kept in a short, deep pile of about 3 ft., the bottom fish lose 15% of their weight compared to a normal weight loss of 3-8%, which is entirely due to biochemical changes that cause a loss of water holding capacity leading to drip (Regenstein and Regenstein, 1991). Crushing of the fish by ice or other fish can seriously affect the quality of fish by releasing enzymes from the gut into the fish muscle thereby accelerating autolytic processes.

3. MATERIALS AND METHODS

3.1 Study Area

The Kathmandu Valley, the capital, is the political, commercial and cultural hub of Nepal. Spread across an area of 360 square kilometers and at an altitude of 1336 meter above the sea level, Kathmandu is an exotic and fascinating showcase of a very rich culture, art and tradition. The valley, roughly oval bowl measuring 24 km east-west and 19 km north-south, is encircled by a range of green terraced hills and dotted by compact clusters of red tiled-roofed houses.

Kathmandu Valley lies between the latitudes 27º 32' 13" and 27º 49' 10" north and longitudes 85º 11' 31" and 85º 31' 38" east and is located at a mean elevation of about 1,300 meters (4,265 feet) above sea level. The climate of Kathmandu Valley is sub-tropical cool temperate with maximum of 35.6°C in April and minimum of –3°C in January and 75% annual average humidity. The temperature in general is 19°C to 27°C in summer and 2°C to 20°C in winter. The average rainfall is 1400 millimeters, most of which falls during June to August. Administrative Division Kathmandu Valley comprises of three districts, Kathmandu, Lalitpur, and Bhaktapur, together which cover an area of 899 square kilometers, whereas the area of the Valley as a whole is 665 square kilometers. The Valley encloses the entire area of Bhaktapur district, 85% of Kathmandu district and 50% of Lalitpur district. With more than 1.5 million people, (220,000 households) the Kathmandu Valley is the most important urban concentration in Nepal.

Total 5 stations within Kathmandu District were chosen as a study area. Among five stations, four were the retail markets and one was a wholesale market. The study sites were as follows:

Table 1: Study stations within Kathmandu Valley

S.N	Study Stations	Remarks
1	Kalimati	Wholesale market
2	Balkhu	Retail market
3	Khicchapokhari	Retail market
4	Lagankhel	Retail market
5	Bhaktapur	Retail market

3.2 Research materials

Autoclave Incubator Cotton swabs

Weighing machines	Petri Dishes	Mortar and pistle
Sterilizing agent: Alcohol	Sterile zipper plastic bags	
Salmonella/Shigella agar	Total plate count agar	

3.3 Research methods

3.3.1 Microbial Analysis

3.3.1.1 Sample collection

- Samples were collected from March 2016 to June 2016.
- Fifteen fish samples were randomly collected from three substations of each station. Total 225 samples were examined during the research.
- Samples were collected aseptically in a clean plastic bag and carried to the Fisheries Research Division, NARC situated at Godawari for examination inside a sterile cooler box.

The samples from three major fish body parts susceptible to the microbial contamination were collected as follows:

Gills: Sample from the skin of raw fish was taken by cutting out the gills with sterile scissors.

Muscle: Muscle from the dorsal part below the fin region was cut out with a sterile scalpel.

Intestine: All the parts of alimentary canal including Intestine were cut out with sterile knife.

3.3.1.2 Enumeration of Total Bacterial Load in Gills, Muscle and intestine.

- Samples from different parts of fresh fish were ground using sterile mortar and pistle and then transferred to saline solution.
- Preparation of the media, Isolation and identification of the bacteria was done according to Cheesbourgh (1984).
- Sterilization of the media and other apparatus was done by autoclaving at 121°C for 15 min.
- Pour plate method was employed for the determination of microbial load of samples using Total plate count agar.
- Ten folds serial dilutions of the samples was made and $10^{-3,}$ 10^{-5} and 10^{-7} dilution of the samples from different location was plated out on TPCA.

- ❖ All samples were incubated at 37°C for 24 h.
- ❖ Counting was done according to Plate Count Method.

3.3.1.3 Isolation of *Salmonella* spp.

- ❖ Preparation of the media, Isolation and identification of the bacteria was done according to Cheesbourgh (1984).
- ❖ Sterilization of the media and other apparatus was done by autoclaving at 121°C for 15 min.
- ❖ The samples from different parts were inoculated in Selenite F-Broth for enrichment.
- ❖ After incubation of 24 Hours, the samples were streaked in Salmonella/Shigella Agar using sterile inoculating loop.
- ❖ After obtaining the pure culture, the organism was identified by using standard microbiological techniques as described in BERGEY'S MANUAL OF SYTEMATIC BACTERIOLOGY-1986which involves morphological appearance of the colonies, Gram Staining, and Biochemical test (Cheesbrough, 1984).

Table 2: Biochemical Tests performed for Identification of Bacteria

S.N	TEST	BIOCHEMICAL MEDIA
1	Sulphur Reduction, Indole Production and Motility(SIM) Test	Sulfide- Indole- Motility medium (SIM)
2	(TSI) Test	Triple Sugar Iron (TSI) Medium .
3	Citrate utilization test	Simmon's citrate agar.

3.3.2 Sensory Analysis

Sensory evaluation of all samples was done by using the QIM. Evaluation of the appearance, smell, shape and texture of all samples were given demerit scores (0-3) and recorded in the QI form shown in Appendix 1. These scores were added up to get a freshness score expressed as a QI value and comparisons were made with various alternative methods (QIM_eurofish, 2005).

3.4 Sources of data

Primary data as well as Secondary data was collected for the research purpose. The primary data was collected by visiting the study station, interviewing the stakeholders and laboratory procedures. Similarly, secondary data were collected from different sources like websites, related publications, governmental and non-governmental organizations.

3.5 Data interpretation and analysis

The data were presented in the form of tables, graphs, bar diagram etc. Similarly, the data were analyzed using different statistical tools like Arithmetic mean, mode etc.

3.6 Photography

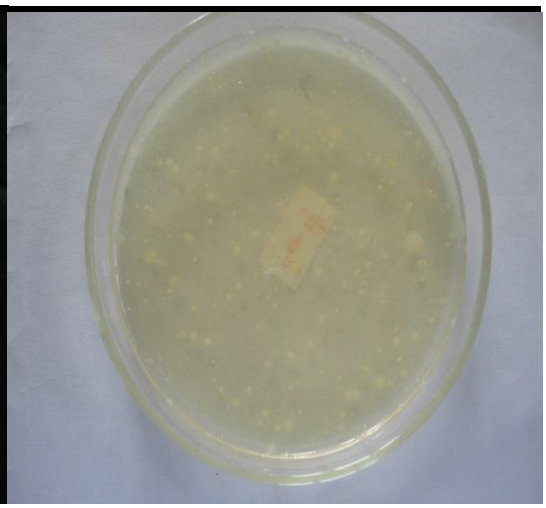
The photographs during the research activities were taken using Sony Shot 8.1 camera.

1. Sample collection by researcher

2. Sensory analysis of samples

3. Extraction of muscle portion for analysis

4. Autoclaving of media and materials



5. Pouring of media under sterile condition 6. Growth of microorganisms in TPCA

4 RESULTS

4.1 Sensory Analysis

From the sensory analysis of the samples from different stations, it was found that the

Quality Parameter	**Character**	**Observation**	**Stations**													
			Kalimati			**Balkhu**		**Khichapokhari**			**Lagankhel**			**Bhaktapur**		
			1	**2**	**3**	**1**	**2**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Body	Texture	Bright and Shining														
		Bright	1	1	1	1	1	1	1	1	1	1	1	1		
		Dull													2	2
	Blood Spot on Gill Cover	None	0	0	0		0			0				0		0
		Small (10-30%)				1					1	1	1		1	
		Big (30-50%)						2	2							
		V. Big (above50%)														
	Stiffness	Stiff														
		Elastic		1								1				
		Firm	2		2	2	2	2	2	2	2		2			2
		Soft												3	3	
	Belly	Firm			0					0	0					
		Soft	1	1		1	1	1	1			1	1			1
		Belly burst												2	2	
	Smell	Fresh														
		Neutral	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		Musty/Sour														
		Stale meat/ Rancid														
Eyes	Clarity	Clear					0						0			
		Cloudy	1	1	1	1		1	1	1	1	1		1	1	1
	Shape	Plain	0	0	0	0	0	0			0			1	0	
		Sunken							1	1		1	1			1
Gills	Colour	Red	0	0	0	0	0	0	0	0	0	0	0			
		Faded												1	1	1
	Smell	Fresh														
		Neutral	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		Sweaty / Slightly Rancid														
		Sour Stink/ stale Rancid														
Total score			7	6	6	8	6	9	10	7	7	8	8	11	12	10
Average QI			**6.3**			**7.0**		**8.7**			**7.7**			**11**		

Quality Index Score for the samples from Kalimati station was the lowest. The Quality Index for the samples was in increasing order for Kalimati, Balkhu, Lagankhel, Khicchapokhari and Bhaktapur with the value of 6.3, 7.0, 7.7, 8.7 and 11 respectively.

Table 3: Quality Index values for samples from different Sampling Stations and Substations.

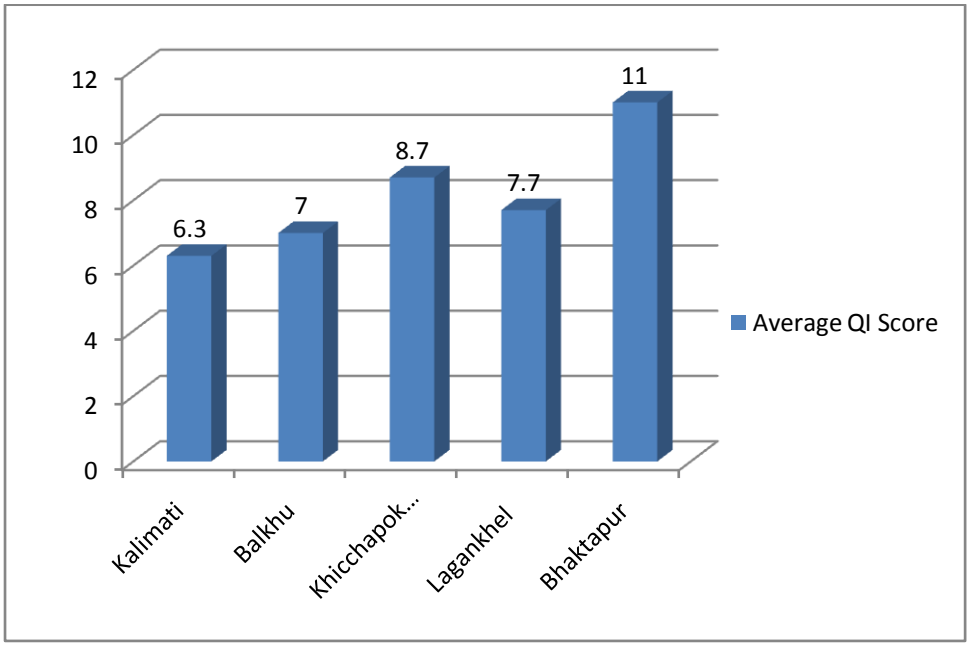


Figure 1:Average QI Score of Samples at different study stations

4.2 Microbial Analysis

4.2.1 Enumeration of Total bacterial loads in gills, muscle and intestine.

The results obtained for the enumeration of the total bacterial loads in different body parts of the fishes are shown in Table 4.

	Body parts susceptible to infection	**Stations**				
		Kalimati Cfu/gm	**Balkhu Cfu/gm**	**Khicchapokhari Cfu/gm**	**Lagankhel Cfu/gm**	**Bhaktapur Cfu/gm**
Sub Station 1	Gills	1.28×10^9	2.89×10^8	3.8×10^6	3.98×10^7	1.26×10^7
	Muscle	1.9×10^8	3.42×10^7	4.0×10^5	1.78×10^6	2.50×10^9
	Intestine	8.0×10^9	5.84×10^9	5.0×10^7	2.62×10^7	Nil
Sub Station 2	Gills	3.0×10^6	5.6×10^7	9.0×10^6	1.0×10^6	1.80×10^9
	Muscle	3.14×10^5	3.26×10^5	2.0×10^5	1.12×10^6	7.6×10^8
	Intestine	5.42×10^9	7.02×10^7	1.8×10^8	2.74×10^7	7.0×10^7
Sub	Gills	2.7×10^6	5.12×10^7	3.0×10^7	4.16×10^7	1.14×10^8

Station 3	Muscle	6.0×10^5	3.55×10^7	1.7×10^6	1.68×10^7	4.8×10^9
	Intestine	3.0×10^7	5.52×10^9	1.56×10^7	2.31×10^7	Nil

Table 4: Total Plate Count for the Microbial analysis of samples in different study stations

Table 5: Average microbial loading in different body parts of samples with total load in edible parts

S.N	Stations	Average microbial loading at different body parts of samples			Total Microbial Load in Edible parts (Gills and muscle) Cfu/gm
		Gills Cfu/gm	Muscle Cfu/gm	Intestine Cfu/gm	
1	Kalimati	4×10^8	6.36×10^7	4.48×10^9	4.64×10^8
2	Balkhu	1.73×10^8	1.73×10^7	2.96×10^9	3.46×10^8
3	Khichapokhari	1.43×10^7	7.67×10^5	8.19×10^7	1.51×10^7
4	Lagankhel	2.75×10^7	6.57×10^6	2.56×10^7	3.41×10^7
5	Bhaktapur	6.42×10^8	2.69×10^9	7.0×10^7	3.33×10^9

The microbial load in the edible portions (muscle and head with gills) of *Chirrinusmirgala*had the lowest value 1.51 $x10^7$for the samples from Khicchhapokhari and highest microbial load was found in the samples of Bhaktapur with the value 3.33 $x10^{9.}$

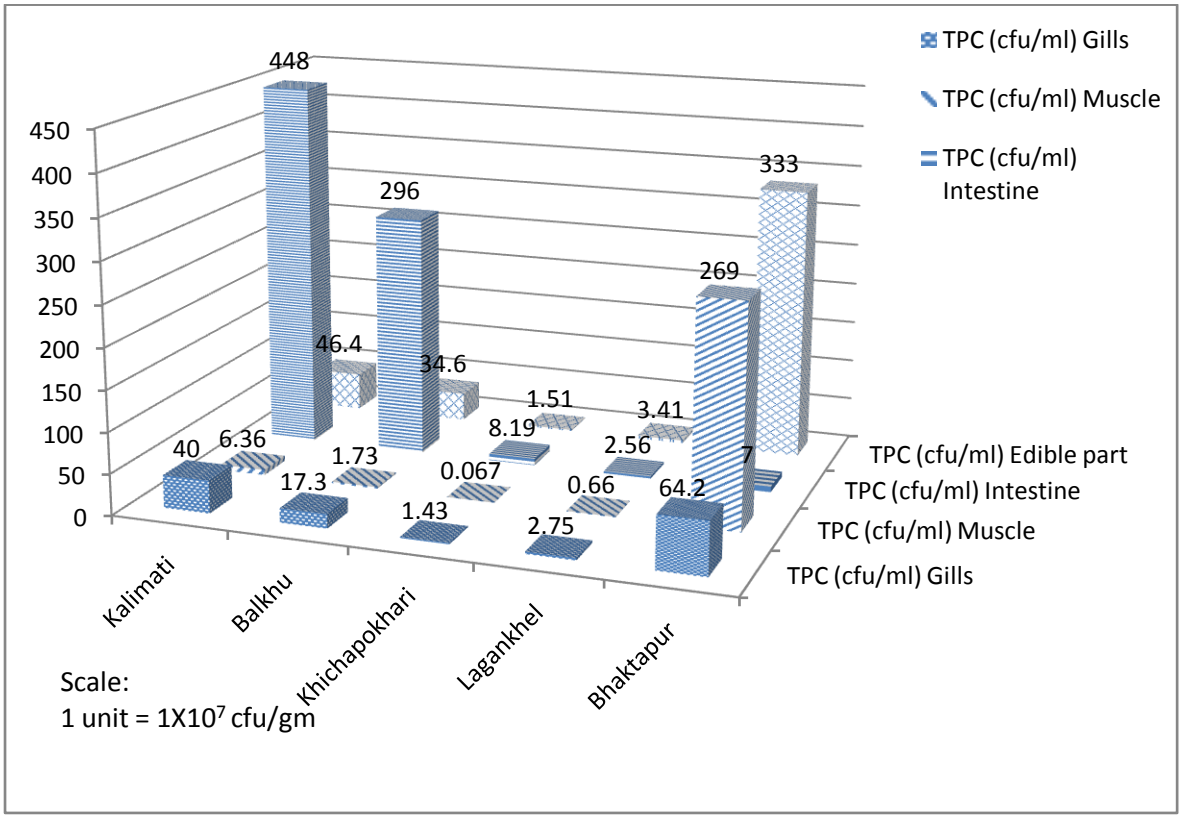


Figure 2: Average microbial loading in different body parts of samples with total load in edible parts

4.2.2 Isolation of *Salmonella* spp.

Table 6 shows that isolation of *Salmonella* spp.was found to be negative for the gills. Similarly, the muscle portion of the fish was also found to be negative for the presence of *Salmonella* spp.except for the samples of Bhaktapur substations 1 and 2 which were found to contain the pathogenic microorganism in their muscle as well. Similarly, the samples were found to be positive for the presence of the *Salmonella* spp. in the intestinal part of the fishes.

Table 6: Isolation of *Salmonella* spp.on different parts of samples at different study stations.

S.N	Stations	Substation 1			Substation 2			Substation 3		
		Gills	Muscle	Intestine	Gills	Muscle	Intestine	Gills	Muscle	Intestine
1	Kalimati	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve
2	Balkhu	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve
3	Khichapokhari	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve
4	Lagankhel	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve
5	Bhaktapur	-ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve

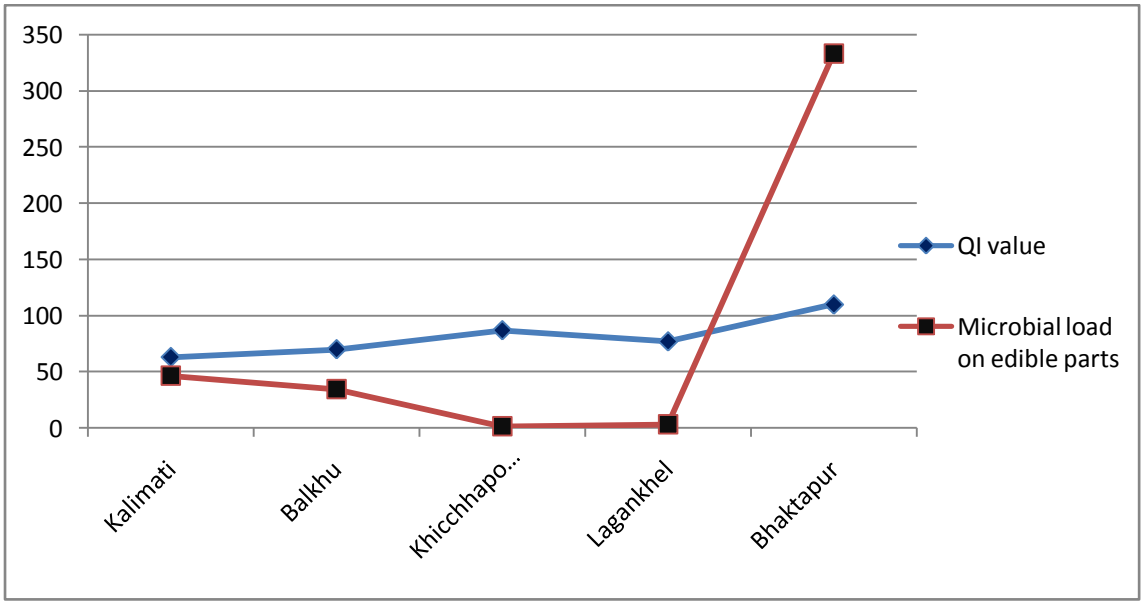


Figure 3: Relationship between QI Score and Microbial Quality of the samples

Figure 3 shows the relationship between QI score and the microbial quality of the samples. It illustrates that the increasing value of QI accompanies the decrease in the

microbial quality in the study stations Kalimati, Balkhu, Khicchhapokhari and Lagankhel. But for the samples from Bhaktapur, though the QI score increases, the microbial load increases highly.

5. DISCUSSION

5.1 Sensory Analysis

Sensory evaluation is one of the most important methods for assessing freshness and quality in the fishing sector and in fish-inspection services. Sensory methods performed in a proper way are a rapid and accurate tool providing unique information about the food(Hyldig*et al.,* 2007). In the study of sensory analysis score of the samples from different study stations, the Quality Index for the samples were in increasing order for Kalimati, Balkhu, Lagankhel, Khicchapokhari and Bhaktapur with the value of 6.3, 7.0, 7.7, 8.7 and 11 respectively.As demonstrated by Meilgaard*et al.*, (1991), the increasing value of QI denotes the decreasing trend in the quality of fishes.

Kalimati station is a wholesale market. The fishes from different routes reach to Kalimati stations and are supplied to other different retail markets included under the study sites.

After Kalimati station, Balkhu is the next station with lower QI Sore. The reason being Balkhu is the second largest market for vegetables and fishes as well. Similarly, due to the shorter transportation distance among remaining stations from Kalimati, the QI Score may be less. Bhaktapur which lies at the greatest travelling distance from the wholesale market had the highest Score of 11 signifying the lowest sensory quality standards.

When the QI is above 20, the spoilage of fish becomes evident (Sveinsdottir*et al.*, 2002). Thus from the analysis of results obtained it can be said that the fishes are not evidently spoiled to reject for the consumption.

5.2 Microbial Analysis

5.2.1 Analysis in terms of differential loading of the body parts of the sample

Bacteria naturally exist on the skin, gills, and in the gut but they are not able to cause spoilage because of the natural defensive mechanism in healthy living fish. After slaughter of fish, bacteria find access into the fish flesh not only due to lost sterility, but because of the various mechanical and autolytic changes that rupture gut walls and soften fish flesh, making it easy for bacteria to access fish tissue (Bremner, 2002). Figure 2 illustrates that the microbial loading of intestine was found to be the highest followed by gills and muscles. Yakinori and Hirofami (1984) reported higher bacterial count in the intestine of carps. The intestine of pearl spot, *Etroplus suratensis* carried heavy bacterial load when compared with skin and gill samples (Surendran and Mahadeva, 1985).

Microbial characteristics of ice stored *Labeo gonius* along with their biochemical parameters were investigated by Leelabati and Viswanath (1999). They also reported higher bacterial count in the intestinal region. The result of the present study is in agreement with the inference made in the work done by Leelabati and Viswanath (1999). Surendran*et al.*,(1976) recorded that in oil sardine the higher magnitude of bacterial population was seen in gut samples and TPC ranged from 10^5 to 10^8/g when compared to skin with muscle which had values from 10^3 to 10^5/g and gill samples which had TPC in the range of 10^5 to 10^6 .The result of the present study showed that the population of bacterial flora of the whole digestive tract is comparatively higher than the corresponding skin with muscle samples. The total aerobic plate count varied between 1.51 $X10^7$to 2.69 $X10^9$cfu /g for muscle and 2.56 $X10^7$to 4.48 $X10^9$cfug for gut samples. So it is inferred that the aerobic bacterial load was higher in the region of gut or digestive tract. Gut provides favourable ecological niche for the growth of microorganisms. It is therefore suggested that evisceration of fishes will enhance the keeping quality of fish.

5.2.1 Analysis with reference to the differential loading of samples in study sites

Due to relatively smaller size of *Chirrinus mirgala*, whole fish is consumed by removing the alimentary canal and visceral mass. While analyzing the total microbial load in edible parts (Head along with gills and muscles) with reference to differential loading of samples in the study sites, it was found that the lowest microbial load was present in the fishes of Khicchapokhari with the value of 1.51 $x10^{7.}$ Likewise, the highest value of microbial load 4.64 $x10^8$ was found in the samples of Kalimati.

The research result seems to be consistent with the researches carried out previously. According to Ali and Mohammad(2012), during the first 30 days of frozen storage, simultaneous with slight changes of biogenic amines, bacterial load significantly decreased ($P < 0.05$), but as frozen storage time lengthened, progressive development of biogenic amines and microbial load (except for *Pseudomonas* spp.) was observed. Gebremariam (2010) reported that the total bacterial loadand the total coliforms of Nile tilapia during frozen storagedecreased in fish samples until the 60th day of frozenstorage, but started to increase on the 75th day of storage.During ice storage, the Total Plate Count (TPC) of mesophilic bacteria decreased continuously from 5.56 X 103 cfu/g of meat (0 day) to 1.73 X 102 cfu/g ofmeat (22nd day) and 5.83 X 103 (0 day) to 1.90 X 102 cfu/g of meat (22nd day) during 24 h and 48 h culture respectively(Praveen Kumar *etal.*, 2015).

The decrease in Microbial loading in the samples from retail stations was due to the washing with water from the melted water.More-over, during the first 30 days of frozen storage, a reduction in mesophilic ($P < 0.05$) and psychrotrophic ($P > 0.05$) bacteria was observed, which might be due to the cold shockduring storage under freezing condition(Ali and Mohammad,2012).

With representative sample units not less than five, plate counts below $5X10^5$are considered of good quality; between 7.5 – $10X10^5$ and marginally accepted quality (sample units with plate counts between $5X10^5$ and 10^7 not exceeding three) and plate

counts at or above 10^7 are considered unacceptable in quality (ICMSF, 1986). The fishes available in the market of Kathmandu valley seems to be of unacceptable quality.

The microbial loading in the samples of Bhaktapur is inconsistent with the trend seen in the result. When table 4 is observed, it can be seen that the samples from the Bhaktapur sub stations 1 and 2 had the bursted belly. Due to the same reason, the microbial load in the muscle of the samples from those sub stations may have been relatively greater in comparison to other samples in Table 4 which eventually broke the consistency of the result.

5.2.3 Isolation of Salmonella spp.

Barbara (2003)mentioned that *Salmonella* spp. can be found in the digestive tracts of humans and animals, especially reptiles. *Salmonella* spp. on the skin of reptiles or amphibians can be passed to people who handle the animals. Food and water can also be contaminated with the bacteria if they come in contact with the feces of infected people or animals.

According to Table 6, isolation of *Salmonella* spp.from the alimentary canal of the samples may indicate the unsatisfactory hygienic conditions during catching, handling and marketing of the fish.

Salmonellosis has an epidemiological importance as some of its members are pathogenic and may cause serious infections and food poisoning to man. Moreover, Salmonellosis count can be taken as an indicative of possible enteric contamination in the absence of Coliform bacteria (Pogorelova *et al*., 1993).

6. CONCLSION

From the analysis of results obtained from sensory analysis, it can be said that the fishes are not evidently spoiled to reject for the consumption. The absence of pathogenic Microorganism *Salmonella* spp.in the edible part and only presence in the alimentary canal also supports the conclusion that the fishes are not evidently spoiled to reject for consumption. But due to the presence of high bacterial load in the edible portions of the samples, it can be clearly said that the handling and preservation techniques of the fishes employed in Kathmandu Valley has not been standardized as per the international system and requirement. There always lies the risk of cross contamination and the outbreak of a serious disease caused due to the pathogenic microorganisms related to the fresh fish. The fish act as a reservoir of human pathogens and the presence of highly pathogenic agents such as *Salmonella, Shigella* spp. and of opportunistic pathogens is a potential health risk/hazard to human beings and may cause diseases to susceptible individuals especially the immune-compromised consumers. Moreover the recoveries of various organisms, which are potentially pathogenic to humans, in the fish suggest that if they are improperly handled, undercooked or consumed raw may contribute to the spread of the pathogens in the community. Further examination of fish especially for the presence of pathogens, during handling, storage and up to the very point of consumption is needed for the protection and maintenance of community health by keeping food borne diseases to a minimum.

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APPENDIX 1

1. Sensory Analysis

1.1 Material

- Samples — Whole fishes
- Aluminium Foil — 1 unit
- Table For evaluation — 1 unit
- Tags — 1 unit
- Sensory form — 15 units

1.2 Method

Samples from the study sites were put on the sterilized aluminium foil.

Apperance of different body features were studied according to the Quality Index Method(QIM).

The data was recorded in the sensory form.

1.3 Sensory Form

Table 7: Quality assessment scheme used to identify the quality index demerit score (Larsen et al. 1992)

Quality parameter	Character	Score (ice/seawater)
General appearance	Skin	0 Bright, shining 1 Bright 2 Dull
	Bloodspot on gill cover	0 None 1 Small, 10-30% 2 Big, 30-50% 3 Very big, 50-100%
	Stiffness	0 Stiff, in *rigor mortis* 1 Elastic 2 Firm 3 Soft
	Belly	0 Firm 1 Soft 2 Belly burst
	Smell	0 Fresh, seaweed/metallic 1 Neutral 2 Musty/sour 3 Stale meat/rancid
Eyes	Clarity	0 Clear 1 Cloudy
	Shape	0 Normal 1 Plain 2 Sunken
Gills	Colour	0 Characteristic, red 1 Faded, discoloured
	Smell	0 Fresh, seaweed/metallic 1 Neutral 2 Sweaty/slightly rancid 3 Sour stink/stale, rancid
Sum of scores		(min. 0 and max. 20)

APPENDIX 2

2. Microbiological Analysis

2.1 Media used for the culture and enumeration of microorganisms:-

Different type of culture media such as enrichment media, selective media, and differential media were used. Composition and preparation of different type of culture media are given below:-

1. Total plate count agar (TPCA)

The total plate count agar is used for the enumeration of bacteria in foods and water.

Composition:-

Ingredients	gm/liter
Tryptone	5.0
Yeast	2.5
Dextrose	1.0
Agar	15.0
Final pH	7.00±.2

Direction for preparation:-

23.5 grams of TPCA was dissolved in 1000 ml of distill water, boiled to dissolve the medium completely and sterilized at 15 lbs pressure (at 1210C) for 15 minutes. 2. 2.

2. Selenite F enrichment Broth

Selenite F Broth is used as an enrichment medium for the isolation of Salmonella groups and few spp. of Shigella groups, when isolating these organism from foods, dairy products etc.

Composition:-

Ingredients	gm/litre
Part A	
Tryptone	5.0
Lactose	4.0
Sodium phosphate	10.0
Part B	
Sodium acid selenite	4.0
Final pH (at 25^0C)	7.4±0.2

Direction for preparation:-

19 grams of part A and 4 grams of part B was suspended in1000ml of distilled water. It was warmed to dissolve the media and mixed well. It was then dispensed or sterilized in a boiling water bath or in free floating steam for 10 minutes. DO NOT AUTOCLAVE, EXCESSIVE heating is detrimental.

5.Sulfide-Indole-Motility Medium (SIM)

Sulfide-Indole-Motility is a semi solid medium used for the determination of sulfide production, Indole formation and motility of enteric bacteria.

Composition	gm/liter
Beef extract	3.0
Peptone	30.0
Peptonized iron	0.2
Sodium thiosulfate	0.025
Agar	3.0
Final pH (at 25^0C)	7.3±0.2

Procedure

36 grams was suspended in 100ml-distilled water. It was heated to boil to dissolve the medium completely. It was dispense in tubes and sterilized by autoclaving at 15 lbs. pressure (121^0C) for 15 minutes. The medium was allowed to solidify in vertical position.

Reagent: Kovac's reagent

Composition	gm/liter
P-Dimethyl- aminobenzaldehyde	5.0
Amyl or Isoamyl alcohol	75.0 ml
Conc. Hydrochloric acid	25.0 ml

Procedure

The test organisms was stabbed into the medium and incubated at 37^0C for 24 hours. Motile organism show diffuse growth or turbidity away from the line of inoculation and non-motile grows only along the line of inoculation. Blackening along the line of inoculation indicates H2S positive test. 0.2ml of Kovac's reagent was added to the tube and allowed to stand for 10 minutes. A dark red colour in the reagent indicates a positive indole test.

4. Citrate Utilization Test

Citrate utilization test is performed to determine if an organism is capable of utilizing citrate as the sole source of Carbon for metabolism and energy source for the growth,

Medium: – Simmon's Citrate Agar

Composition:-

Ingredients	gm/liter
Monoammonium phosphate	1.0

Potassiumphosphate	1.0
Sodium Chloride	5.0
Sodium Citrate	2.0
Magnesium Sulphate	0.2
Bromothymol blue	0.08
Agar	15.0
Final pH (at 25^0C)	6.8±0.2

Preparation

24.2 grams was suspended in 1000ml-distilled water. It was heated to boil to dissolve the medium completely. It was distributed in tubes and sterilized by autoclaving at 15 lbs. pressure (121^0C) for 15 minutes. The mediums in tubes were solidified in slanted position.

Procedure

The slant was streaked with test organism and incubated at 37^0C for 48 hours. Growth of organism with an intense blue colour on slant is the indicative of positive test. No growth no change in colour (green) is the negative test.

5. Triple Sugar Iron Agar Test

The test is done to determine the ability of an organism to utilize specific Carbohydrate incorporated in the medium, with or without the production of gas, along with determination of possible hydrogen sulfide production

Composition	gm/liter
Peptone	10.0
Tryptone	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulfate	0.2
Sodium Chloride	5.0
Sodium Thiosulfate	0.3
Phenol- red	0.024
Agar	12
Final pH (at 25^0C)	7.4±0.2

Preparation

6.5 grams was suspended in 1000ml-distilled water. It was boiled to dissolve completely. It was distributed in tubes and sterilized by autoclaving at 15 lbs. pressure (121^0C) for 15 minutes. The medium was allowed to set in slopped from with a butt about 1 inch long.

Procedure

The test organism was stabbed in the butt and streaked on the slant. The tubes were incubated at 37^0C for 24 hours. Black coloration of butt was indicative of H_2S formation. The change in colour of butt, slant and gas formation was also noted and recorded as alkali/alkali, alkali/acid and acid/acid for the growth of fermenters and all sugar fermenters.