**CHAPTER 1: MICROBIOLOGY AS APPLIED SCIENCE**

Applied microbiology deals with the practical application of microorganisms to human uses. Microbial activities have been important since the discovery of fermentations. Beer, wine and leavened bread were important fermented products even in early Sumerian cultures (4th century B.C.).Microorganisms are very useful to our day to day activity. For instance, in industry, food preparation, beverage production, water treatments, soil formation and enrichments, pharmaceuticals, mining, energy and so on.



 **Fig. 1.1:** Scope and applications of microbial products

**Some of the applications of microorganisms are briefly discussed below.**

1. **Biofuels from Microbes**

It is known that microorganisms can produce fuels, such as ethanol, methane and hydrogen, from organic matter. Hence, these biofules and others can be produced by microbes at industrial level.

**Hydrogen**: It is expected as an ideal fuel for future transportation because it can be converted to electric energy in devices known as microbial fuel cells and converted to mechanical energy without production of CO2.

**Methane/biogas:** Biogas plants produce methane gas sustainably along with carbon dioxide from organic substances basically plant biomass or/and industrial wastes.

**Ethanol**: Bioethanol fermentation is by far the largest scale microbial process. Industrial ethanol production uses sugar cane molasses or enzymatically hydrolysed starch (from corn or other grains) or/and acid hydrolysis of cellulose and starch, and batch or continuous fermentation with yeast *Saccharomyces cerevisiae* to produce ethanol. By-products of this process are CO2 and low amounts of methanol, glycerol etc. Ethanol does not need to be rectified to high purity if it is to be used as a fuel. Acetone is produced together with butanol and ethanol.

1. **Electricity Generation by Microbes**

It is known that microorganisms can also convert organic matter into electricity in devices known as microbial fuel cells. As a result, interest in microbial fuel cells is increasing from time to time. Microbial fuel cells offer the possibility of harvesting electricity from organic waste and renewable biomass. These are attractive sources of energy because most of them are ‘carbon-neutral’.

1. **Biomining (Bioleaching)**

Although mining is one of the humankind's oldest activities, the techniques used to extract minerals haven't changed substantially for centuries. Ores are dug from the earth, crushed, and then minerals such as copper and gold are extracted by extreme heat or toxic chemicals. These traditional mining technologies lead to the environmental and health effects.

As the availability of mineral-rich ores decreases, methods are needed to extract minerals from less concentrated sources. This need new discipline known as **biohydrometallurgy**, the use of microbes to extract metals from ores. Generally, mineralytic effects of bacteria and fungi on minerals are based mainly on three principles, namely acidolysis, complexolysis, and redoxolysis. Hence, microorganisms are able to mobilize metals by (1) the formation of organic or inorganic acids; (2) the excretion of complex agents; and (3) the oxidation and reduction reactions. Sulfuric acid is the main inorganic acid found in leaching environments. It is formed by sulfur-oxidizing microorganisms such as thiobacilli. Organic acids are formed by bacterial as well as fungal metabolisms which result in acidolysis.

1. **Microorganisms in Petroleum Recovery Technology**

There are **3** ways in which microorganisms may contribute to enhanced oil recovery: (a) microorganisms can produce biosurfactants and biopolymers on the surface; (b) microorganisms grow in reservoir rock pore to produce gases, biosurfactants, and other chemicals to recover and trapped oil in reservoirs; and (c) microorganisms can selectively plug high-permeability channels in reservoir rock so that the sweep efficiency of the recovery process can be increased. Bacteria actually produce the polymer, much as humans make and store fat. Normally, the bacteria use the polymer for their own energy needs. But the bacteria can be "harvested" before using the polymer they have stored, and it can be used by people.

1. **Microbes in Food**

Microorganisms are widely used in the food industry to produce various types of foods that are both nutritious and preserved from spoilage because of their acid content.

**Dairy foods:** In the dairy industry, many products result from fermentation by microorganisms in milk and the products of milk. For example, **buttermilk** results from the souring of milk by lactic acid bacteria. The flavor is due to substances such as diacetyl and acetaldehyde, which are produced by species of *Streptococcus, Leuconostoc* and *Lactobacillus* as they grow.

Milk can be fermented to produce a variety of products, including butter, buttermilk, yoghurt and cheese. In each case, acid produced by the fermentation process causes coagulation or curdling of the milk proteins. In cheese-making, this coagulation is effective by the addition of the *rennin,* or by the action of lactic acid bacteria (e.g *Streptococcus lactis* and *S. cremoris*). Coagulation allows the separation of the semisolid *curd* from the liquid *whey*. The subsequent steps in the cheese-making process depend on the specific type of cheese. Following separation, the curd of most cheeses is pressed and shaped, removing excess liquid and firming the texture. During the ripening process, salt is often added, and flavour develops due to continuing microbial action on the protein and fat components of the cheese. The length of the ripening period varies from a month to more than a year according to type, with the harder cheeses requiring the longer periods.

Yoghurt is another milk derivative. Thickened milk is exposed to the action of two bacteria, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, both of which ferment lactose present in milk into lactic acid. In addition, *L. bulgaricus* contributes aromatics responsible for imparting flavour to the yoghurt. Other dairy products, such as soured cream and buttermilk, are also produced by means of the fermentative properties of species of streptococci and lactobacilli.

**Other fermented foods:** These are also the product of microbial action. For example, **sauerkraut** is produced by *Leuconostoc* and *Lactobacillus* species growing within shredded cabbage. Cucumbers are fermented by these same microorganisms to produce **pickles.**

**Bread:** It is also another product of microbial action. Flour, water, salt, and yeast are used to make the dough. The biological agent responsible for bread making is yeast. In fact baker’s yeast and brewer’s yeast are just different strains of the same species, *Saccharomyces cerevisiae*. This organism ferments the carbohydrates in the dough and produces carbon dioxide, which causes the dough to rise and creates the soft texture of bread. In bread making, aerobic, rather than anaerobic conditions are favored, so sugar present in the dough is converted all the way to carbon dioxide rather than to alcohol. This causes the bread to rise. Any small amount of ethanol that may be produced is evaporated during the baking process. Unleavened bread is bread that contains no yeast. Sourdough bread can be made by using lactic acid bacteria to contribute a sour flavor to the dough.

In Ethiopia, *enjerra, difo dabo, kocho, ergo, ayib, awaze, data*, etc are product of microorganisms.

1. **Microbes in Soil (Roles of Microbes in Agriculture)**

We might think of the soil we walk on as an inert substance, but nothing could be further from the truth. Soil, in fact, is teeming with microscopic and small macroscopic organisms, and it receives animal wastes and organic matter from dead organisms. Microorganisms act as decomposers to break down this organic matter into simple nutrients that can be used by plants and by the microbes themselves. Soil microorganisms are thus extremely important in recycling substances in ecosystems. Microorganisms play several key roles in the environment. For instance, they are well-known for their activities in biogeochemical cycling and soil fertility maintenance. Besides, several microbes could be shown very helpful in maintenance of environmental quality through biodegradation of wastes (urban, municipal and industrial) into useful products. They are also used in biodegradation of harmful pesticides such as DDT, lindane heptachlor, chlordane, malathion, etc. Thus, microbes are purifiers of the environment.

The use of *mycorrhizae* to enhance the uptake of nutrients and water for establishment of seedlings on degraded lands. This will, however, not lead to the improvement of soil fertility by mycorrhizae as such, although the success of plant growth will eventually lead to reclamation of degraded lands. However, nitrogen fixing bacteria like *Rhizobium* or *actinomycete* genus *Frankia* can be used to induce nodule formation in a variety of plant species, so that these can be used for improving soil fertility of degraded lands. This nodule formation can be induced, both in leguminous and non-leguminous plant species comprising annuals (cereal and legume crops) and perennials (trees). Efforts are underway to manipulate the genes of both host and *rhizobia* to obtain maximum efficiency of nodule formation. Strains are also being adapted for unusual soil environments representing degraded lands.

Generally, microbes are very important for agriculture since they solubilize inorganic phosphate; they enhance nutrient uptake of plant root; they increase soil nutrient by composting, mineralization, solubilization and nitrogen fixation; they suppress pathogens in soil; and they condition the soil.

1. **Microbes in Industry**

Today, hundreds of different substances are manufactured with the aid of microorganisms. Various species of yeasts, molds, bacteria, and actinomycetes are used in manufacturing processes. The organisms themselves are sometimes useful when they can serve as a source of protein. For instance, animal feed consisting of microorganisms is called single-cell protein (SCP) used as source of protein. This protein is an important high-yield, relatively inexpensive source of protein-rich food. More often the valuable substance is a product of microbial metabolism.

Antibiotics are among the most important compounds produced by industrial microbes. The most useful ones are secondary metabolites produced by **filamentous fungi**, **bacteria** and **actinomycetes**. The new antibiotics are discovered by screening microbes isolated from natural samples for the production of chemicals that inhibit specific test bacteria. The test bacteria are related to bacterial pathogens. Most of the positive results are likely to be currently known antibiotics but new ones are still discovered. The new substance is tested for toxicity and effectiveness by infected in model animals before commercial production is contemplated. Most new substances are likely to be either toxic to the animal, or relatively ineffective in killing pathogens in the body. Thus, an enormous amount of effort must be expended to find the rare substance that is novel and effective. Generally, enzymes, organic acids, ethanol, acetone, glycerol, foods, beverages, antibiotics and so on can be produced by microbes at industrial level.

1. **Microbes in Wastewater Treatments**

The use of microbes in wastewater treatment plants is well documented and is an integral piece of the wastewater treatment process. Traditionally, microorganisms are used in the secondary treatment of wastewater to remove dissolved organic matter. The microbes are used in fixed film systems, suspended film systems or lagoon systems depending upon the preference of the treatment plant. All of which are stages that microbial supplementation can be added with benefit. A higher concentration of microbes is going to be able to quickly remove the organic matter from the water, particularly in the case of lagoon systems where it can take several months for the degradation of waste to be completed.

Microbes can also be of benefit in other stages of the process. Microbes added into the primary treatment phase can degrade bottom and surface solids resulting in less production of sludge. This can lead the secondary treatment phase to be more effective. In some wastewater treatment plants, an advanced treatment stage is necessary to remove excess nutrients that can result in algal blooms and other downstream issues. Microbes can be substituted for chemicals to keep the treatment process as natural as possible and minimize further pollution. Finally, microbes aid in the treatment and disposal of the sludge by decomposing additional organic matter and reducing volume while also limiting the noxious odors emitted by the sludge.

1. **Microbes in Medicine**

Microbes are very important in medicine since they are used for synthesis of antiviral, antibacterial and antifungal agents. In addition, microbes produce antioxidants and anti-inflammatory chemicals. Recent development in antibiotic and other chemotherapeutic agents’ production has been used microbial biotechnology in transformations and biotransformation of natural pencillin G to several semi-synthetic penicillns. Penicillin acylase produced by *Saccharomyces cerevisiae* and *Kluyvera citrophila* is used in biotransformation of penicillin G to semi-synthetic penicillins.

**Probiotics**: are living organisms which upon ingestion in certain numbers; colonize the body and have beneficial health effects for the host. The most common probiotic bacteria are lactic acid bacteria (LAB). Although there are many selection criteria for microbes to be used as probiotics, the most common selection criteria for probiotics are**:**

* Antimicrobial activity
* Acid and bile tolerance
* Adhesion to the intestinal epithelium
* Non-toxic and non-pathogenic to human and animals
* Enhancing immune system
* Antibiotic resistance

Probiotics can help to prevent and treat disease through a number of mechanisms. For instance, by production of bacteriocins that can inhibit pathogenic microorganisms. Besides, by interacting directly with the disease-causing microbes and making it unsuitable condition for them to cause disease.

Generally, beneficial health effects of probiotic LAB include: improving the intestinal microflora balance, inhibiting the growth of pathogenic bacteria, promoting good digestion, immunostimulating and increasing resistance to infection, prevention and treatment of diarrheal disease, prevention of systemic infections, reduction of serum cholesterol, reduction in blood pressure, prevention and allergy lowering effect, removal of toxins, synthesis and enhancing the bioavailability of nutrients and alleviation of lactose intolerance. The organisms help reinforce the natural bacterial barrier that exists on the lining of the digestive tract providing additional protection against pathogenic organisms that can cause diarrhea. However, most of the mechanisms behind the beneficial health effects are not fully known and understood.

**CHAPTER 2: FOOD MICROBIOLOGY**

**Introduction**

Foods, microorganisms and humans have had a long and interesting association that developed long before the beginning of recorded history. Foods are not only of nutritional value to those who consume them but often are ideal culture media for microbial growth.

The interaction between microorganisms and foods may be understood from 3 aspects: (i) microbial fermentation introduces desirable flavor and physical characteristics in many food products; (ii) food products may become contaminated with pathogens or microbial toxins and thereby become vehicles for the transmission of disease to humans and other animals; and (iii) many spoilage microorganisms are capable of causing off-flavor and physical changes in food products.

Microorganisms have been playing a pivotal role in the fermentation of foods and beverages since the beginning of human civilization. Fermented foods and beverages are defined as products obtained through the desirable biochemical changes caused by the action of microorganisms or their enzymes. In indigenous fermented foods, the microorganisms responsible for the fermentation are usually the microflora naturally present on the raw substrates.

This chapter focuses on the principles of food microbiology such as factors affect the ability of microorganisms to proliferate in foods, food spoilage microorganisms, food toxins and their sources, mechanisms of food preservation and traditional fermented foods and their advantages. Food microbiology deals with the study of source, behavior, identification and characterization of microorganisms with beneficial as well as deleterious effect on raw as well as processed foods, food preparation using microorganisms, and mechanisms of food preservation.

* In general, food microbiology encompasses the study of:
	+ - Microorganisms which have both beneficial and deleterious effects on the quality and safety of raw and processed food.
		- The general characteristics of microorganisms found in food such as
			* their growth characteristics, identification, and pathogenesis.
			* food poisoning, food spoilage, food preservation, and food legislation.

**2.1. Factors that Affect Microbial Growth in Foods**

* Factors that affect the growth of microorganisms in food are divided into **Intrinsic** and **Extrinsic** parameters/ factors.
1. **Intrinsic factors:**
	* Properties that exist naturally as part of the food product itself.
	* They are also called inherent characteristics.
	* May affect the growth of microorganisms positively or negatively.
2. **pH**
* Most microbes grow best at or around pH 7.0 (6.5 – 7.5), although different microorganisms have different capabilities to survive and proliferate at different pH values.
* Few can grow below pH 4.
* In general, yeasts and molds can survive wide range pH than bacteria.
* Moulds are able to survive pH 1.5 – 9.0 and yeasts can survive pH 2 – 8.5.
* Generally, gram negative bacteria are more sensitive to low pH than gram positive bacteria.
* The approximate pH ranges for:
	+ - most bacteria ( minimum 4.5, optimum 6.5 – 7.5 and maximum 9.0 ).
		- pH range for gram positive bacteria is (4 – 8.5) and for gram negative bacteria (4.- 9.0).
		1. **Moisture content (water activity [aw])**
* The amount of free / unbound water in food which can be utilized by microbes.
* Foods with higher water activity are easily perishable as favor the growth of spoilage microorganisms.
* Therefore, it is important to predict food safety, stability and quality.
* The value of water activity ranges from 0.0 to 1, with the value of 1 for pure water.
* It is expressed by the following formula Water Activity (**aw**) = RH / 100.
* The water activity of most dry foods is around 0.2 whereas for most fresh foods the value is around 0.99.
* If a food has a water activity value of 0.85 or below, it is generally considered as non -hazardous.
* Most moulds and yeasts can grow at a minimum water activity value of around 0.80 with xerophilic molds capable of surviving at water activity of 0.65 and osmophilic yeasts at 0.60.
* Most bacteria including *Clostridium botulinum* cannot grow at water activity below 0.91, however, *Staphylococcus aureus* can grow at water activity of 0.86. Therefore, dry food like bread is spoiled by moulds and yeasts rather than by bacteria.
* Requirement of water activity by microorganisms is in the following order *Bacteria > Yeast > Mold.*

 **Table 2.1:** Minimum water activity values of most spoilage microorganisms.

|  |  |  |
| --- | --- | --- |
| **Microbial group**  | **Minimum aW** | **Examples**  |
| Most bacteria  | 0.91 | *Salmonella* spp*., Clostridium botulinum*  |
| Most yeasts  | 0.88 | *Torulopsis* spp*.*  |
| Most molds  | 0.80 | *Aspergillus flavus*  |
| Halophilic bacteria  | 0.75 | *Wallemia sebi*  |
| Xerophilic molds | 0.65 | *Aspergillus echinulatas* |
| Osmophilic yeasts | 0.60 | *Saccharomyces bisporus* |

* + 1. **Redox potential (Eh)**
* It is the measure of tendency of a revisable system to give or receive electrons.
* It is measured in electrical units of millivolts (mV).
* It measures the availability of oxygen in foods.
* If free oxygen is present in the system, then it can act as an electron acceptor.
* In the absence of free oxygen, oxygen bound to some other compound, such as NO3 and SO4, can accept the electron.
* The redox potential of food is affected by the chemical composition of food, specific processing treatment and storage conditions.
* Fresh foods of plant and animal origin are in their reduced form due to several reducing factors found in fresh foods such as ascorbic acid, reducing sugars, and sulfhyridl group (–SH) of proteins.
* Heating can change the Eh state of a given food and foods stored in air will have more positive redox potential than the one stored under vacuum and modified atmosphere (CO2 and N2).
* Based on the growth of microorganisms in the food under different redox potential they could be:

Strict aerobes, Eh (+500 to +300 mV).

Strict anaerobes, Eh (+100 to –250 mV or lower).

Facultative anaerobes, Eh (+300 to +100 mV).

Aerotolerant anaerobes.

* Molds, yeasts, and *Bacillus*, *Pseudomonas*, *Moraxella*, and Micrococcus are aerobic.
* Facultative anaerobes are the lactic acid bacteria and those in the family *Enterobacteriacae.*
* The most common anaerobe in food is *Clostridium*.
* Therefore, the Eh of food determines the growth of microorganisms in food.
	+ 1. **Antimicrobial components and barriers of food**
* Certain food types contain naturally occurring substances to resist attack by microorganisms such as:
* Essential oils in spices:
	+ eugenol in cloves and cinnamon
	+ allicin in garlic
	+ cinnamic aldehyde in cinnamon
* Lactaferrin, lactoperoxidase, lysozyme and free fatty acids found in milk exhibit antimicrobial activity.
* Ovatransferrin, avidin, lysozyme and ovoflavoprotein in hen’s egg albumin.
* The hydroxycinnamic acid derivatives (p-coumaric, feluric, caffeic and chlorogenic acids) found in fruits, vegetables, tea and other plants possess antibacterial and antifungal activity.
* Physical structure such as shells of eggs and nuts, testa of seeds and the outer cover of fruits.
* Therefore, the presence of these antimicrobial substances and physical structure of foods prevent from microorganisms attack.
	+ 1. **Nutrient composition**
* In order to grow and function normally, microorganisms require the following important nutrients in foods:
* Water, carbon source for energy, source of nitrogen, vitamins, minerals and related growth factors.
* Thus, foods with the proper nutrient composition can easily be spoiled by microorganisms.
1. **Extrinsic factors:**
* Factors which are not the inherent part of the food.
* Environmental factors that could affect the growth of microorganisms in food.

**I) Temperature of storage**

* Microorganisms can grow in a wide variety of storage temperatures.
* The maximum and minimum temperatures at which microorganisms grow in foods depend on other intrinsic and extrinsic growth factors.
* Based on their growth temperature, microorganisms are generally classified as psychrophiles (low temperature), mesophiles (intermediate temperature),
* thermophiles (high temperature) and hyperthermophiles (extreme thermophiles).
* Spore forming bacteria can survive high temperature than non – spore formers, molds and yeasts.

 **II) Relative humidity (RH) of the storage environment**

* + - * + It is the amount of moisture in the food storage environment which affects the inherent water activity.
				+ Relative humidity and water activity are interrelated.
				+ If food with low water activity which is not suitable for most microorganisms is stored in an environment with high relative humidity, the water activity increases in the food leading to spoilage by microorganisms.
				+ Hence, food should be stored in an environment with low relative humidity in order to reduce spoilage by microorganisms.
				+ Relative humidity is also related to the storage temperature.

 **III) Gaseous atmosphere**

* The atmosphere under which food is stored also affects the growth of spoilage microorganisms.
* Oxygen is one of the most important gases that can affect the redox potential of foods.
* Modified atmosphere storage of foods, controlled atmosphere (storage of foods in atmosphere containing 10% of CO2).
* Storage of food at high concentration of CO2 affects the growth of moulds and gram negative bacteria.
* Because, CO2 tends to decrease the pH of foods through production of carbonic acid which can adversely affect the solute transport, inhibition of key enzymes involved in carboxylation and decarboxylation reactions.
* Molds and gram negative bacteria are the most sensitive to high CO2.
* However, gram positive bacteria tend to be more resistant to high concentration of CO2.

**2.2. Fermentation**

**Definition**

Biochemically, fermentation is the metabolic process in which carbohydrates and related compounds are partially oxidized with the release of energy in the absence of oxygen as electron acceptors. The final electron acceptors are inorganic compounds produced from the breakdown of the carbohydrates. Consequently, incomplete oxidation of the parent compound occurs, and only a small amount of energy is released during the process. The products of fermentation consist of some organic compounds that are more reduced than others. Fermentation is one of the oldest and most economical methods of producing and preserving food.

* **Advantages of fermentation:**
1. Enhance flavor and taste of the fermented food
	* Fermentation makes the food palatable by enhancing its aroma and flavor.
2. Enhance nutritional quality/value
	* Fermentation also reduces the levels of antinutritive factors such as phytic acid and tannins in food.
	* This help to increase bioavailability of nutrients such as iron, protein and simple sugars in fermented food.
3. Preservative properties by destroying undesirable spoilage and pathogenic microorganisms.
	* The lowering the pH to below 4 through acid production, inhibits the growth of pathogenic spoilage microorganisms which can cause food spoilage and food poisoning.
4. Detoxification process
	* Lactic acid fermentation detoxifies toxic substance such as mycotoxins in foods.
* **Types of fermentation process**

Traditionally fermented foods are classified as alcoholic and non alcoholic products that are prepared locally at the house hold level using back slopping of substances from previous fermentation processes. Based on the end product there are four main fermentation processes: alcoholic, lactic acid, acetic acid and alkali fermentation. Alcoholic fermentation results in the production of ethanol, and yeasts are the predominant organisms (e.g. wines and beers). Lactic acid fermentation (e.g. fermented milks and cereals) is mainly carried out by lactic acid bacteria (LAB). Acetic acid fermentation, in which *Acetobacter* convert alcohol to acetic acid in aerobic condition results in the production of vinegar. Alkali fermentation is basically a proteolytic type and often takes place during the fermentation of protein rich substrates. These are popularly used as condiments.

**2.3. Fermented foods**

Fermented foods are produced world-wide using various manufacturing techniques, raw materials and microorganisms. The microorganisms used for fermentation are bacteria, yeasts and molds. The raw materials traditionally used for fermentation include fruits, cereals, honey, vegetables, milk, meat and fish. Fermented products encompass wine, beer, vinegar, bread, *enjera*, *tella*, *tej*, *qotchqotcha*, *awaze*, *borde*, *shamita*, *data,* *siljo*, soy sauce, sauerkraut, kimchi, pickle and different fermented milk products.

Most fermented foods are dependent on lactic acid bacteria (LAB) to mediate the fermentation process, although yeasts are involved in different fermentations. Lactic acid fermentation contributes towards the safety, nutritional value, shelf life and acceptability of a wide range of fermented foods. Many fermented foods extend their shelf life considerably over that of the raw materials from which they are made. In addition to being made more shelves stable, all fermented foods have aroma and flavor characteristics that result directly or indirectly from the fermenting organisms. For instances, the vitamin content of the fermented food is increased along with an increased digestibility of the raw materials. The fermentation process reduces the toxicity of some foods, whereas others may become extremely toxic during fermentation.

When the natural raw materials are acidic and contain free sugars, yeasts grow readily, and the alcohol they produce restricts the activities of most other naturally contaminating organisms. On the other hand, if the acidity of the product permits good bacterial growth and at the same time the product is high in simple sugars, lactic acid bacteria may be expected to grow.

Substrates that contain polysaccharides but no significant levels of simple sugars are normally stable to the activities of yeasts and lactic acid bacteria due to the lack of amylase in most of these organisms. Thus, to increase the efficiency of fermentation, exogenous source of amylase enzymes must be supplied. The use of barley malt in the brewing industries is an example of this.

**Lactic Acid Bacteria (LAB)**

The lactic acid bacteria classified into two groups based on end products of glucose metabolism. These are the homofermentative and heterofermentative. Lactic acid bacteria that produce lactic acid as the major or sole product of glucose fermentation are called *homofermentative.* The homofermentative pattern is observed when glucose is metabolized but not necessarily when pentoses are metabolized, for some homolactics produce acetic and lactic acids when utilizing pentoses. Sometimes, the homofermentative character of homolactics may be shifted for some strains by altering growth conditions such as glucose concentration, pH, and nutrient limitation. Lactic acid bacteria that produce equal molar amounts of lactic acid (lactate), CO2, and ethanol from glucose are known as heterofermentative*.*

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**Fig.2.1:** Generalized pathways for the production of some fermentation products from glucose by various organisms. (A): homofermentative lactics; (B): heterofermentative lactics; (C) and (D): *Propionibacterium*; (E): *Saccharomyces* spp.; (F): *Acetobacter* spp.; (G): *Acetobacter* "overoxidizers".

In terms of their growth requirements, the lactic acid bacteria require amino acids, vitamins, and purine and pyrimidine bases— hence their use in microbiological assays for these compounds. Although they are mesophilic, some can grow below 5oC and some as high as 45°C. With respect to growth pH, some can grow as low as 3.2, some as high as 9.6, and most grow in the pH range 4.0-4.5. The lactic acid bacteria are weakly proteolytic and lipolytic.

**2.4. Ethiopian Traditional Fermented Foods**

A wide variety of fermented foods and beverages are consumed in Ethiopia. Although some of the food items may be consumed in their raw forms, processing of one type or another is usually a rule than an exception. This usually includes salting and drying, boiling, roasting, frying, baking, cooking, fermenting or various combinations of these. The type of microbial flora developed in each traditionally fermented food depends on intrinsic and extrinsic factors.

**2.4.1 Traditional Fermented Dairy Products**

In Ethiopia, a considerable proportion of milk is consumed in the fermented form. The fermented product has different vernacular names such as *ergo*, *ititu*, *geinto* or *meomata* among the Amhara, Oromo, Sidama, or Wolayta people, respectively. The fermentation is usually natural or spontaneous, with no defined starter cultures used to initiate it. In most cases, this is made possible through the proliferation of the initial milk flora, with microbial succession determined by ambient temperatures and chemical changes in the fermenting milk. In most urban homes, no attempt is made to control the fermentation. Raw milk is left either at ambient temperatures or kept in a warmer place to ferment. In rural areas, particularly among the pastoralists, raw milk is usually kept in a well-smoked container and milk from a previous fermentation used as source of inoculum. Lactic acid bacteria also become established in the container and serve as undefined starter culture. Incubation temperature does not usually vary significantly, particularly in the lowlands, and the taste of the fermented product may, in general, be more or less uniform. The fermented product processed into traditional butter (*qibe*) and buttermilk (*arrera*). The buttermilk may further be processed into traditional cottage cheese (*ayib*) and whey (*aguat*).

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**Fig.2.2:** Flow scheme for milk and milk products available to rural small-holder milk producers in Ethiopia.

1. ***Ergo* (Sour milk)**

Lactic acid bacteria such as *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Lactobacillus* carryout the souring process in *ergo* formation. *Ergo* is acidic fermented product. Milk in smoked containers has a lower rate of pH drop and the fermented product has good flavor for a longer time after coagulation. Similarly, the growth of coliforms and lactic acid bacteria is slow in milk in smoked containers, thus assuring good and slow development of flavor components, safety of finished product and better keeping quality.

Milk incubated at lower temperatures had a better *ergo* flavor. As the temperature of incubation raise, the rate of pH drop will become faster, and the time of coagulation became shorter. In most case, the rate of growth of the various groups of bacteria increases with an increase in incubation temperature. At lower incubation temperatures, lactococci dominate the lactic flora, while lactobacilli dominate at higher incubation temperatures. Smoking of containers may help to produce a safer and tastier *ergo* with better keeping quality at household level. In addition, lower incubation temperatures (around 20oC) may favor a gradual proliferation and succession of lactic acid bacteria and thus guarantee for a desirable fermentation.

**Microbiology of yogurt**

Yogurt has a semisolid mass due to coagulation of milk by starter-culture lactic acid bacteria. Milk is exposed to the action of two lactic acid bacteria: *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, both of which ferment lactose present in milk into lactic acid or lactate. In addition, they contribute aromatics responsible for imparting flavour of the yoghurt. Yogurt has a sharp acid taste and flavor. The flavor is due to the combined effects of acetaldehyde, lactate, diacetyl and acetate, but 90% of the flavor is due to acetaldehyde.

**Biochemistry of yogurt: Lactose Metabolism**

Both *Lactobacillus bulgaricus* and *Streptococcus thermophilus* species have a constitutive β-galactosidase system, and lactose is hydrolyzed to glucose and galactose. *Lab.* *bulgaricus* strains have enzymes for the Leloir pathway to metabolize galactose, but when actively metabolizing glucose they do not utilize galactose well. Most *Str.* *thermophilus* strains do not have the enzymes of the Leloir pathway (or have a very weak system) and thus do not metabolize galactose well. As a result, galactose is excreted outside and accumulated in yogurt.

 β-galactosidase

Lactose---------------------→ glucose→EMP→*Lab*.→D(-)lactate

 *Str.* →L(+)lactate

 +

 galactose→*Lab*. →D(-)lactate or accumulated in yogurt

 *Str*. → excreted and accumulated in yogurt

**Flavor Production**

The major flavor compound in yogurt is acetaldehyde, with some diacetyl, lactate and acetate. Acetaldehyde is produced in two ways: from glucose via pyruvate by *Streptococcus* spp. and from threonine (supplied or produced through proteolysis in milk) by *Lactobacillus* spp.

**Proteolysis**

Proteinases (*Lab.*) Peptidase

Milk protein------------------------→Peptides--------------------→ Amino acids

* Excess accumulation of peptides cause bitter flavor in yogurt
1. ***Qibe* (traditional butter)**

*Qibe* is a traditional Ethiopian butter which is made from *ergo*. It has a white to yellowish color, depending on age, and is semi-solid at room temperature. It has a typical diacetyl taste and flavor when fresh, but extended storage at ambient temperatures results in putridity and rancidity. *Qibe*, without further processing, is used for hairdressing and as a skin cosmetic mainly by women. A small amount of the fresh form is traditionally fed to infants of weaning age. Generally, *qibe* is used in the diet after processing into ‘*nitir* *qibe*’ (traditional ghee), by heating it to boiling after selected types of spices are added to it. *Nitir qibe* is basically used for the preparation of stews made of legumes or meat, which are eaten with *enjerra*, fermented pancake-like bread. In Addis Ababa, where consumption of *qibe* is believed to be high, over 54% of milk is converted to *qibe.*

*Qibe* is produced by churning *ergo* in traditional utensils. Milk for churning is accumulated over several days in the utensil and allowed to sour into *ergo*. Traditionally, *qibe* production is the responsibility of women and the processing of producing *ergo* mayneed1 to 2 hours of churning time. *Qibe* has 17.2% moisture, 1.3% protein, 81.2% fat, 0.1% carbohydrate, 0.2% ash, 0.024% calcium and 0.0015% iron.

1. ***Arrera* (defatted buttermilk)**

*Arrera* is another by-product of *ergo* obtained after churning and removal of *qibe*. It has a thin consistency and basically contains the casein portion of milk. Its taste and odor are similar to those of *ergo*. There is possibility to use as a source of cream production. It is either consumed in that form or cooked to produce *ayib*. In contrast to other traditional dairy products, *arrera* has fewer calories. It contains 91.5% moisture, 3.1% protein, 1.4% fat, 3.4% carbohydrate, and 0.6% ash. A hundred grams of *arrera* give 95 mg calcium, 84 mg phosphorus, 1.0 mg iron, 0.03 mg thiamine, 0.21 mg riboflavin and 0.10 mg niacin.

1. ***Ayib* (cottage cheese)**

*Ayib* is a traditional Ethiopian cottage cheese made from sour milk after the fat is removed by churning. It is an acidic product. *Ayib* is an important source of nutrients and serves as a staple diet. It may be consumed fresh as side dish, or it may be spiced with hot spices, salt and other herbs. Raw milk is collected in a clay pot and kept in a warm place (about 30oC) for 24 to 48 hours to sour spontaneously. The pH of sour milk is usually about 4. Churning of sour milk is carried out by slowly shaking the contents of the pot until the fat is separated. The fat is then removed and the defatted milk is heated to about 50oC until a distinct curd mass forms and floats over the whey. The whey is traditionally known as *aguat*. Temperature, however, can be varied between 40oC and 70oC without markedly affecting product composition and yield. After gradual cooling, the curd is recovered from the whey. *Ayib* comprises 79% water, 14.7% protein, 1.8% fat, 0.9% ash and 3.6% soluble milk constituents and the yield should be at least 1 kg of *ayib* from 8 liters of milk.

The most common microorganisms in ayib include *Microbacterium,* *Brevibacterium*, *Pseudomonas*, *Lactobacillus fermenti* and *Lactobacillus plantarum*;andthe yeast *Kluyveromyces lactis*, *Kluyveromyces bulgaricus* and *Candida pseudotropicalis* that could ferment lactose.

Heat precipitation of curd at 70oC (pH 4.0) was recommended as it resulted in a less contaminated and more wholesome *ayib*.

**Milk chemistry**

Milk is a very complex fluid. Most of the mass of milk is an aqueous solution of lactose (main carbohydrate source in milk), lipids, milk fat, milk protein, organic and inorganic salts, vitamins, and other small molecules. In this aqueous solution, some dispersed proteins at the molecular level (whey proteins), others as large colloidal aggregates (the caseins), and lipids, which exist in an emulsified state as globules are present.

The proteins of milk belong to two main categories that can be separated based on their solubility at pH 4.6 at 20 0C. Milk proteins insoluble under these conditions are known as **casein proteins**. The proteins soluble under these conditions are known as **serum** or **whey proteins**. Casein proteins consist of four types with substantially different properties: αs1-casein, αs2- casein, β-casein and *K*-casein.

**There are 3 types of cheese based on the manufacturing process:**

* 1. **Rennet Curd Cheeses**

Rennet curd cheeses are those in which the coagulum is formed by activity of coagulants, which are mixture of enzymes called **rennets** and they have proteolytic activity. Chymosin (also known as rennin) is the protein that is active enzyme in rennet used in cheese making. Chymosin is the preferred coagulant, because it has specificity toward one peptide bond in *K*-casein. Caseins exist in complexes of discretely arranged molecules called **micelles**. At the normal pH of milk (6.6–6.7), micelles carry a net negative charge because of the non-protonated amino acid, carboxyl and phosphate groups on caseins. Through electrostatic repulsion and stearic hindrance via the hairs of *K*-casein, micelles are stable (show no tendency to flocculate or gel) and remain as individual entities. Activity of the coagulant (enzyme) removes the protruding and hydrophilic region on the *K*-casein molecule. This eliminates stearic hindrance and reduces the negative charge at the micelles surface. With loss of these properties, micelles begin to come together (clot formation).

* 1. **Acid Curd Cheeses**

Acid curd cheeses do not rely on activity of coagulant enzymes to clot milk. Instead, milk is acidified by direct addition of acid or through lactic acid produced by defined starter culture of lactic acid bacteria added in the milk. At a pH of approximately 5.2, caseins in milk begin to flocculate and eventually gel as the pH decreases. Gelation is the consequence of acidification-induced physicochemical changes to caseins. At neutral pH, casein micelles remain as individual entities and are unable to interact or form aggregates. This is, in part, caused by charge repulsion (micelles are negatively charged). In addition, hydrophilic regions of K-casein molecules protrude from the micelle core and prevent hydrophobic cores of adjacent micelles from interacting (stearic repulsion).

As the pH is lowered (below 5.2), the calcium-phosphate complex disintegrates and some casein molecules dissociate from micelles. There is also a reduction of the net negative charge on casein molecules, an increase in hydrophobic interactions, and it is thought that the protruding portion of casein molecules falls back onto the casein micelle core. The net result is that micelles and solubilized casein molecules begin to form aggregates, eventually leading to formation of a continuous network of aggregates and visible gel.

* 1. **Acid-Heat Coagulated Cheese**

The manufacturing of acid-heat coagulated cheeses is by heating milk at 78–80°C and then acidifies milk by direct addition of citric, acetic, or lactic acid to the desired pH (5.8–5.9 for ricotta, 5.2–5.3 for queso blanco). Milk can also be first acidified by direct addition of citric, acetic, or lactic acid to the desired pH and then heated. Thus, heating of the milk causes coagulation and flocculation of caseins and whey proteins.

**2.4.2. Traditional Fermented Plant Foods**

* + 1. ***Enjerra* fermentation**

*Enjerra* is fermented, sour leavened pancake-like bread made from *teff* (*Eragrostis tef*), wheat, barley, sorghum or maize or a combination of some of these cereals. *Enjerra* can be produced from any of the various cereals depending on availability and abundance of the cereals, which are cultivated in the agro-ecological zones suitable for their growth. Generally, people on the highlands prepare *enjerra* from barley and wheat whereas those on the lowlands prepare it from maize, sorghum or millet. Wherever the soil type and rainfall patterns are suitable for cultivation of *teff*, *enjerra* madefrom *teff* is more favored than from the other cereals. *Enjerra* made form *teff* is the most common and the main staple food in much of the central and northern highlands of Ethiopia as well as among the urban community.

The various *enjerra* types produced from the different varieties of cereals do not have significant variation in their calorie, moisture, protein, carbohydrate and phosphorus contents. Significant variations are, however, observed in the other nutrients such as minerals. The fermentation process results in significant reduction of most of the nutrients found in the cereal flour. However, in general, *enjerra* can be considered as good sources of energy, fiber, iron and vitamins.

The preparation of teff *enjerra* consists of two stages of natural fermentation, which last for about 24 to 72 hours, depending on ambient temperatures. Temperature in the highlands of Ethiopia is generally between 17 and 25oC. The only required ingredients are the teff flour and water. An appropriate amount of flour is mixed with twice its weight of water. This is kneaded thoroughly to produce a thick paste. Inoculation is accomplished by consistently using partially cleaned fermentation container and by adding some *ersho*, the clear, yellow liquid that accumulates on the surface of the batter towards the final stage of a previous fermentation.

The fermentation processes is as follows. The initial 18 hours are characterized by vigorous evolution of gas and maximum dough-rising. This is followed by the appearance of an acidic yellowish liquid on the surface of the dough at about 30-33 hours of fermentation. Gas evolution decreases after the pH has fallen below 5.8 (31 hours). The liquid layer is discarded at the end of the first stage of fermentation. As soon as the liquid layer is poured off, about 10% of the fermenting dough is mixed with three parts of water and boiled for 2 to 5 minutes. This is called ‘absit’, a dough enhancer, and it is mixed with the rest in the fermentation vat. This process signals the initiation of the second stage of fermentation. By mixing the boiled dough with the rest in the vat, the dough-rising and gas formation processes are enhanced so they occur in a short time. Maximum dough-rising, which normally takes 30 minutes to 2 hours, signals the termination of fermentation. At this stage the fermenting dough is thin enough to pour onto the hot flat pan, locally known as ‘mitad’ for steam-baking into *enjerra*. The *mitad* pan is made of clay and has a diameter of 45-60 cm. Baking is preceded by cleaning the heated pan with piece of cloth after greasing the pan with kale and cotton seeds. Pouring of dough starts from the outer part of the pan to the center moving clockwise direction. Bubbles start forming within seconds and the pan is then covered with a lid traditionally called ‘akabalo’. The total baking time for one *enjerra* is 2- 3 minutes. The temperature in the middle of the *enjerra* during the baking process would reach around 90oC. The *enjerra* is removed from the baking pan with the help of a straw plate and allowed to cool down. The weight of one teff *enjerra* is 350-450 g. *Enjerra* can be kept for 3 to 4 days. Longer keeping results in drying and moldiness.

*Absit* ensures that *enjerra* will have the proper texture and consistency. *Enjerra* baked without ‘absit’ or with less ‘absit’ than the required will have fewer amounts of eyes on the upper surface. A higher number of larger eyes are a very desirable attribute of an attractive *enjerra*. It also tends to be brittle after few hours of baking. Too much ‘absit’ makes baking difficult. *Enjerra* baked at 24 hours or less is called ‘aflegna enjerra’ and has sweet taste. It is recommended for people suffering from gastritis and, thus, do not tolerate acidic foods. A major source of inoculum for teff fermentation is the teff flour itself. The traditional threshing processes of teff would result in the contamination of the teff seeds with a wide variety of microorganisms of soil origin.

*Ersho* is supposed to be a starter for teff fermentation. A study on the microbial flora and chemical properties of *ersho* showed that *ersho* had a pH of 3.5 and titratable acidity of 4.46%. It, thus, does not support the survival of various groups of microorganisms. The mean aerobic mesophilic bacterial count of *ersho* is around 106 cfu/ml and this consisted of only *Bacillus* spores. Yeast counts ranged between 105 and 106 cfu/ml and *Candida milleri*, *Rhodotorula mucilaginosa*, *Kluyveromyces marxianus* and *Pichia naganishii* were the major yeast species. *C*. *milleri* was found in over 80% of the *ersho* samples from every household. *R*. *mucilaginosa*, the second most abundant, was encountered only in <40% of the samples. Only *Candida* and *Kluyveromyces* species were active gas producers from glucose, sucrose and a variety of other sugars.

Removal of the liquid layer (*ersho*) at the end of the primary fermentation removes soluble compounds. About 4-13% of teff nitrogen was lost depending on the stage of fermentation and thus this could be avoided by stopping the fermentation process before the liquid/solid separation.

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 **Fig.2.3:** Flow chart of *enjerra* production

**B) *Kotcho* Fermentation**

Almost 10 million people in Ethiopia are dependent on enset (*Ensete ventricosum*), also known as 'false banana. *Enset* is the main source of food in the densely populated areas of central and southwestern Ethiopia.

The job starts with the digging of a pit around the homestead. The inner part of the pit is lined with enset leaves to collect and prevent the juicy part from leaking into the ground. Pseudostem, removed from the enset plant, is tightly secured on a wooden pole and, using a bamboo scraper, the woman scrapes the fleshy part of the pseudostem down towards the pit. After a short while the juicy part sediments into a moist sticky product known as *bulla*. The clear solution over the *bulla* sediment is discarded. The remaining thick white *bulla* is thinly spread for dehydration, and a handful is then wrapped in fresh enset leaves, tightly tied with fiber and allowed to ferment. The fermenting *bulla* is protected from air and light to avoid undesirable color change, as white *bulla* has a higher market value. A serrated animal shoulder bone is usually used to pulverize the corm (root part) of the plant. The grated pieces of the corm are mixed with the fleshy scrapped pseudostem and buried in the pit. A pre-fermented *kotcho* is used as a starter to initiate the fermentation. Four to eight mature enset plants are required to obtain sufficient *kotcho* to fill a pit. The *kotcho* is then pressed by hands or feet, covered with fresh enset leaves, and layered with discarded enset parts. Heavy materials such as large stones are put on top of the layering to ensure the creation of airtight conditions in the pit.

The length of fermentation time varies from a few weeks, to several months or years depending on ambient temperatures of incubation. In the cooler regions, it is kept in a pit for years and the quality is said to increase with increasing fermentation time. In warmer regions, fermentation is rapid and is therefore terminated within 3 to 6 months. After the fermentation is completed, a portion is removed from the pit and the liquid is squeezed out of it resulting into a moist fibrous *kotcho*. This is kneaded and shredded. Final sifting removes the remaining fibers and gives a finely powdered substance, the *kotcho* powder.

The content of the various nutrients decrease during fermentation, possibly due to excessive leaching following the peak of microbial activity. The *kotcho* protein is generally higher in lysine than most cereals. Enset products contain more calcium than most cereals, tuber and root crops. This may be the reason why enset products are used for healing fractured bones.

 The fermented products of the enset plant are used to make different dishes such as thin, unleavened *kotcho* bread (simply called *kotcho*), bulla porridge (*genfo*), thick cooked bulla gruel (*atmit*), and a shredded flake made of a mixture of *kotcho* and *bulla* (*firfir*). The fermented products of the enset plant are basically energy foods and are often blamed for causing protein deficiency disease when eaten alone as staple. However, it should be noted that dishes made of these products are traditionally served with other protein and vitamin sources. The various *kotcho* and *bulla* dishes are supplemented with milk, *qibe* (traditional butter), *ayib* (traditional cottage cheese), meat, beans separately or in combination. Unfermented, fermenting or completely fermented *kotcho* is baked or cooked and eaten alone or in combination with various indigenous foods.

*Leuconostoc mesenteroides* initiate the fermentation of *kotcho* and dominated the lactic flora with counts of 107 cfu/g on day 8. The pH of the fermenting mass drop from 6.5 to 5.6 in 8 days. *Lactobacillus coryneformis* and *Lactobacillus plantarum* dominate thereafter and further reduce the pH to 4.2 after 50 days. Spore formers are present at levels of <103 cfu/g during the first 15 days. Generally the population of *Clostridium* spp. is more abundant than *Bacillus* spp. *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium sticklandi*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus licheniformis* and *Bacillus cereus* are among the spore-formers which appeared to show active growth in fermenting *kotcho*. Yeasts reach highest counts (103 cfu/g) between 22 and 43 days and the yeast flora consists of the *Trichosporon*, *Torulopsis*, *Rhodotorula* and *Candida*.

### 2.4.3. Traditional Fermented Condiments

1. ***Siljo* Fermentation**

The majority of traditional fermentations are accompanied by certain biochemical changes of nutritional importance. Some fermented foods produce strong flavor such that the product is not consumed alone, but is added as a condiment to make the food more tasty and enjoyable. A typical example of legume fermentation practiced in Ethiopia is *siljo* fermentation. *Siljo* is a fermented product made from safflower (*Carthamus tinctorius*) extract and faba bean (*Vicia faba*) flour. It is a popular condiment during the long fasting period before Easter. Faba bean flour is thoroughly mixed with safflower extracts and cooked well to a gruel consistency. This is cooled to about 55oC and black mustard powder, homogenized in warm water, is added to it. After a thorough mixing, the gruel is left to ferment at ambient temperature. At around 48 hours of fermentation, peeled garlic, rue leaves and other spices are added to it. Its fermentation is spontaneous and the product is usually ready for consumption after three to five days of fermentation. The fermented product is a gray gruel with a typical acidic and mustard flavor. It is consumed as a side dish to any one of the major legume-based sauces with *enjerra*. *Siljo* is believed to add some variety to the monotonous fasting dishes of the average highland Ethiopian. It is a household product, not produced in large amounts and whatever is produced is usually consumed within a few days.

Since the major substrates are thoroughly cooked during initial preparation, the black mustard powder will be the source of starter microorganisms. It contains *Lactobacillus acidophilus*, *L*. *plantarum* and *L*. *delbruekii* and the yeasts *Saccharomyces cerevisiae*, *Rhodotorula glutinis*, *Yarrowia lipolytica* and *Saccharomyces rouxii*. The fermentation is, however, initiated and later dominated by *L*. *plantarum* and *L*. *acidophilus*.

The pH of the fermenting mass drops to 4.5 within 36 hours and reach 4.0 at day 7. Aerobic mesophilic and lactic acid bacteria are present at levels as high as 1010 cfu/ml after 36 hours of fermentation, but Enterobacteriaceae are not detected. *Micrococcus, Bacillus* and *Lactobacillus* species dominate the flora. Enterococci decrease in number while lactococci, lactobacilli and yeasts increase during the fermentation period. The pH fall from an initial value of 6.1 to 4.2 at 96 h and the initial and final values of titratable acidity are 0.36 and 0.75, respectively.

The moisture content of *siljo* is about 86%. There is a slight increase in crude protein, crude fat and ash during the fermentation, with final values of around 28%, 25% and 7%, respectively. There is marked increase in protein availability and concentration during the fermentation.



 **Fig. 2.4:** Flow chart of *siljo* fermentation

1. ***Awaze***

*Awaze* is a traditional fermented condiment and is consumed with other items on the basis of its desirable aroma and flavor. It is the product of the microbial fermentation of vegetable-spice mixtures. *Awaze* is common in the north and central Ethiopia and is often used to flavor sliced raw or roasted meat and other traditional pancakes. The major ingredient for *awaze* preparation is red sweet pepper (*Capsicum annum)*. The spices added to it include garlic (*Allium sativum*), ginger (*Zingiber officinale*), sweet basil (*Ocimum sanctum*), rue (*Ruta chalepensis*), cinnamon *(Cinamommum zylanicum*), clove (*Eugenia caryophyla*), Ethiopian caraway (*Trachyspermum copticum*), Ethiopian cardamom (*Aframomum anguistifolium*), and salt.

The preparation of the ingredients is as follows. All the seeds of the sweet pepper are first discarded and the fruits are thoroughly washed with tap water and sun-dried. The dried seedless fruits are gently pulverized with a wooden mortar and pestle. Fresh ginger and garlic are peeled, washed and mixed together with small proportions of fresh sweet basil and seeds of rue. These are then mixed with the already dried and pulverized sweet pepper and kneaded together. The whole kneaded mixture is left in a container overnight to form a moist solid mash. The mash is sun-dried by thinly spreading on a clean surface. Small proportions of the dry spices including clove, cinnamon, Ethiopian caraway, Ethiopian cardamom and sweet basil together with certain amount of salt are gently heat-treated separately on a hot metal pan. The heat-treated spice mixture is mixed with the kneaded and dried pepper-spice mixture and is dry-milled. This is finally sieved through a fine wire mesh. The fermentation of *awaze* starts by whipping a portion of the ground pepper-spice ingredient with warm water until it acquires a thick consistency. This is then left to ferment at ambient temperatures.

Ingredients for *awaze* preparation had a microbial load of 106 cfu/g and the flora is dominated by *Bacillus* spp. The count of aerobic mesophilic bacteria decrease during the fermentation period. Yeasts also reach counts of 106 cfu/g at the end of fermentation on day 12. A steady decline in pH is observed in the course of the fermentation and the major drop in pH is noted between 24 and 48 hours. This is also accompanied by increase in titratable acidity. At day 14, pH was around 3.7 and titratable acidity was 0.38%. *Awaze* can be consumed at early or later stages of fermentation. Inoculation of *Salmonella typhimurium* in fermenting *awaze* resulted in a fast elimination of the pathogen. The fermented product also inhibited survival of *E*. *coli* O157:H7 when stored at ambient temperature, whereas cold storage extended the survival to over 7 days.

1. ***Datta* (*Qotchqotcha*)**

*Datta* (also known as *qotchqotcha*) is a condiment of similar use as that of *awaze* mainly in the southern part of the country. The major substrate in the making of *datta* is the small chili pepper (*Capsicum frutescence*) at its green stage. The green pepper, together with the seeds, is thoroughly washed and cut into pieces. Garlic and ginger, in small proportions, are peeled, washed and cut into small pieces. The pepper, garlic and ginger are mixed with small amounts of fresh sweet basil and seeds of rue. The mixed ingredients are manually wet-milled on a flat traditional stone-mill into a greenish paste. This is transferred into a container, tightly closed and left to ferment at ambient temperatures.

In *datta* fermentation, the counts of aerobic mesophilic bacteria remain unchanged during the fermentation. Lactic acid bacteria initiate the fermentation at a level of 104 cfu/g and reach 109 cfu/g at end of fermentation on day 7. Contrary to *awaze* fermentation, the homofermentative lactic acid bacteria initiate and dominate *datta* fermentation for the first two days. The heterolactics dominate thereafter.

*Datta* fermenting condiments had strong bactericidal property against *Salmonella typhimurium*. The fermenting product, when you store at ambient temperature, also has a fast inhibitory property against *E*. *coli* O157:H7, although the pathogen survive for more than seven days at refrigeration storage. In general, the fermentation of *awaze* and *datta* are accompanied by declining pH and increasing titratable acidity.

**2.5. Food spoilage (fungal and bacterial)**

* Food spoilage is any undesirable change in food, which renders food unacceptable for human consumption.
* Spoiled foods are considered as unsafe because high number of foodborne pathogens may present.

**Main causes of food spoilage**

* Food spoilage may occur due to:
1. Physical changes
* Water activity, relative humidity, temperature, light, etc.
* Caused by the inappropriate transport, handling and storage.
* Mechanical damage or physical injury due to freezing, drying, burning, pressure and radiation.
1. Chemical and biochemical factors
* Non-microbial or non-enzymatic changes usually involving oxygen oxidation processes (e.g., rancidity of fats and oils). **Rancidity** is the chemical decomposition of fat and oil, and other lipids.
* e.g. oxidative rancidity of fats in meat leads to discoloration of meat.
* Any chemical changes/interactions not induced by microbial or naturally occurring enzymes.
* Activity of indigenous enzymes in plant and animal tissues (e.g food obtain from vegetable or animal origin).
1. Biological factors
* Microbiological
* Growth and activity of microorganisms such as bacteria, yeasts and molds (most common).
* Macrobiological
* Damage of food by rodents, insects, birds, etc.
* **The main sources of microorganisms in food spoilage include:**
	+ - Soil, water, air, plant and plant products.
		- Food utensils and equipments.
		- Animal hides and feeds.
		- Intestinal tract of humans and animals.
		- Hands of food handlers.
* **The main steps of food spoilage by microbes:**
* Microbes are introduced to food.
* Food is favorable for growth of microbes.
* Food is stored at a temperature that favors for the growth of the introduced microbes.
* Enough time to grow.
* Most food spoilages are caused by bacteria, molds and yeasts.
* Microbial spoilage of food is evidenced by changes in:
	+ - The appearance (color changes, formation of pockets of gas/ swelling).
		- Texture (soft & mushy), odour, change in flavor or slime formation.
* Thus, knowledge about the source of spoilage microorganisms in food helps to protect food from spoilage.
* The major problems of food spoilage are: economic loss, wastage of food and public health problems.
* The rate at which an organism is able to multiply in a food determines whether it will achieve dominance or not.
* Thus, the fastest growing organisms having the greatest opportunity.
* For example, if bacteria, yeasts and moulds are present in a food which is capable of supporting the growth of all the three organisms.
* It is most likely that the bacteria will become dominant first because they are fast grower. Mould or yeast spoilage may occur at a later stage if the conditions in the food at that time permit for growing.

**Classification of food spoilage:**

* **Highly perishable**: Meat, fish, poultry, egg, milk, most fruit and vegetables.
* **Semi-perishable**: Potatoes, nuts, apples.
* **Stable (non-perishable)**: Sugar, flour, rice, dry beans.

**2.5.1. Food spoilage by fungi (moulds and yeasts)**

**Moulds**

The growth of moulds is initiated when a mature spore is able to germinate and start mycelium growth. This leads to food spoilage. The spoiled food becomes colored, musty, softer and sticky or slimy. Moulds are aerobic and spoilage generally begins at the surface, although the mycelium later penetrates deep into the food. Moulds are often associated with the spoilage of 'dry' foods and those foods containing high concentrations of sugar or salt.

* **Moulds important in food spoilage:**
1. **Non-septate moulds reproduce by asexual and sexual**
	* + 1. Genus *Rhizopus*:
* Bread moulds, soft rots in fruits and vegetables, spoilage of chilled meat.
	+ - 1. Genus *Mucor*:
* Spoil wide range of food items
1. **Septate moulds**
2. **Genus *Aspergillus***:

 E.g1. *Aspergillus glaucus*

* Grow in food with low water activity.
* Spoilage of dry foods and foods preserved by sugar and salt.

 E.g2. *Aspergillus niger*

* Spoilage of bread, black rots of fruits and vegetables.
1. **Genus *Penicillium*:**
* Spoilage: soft rots in fruits, 'blue rot'; greenish patches on stored meat, yellow or green spots in eggs, greenish spoilage of cheddar and other cheese and bread.
1. **Genus *Alternaria*:**
* Spoilage: fruit and vegetables.
1. **Genus *Fusarium*:**
* Spoilage: rot fruit and vegetables; cause discoloration in butter.
1. **Genus *Sporotrichum*:**
* Spoil foods with high water activity
* Spoil stored chilled meats

 **Yeasts**

* Yeasts can grow in food with low pH and high sugar concentration, both under aerobic as well as anaerobic conditions. Osmophilic yeasts tolerate conditions of low water activity and are associated with the spoilage of dried fruits, honey and concentrated fruit juices.
* Yeasts can also grow in buttermilk and sour cream and can produce off-flavor of these products.
* **Yeasts important in food spoilage:**
1. **Genus *Saccharomyces***
* *Saccharomyces rouxii* and *Saccharomyces mellis* are fermentative and osmophilic yeasts associated with spoilage of jams, syrups, pickles, brines and alcoholic beverages.

**2. Genus *Candida***

* Composed of some acid tolerant and osmophilic yeasts.
* Are associated with spoilage of high acid foods and brines.
* Lipolytic strains also spoil fats such as butter and margarine.

 **3. Genus *Rhodotorula***

* Associated with spotting of meat and eggs.

 **4. Genus *Torulopsis***

* Some of the members of this genus are fermentative and some are salt tolerant.
* They are found to cause spoilage in brewing.

**2.5.2. Food spoilage by bacteria**

* Spoilage of food by bacteria depends on the suitability of the food items with respect to nutrient composition, availability of free water and the range of pH for their growth. Generally, bacteria cannot spoil food with very low water activity.

 **2.5.2.1. Gram positive aerobic or facultative anaerobic cocci:**

* 1. **Genus *Staphylococcus:***
* *Staphylococcus aureus*, *Staphylococcus epidermidis*.
* Salt tolerant and can grow at temperature lower than 37 OC.
* Spoil foods with relatively high osmotic potential.
	1. **Genus *Streptococcus*:**
* Salt tolerant (6.5% w/v), require complex vitamin rich food for growth.
* *Streptococcus faecalis*, *Streptococcus faecium*, *Streptococcus durans.*
* Able to grow in wide range of temperature 10–450C.
* Associated with spoilage of raw meat, fresh and pasteurized dairy products.

 **2.5.2.2. Gram-positive rods-spore forming**

1. **Genus *Bacillus*:**
* Aerobic spore formers
* Some strains cause spoilage in canned foods.
* Some saccharolytic strains cause rope, for example *Bacillus subtilis* in bread.
1. **Genus *Clostridium*:**
* Anaerobic spore formers
* The thermophilic species are importance in spoilage of foods stored at high temperatures.
* Some are proteolytic and putrefactive, for example (*Clostridium histolyticum,* *Clostridium sporogenes*).
* Some are saccharolytic, for example (*Clostridium butyricum,* *Clostridium perfringens*)*.*

**2.5.2.3. Gram-negative aerobic rods: non spore forming**

* These are psychrophilic organisms can multiply in refrigerator.
* The most common genera: *Erwina*, *Acetobacter*, *Alcaligenes*, *Pseudomonas.*
1. **Genus *Pseudomonas*:**
* Prefer foods with high water activity.
* Many of them are psychotrophs, because they can grow at low temperature (3–70C).
* They can hydrolyze and utilize proteins and lipids for growth.
* Spoilage of fish, poultry, meat, eggs and dairy products.
1. **Genus *Acetobacter*:**
* Oxidize ethyl alcohol to acetic acid.
* Spoilage of alcoholic beverages.
1. **Genus *Erwina*:**
* Causes soft rot in carrots and cucumber.

**2.5.2.4. Gram-negative facultative anaerobic rods**

* 1. **Genus *Escherichia*:**
* Their presence in food can indicate fecal contamination.
* Some species spoil food, fermenting the carbohydrate to acid and gas, and also causing odour.

 **2) Genus *Shigella* and genus *Salmonella*:**

* Pathogenic organisms which may be carried by foods.
* Spoil dairy products.

**2.6. Foods preservation**

* + - Preservation is a process in which foods are prevented from getting spoilage. It extends the shelf life of food by slowing down or stopping growth of microorganisms or by killing them. Preservation can be short-term (for a few days) or long-term (for months or years). In most cases, the colour, taste and nutritive value of the foods are reserved. However, some methods of preservation affect the **structure**, **taste**, **appearance** and **flavour** of the food.
		- Fermentation is enzymatically controlled transformation of organic compounds into fermented products in the absence of oxygen. In most cases, fermentation leads to the food preservation duet o the production of acid, which inhibits most food spoilage and pathogenic microorganisms.
			* Food spoilage can be controlled by controlling the intrinsic and extrinsic factors.
			* The objective of food preservation is to slow down the growth of disease causing and spoilage microorganisms or to kill them at all.
			* This can be done by using sterilization methods, canning, drying, smoking, refrigeration, adding salt or sugar, radiation, high heat processing and chemical preservatives.
		- Removing leftover food on time from work surfaces and utensils in food preparation area.
		- By cooking or applying proper and desired processing treatment to the food before consumption.
		- By avoiding chances of re-contamination of food, hence storing it in proper package.
		- Maintaining good personal hygiene, like food handlers should not have any open cuts and wounds.
		- Maintaining proper sanitation and preventing contamination of water to be used.
		- Use of proper storage condition restricting recontamination and growth.
		- It is vital to eliminate or reduce the populations of spoilage and disease-causing microorganisms.
		- It is essential to maintain the microbiological quality of food with proper storage and packaging.
		- Contamination often occurs after a package or can is opened and/or during handling just before the food is served. This can provide an ideal opportunity for growth and transmission of pathogens, if care is not taken.
		- Generally, food preservation is the mechanisms by which inherent parameters of foods are modified in order to kill or inactivate spoilage microorganisms by modifying the extrinsic factors.
		- Foods can be preserved by a variety of methods. Some of the food preservation mechanisms are removal of microorganisms from foods, use of high and low temperature, reduction of water availability, use of chemical preservatives and radiation, etc.

**2.6.1. Removal of microorganisms from food**

* Microorganisms can be removed from water, wine, beer, juices, soft drinks, and other liquids using filtration and centrifugation methods.
* This can keep bacterial populations to low or eliminate them entirely. Prefilters and centrifugation often are used to maximize filter life and effectiveness.
* Several major brands of beer are filtered rather than pasteurized to better preserve the flavor and aroma of the original products.

**2.6.2. Low temperature storage**

* The use of low temperature in preserving food based on slowing down activities of microorganisms.
* Refrigeration of foods at 4°C retards most microbial growth, but it does not stop all microbial growth. Because, psychrophilic microbes can still grow and cause spoilage.
* Some microorganisms are very sensitive to cold and their numbers will be reduced, but cold does not lead to significant decreases in overall microbial populations.
* Bacteria differ in their capacity to survive during freezing, with gram-positive cocci being generally more resistant than gram-negative rods.
* Of the food-poisoning bacteria, *Salmonella* are less resistant than *Staphylococcus aureus* or vegetative cells of *Clostridium*, whereas endospores and food-poisoning toxins are apparently unaffected by low temperatures.

**2.6.3. Use of high temperature**

* Controlling microbial populations in foods by means of high temperatures can significantly limit the growth of pathogenic and spoilage microorganisms.This help to reduce disease transmission.
* The use of high temperature preserves foods based on destructive effect of temperature on microbes.
* Partial or complete heat inactivation of microorganisms by using canning, pasteurization and commercial sterilization.

**Canning**

* Heating processes provide a safe means of preserving foods, particularly when carried out in commercial **canning** operations.
* It involves heating and sealing under a vacuum to kill microorganisms.
* Canning is two steps process:
* First the food is prepared by being packed into containers, which are then sealed or airtight.
* Then, the containers are heated.
* Two methods of canning/heating:
* Water boiling canning – used for high-acid foods (pH ≤ 4.6).
* Pressure canning – used for low-acid foods (pH > 4.6).
* The precise time and temperature depend on the nature of the food and type of microbes. The canning process kills spoilage microbes, but not necessarily all microbes in food. Because, *Clostridium botulinum* forms spores that require higher temperature for destruction in a reasonable period of time.
* After heat treatment the cans are cooled as rapidly as possible, usually with cold water.
* In general, heat resistance of microorganisms is related to their optimum growth temperatures. Psychrophilic microorganisms are the most heat sensitive, followed by mesophiles and thermophiles.
* Spore-former bacteria are more heat resistant than non-sporeformers, and thermophilic sporeformers are, in general, more heat resistant than mesophilic sporeformers.
* With respect to Gram reaction, Gram-positive bacteria tend to be more heat resistant than Gram-negative bacteria.
* With respect to morphology, cocci, in general, being more resistant than rods.
* Yeasts and molds tend to be fairly sensitive to heat, with yeast ascospores being only slightly more resistant than vegetative yeasts.
* Death of microorganisms occurs due to the damages of some vital functional and structural components. For instance, denaturation of some important enzymes and cell membrane leads to death.
* Bacterial spores, following heating, were found to lose structural components from the spore coat, and develop an inability to use water for hydration during germination. Death results from the inability of a spore either to germinate or to outgrow.
* Despite efforts to eliminate spoilage microorganisms during canning, sometimes canned foods are spoiled. This may be due to spoilage before canning, under processing during canning and leakage of contaminated water through can seams during cooling process.
* Spoiled food can be altered in such characteristics as color, texture, odour, and taste. Organic acids, sulfides, and gases (particularly CO2 and H2S) may be produced.

**Pasteurization**

* It involves heating process of liquid foods at different temperatures for different time in order to kill target pathogens expected in foods.
* The main objective of pasteurization is to destroy all the vegetative cells (non-spore formers) of pathogens and to reduce the levels of spoilage microorganisms (yeasts, molds, bacteria).
* The process of pasteurization is based on food safety and not only on food preservation alone. This means, safety first (elimination of pathogens) and spoilage (extension of shelf life) second.
* The most common pasteurization food is milk. Thus, pasteurization originally is designed to eliminate *Mycobacterium tuberculosis* from milk. However, some brands of beer are pasteurized in addition to filtration.
* The temperature and time are set to the lowest level to meet the microbiological objectives and to minimize thermal damage of the food, which otherwise could reduce the acceptance quality (such as heated flavor in milk) or pose processing difficulties (such as coagulation of liquid egg).
* Depending on the temperature used; thermoduric cells of spoilage bacteria and spores of pathogenic and spoilage bacteria survive the treatment. Thus, additional methods need to be used to control the growth of survivors (as well as post pasteurization contaminants) of pasteurized products, unless a product has a natural safety factor (e.g., low pH in some acid products).
* Effectiveness of pasteurization depends on the combination of time and temperature.
* Pasteurization of milk at:
* 630C → 30min → Low Temperature Long Time (LTLT)
* 720C → 15sec → High Temperature Short Time (HTST)
* 890C → 1sec
* 900C → 0.5sec
* 940C → 0.1sec
* 1000C → 0.01sec

**Commercial sterilization**

* The elimination of all microorganisms through extended boiling/ heating to temperatures much higher than boiling.
* The primary objective of commercial sterilization is to destroy the most heat resistance (spore-forming) pathogenic microorganisms. For instance, *Clostridium botulinum.*
* The secondary objective is to destroy vegetative and spore-forming microorganisms that cause spoilage.
* Spoilage spore-formers are usually more heat resistant than pathogenic spore-formers.
* Any product treated by commercial sterilization is free of vegetative and spore-forming pathogenic, and spoilage microorganisms unless it is re-contaminated.
* The disadvantage of commercial sterilization is, it may affect the colour**,** structure, taste, appearance, flavour and nutritive value of the food.

**2.6.4. Reduction of water activity**

* Microorganisms need water for transport of nutrients, nutrient metabolism, and removal of cellular wastes.
* In a food, the total water is present as free water, or bound water or frozen state.
* The bound water remains bound to hydrophilic colloids and solutes. It is held tightly with other molecules and hard to remove from foods. It is not available for biological functions.
* The frozen state which is present as ice crystal is not also available for biological functions.
* Thus, only the free water which is related to **aw** is important for microbial growth.
* Water activity can be reduced through dehydration, such as drying, freeze-drying (lyophilization) to produce dried foods.
* Drying is the process of removing water from food which inhibits the growth of microorganisms but does not kill microbes. It is the oldest known method of food preservation.
* Water activity can be also reduced by addition of high concentration of solutes such as sugars and salts. This is due to the osmotic pressure.
* It is a common means of eliminating microbial growth.
* The modern process is simply an update of older procedures in which grains, meats, fish, and fruits were dried.
* Lyophilization (freeze-drying) is the process of removing of moisture from food using vacuum space. It is a kind of preservation technology, by which the material cooled at low temperature firstly to be solidified completely, and then dried in vacuum space at low temperature by sublimation to remove moisture. It has become a most important technique for the preservation of heat sensitive pharmaceuticals and foods.

**2.6.5. Food preservation using chemicals**

* Various chemical substances can be used to preserve foods, and these substances are closely regulated by the United State, Food and Drug Administration (FDA) as well as by the WHO.
* Thus, due to restrict rules only few chemicals are used in food preservation, and these are considered as “generally recognized as safe” (**GRAS).**
* They include acetic acid, citric acid, lactic acid, propionic acid, sorbic acid, benzoic acid, sulfur dioxide, sulfite, parabens, nitrate and sodium nitrite at the specified concentration.
* These chemical agents affect the growth of microorganisms by disrupting a critical cell factors. For example, they may damage the plasma membrane or denature various cell proteins. They may interfere with the functioning of nucleic acids, thus inhibiting cell reproduction. The effectiveness of many of these chemical preservatives depends on the food pH.
* Generally, chemical preservatives are used when other control measures are lacking, because they may damage the products.

**Benzoates**

* This includes:
	+ Benzoic acid → C6H5COOH
	+ Sodium benzoate → C7H5NaO2
* Sodium benzoate was the first chemical preservative permitted in foods by the FDA, and it continues in wide use today in a large number of foods.
* The antimicrobial activity of benzoic acid is effective in the undissociated form.
* The antimicrobial activity (effect) of benzoate is related to pH values of foods, the greatest activity being at low pH values of foods.
* Thus, most benzoates are active at low pH values of foods and essentially ineffective at neutral values. Benzoate at pH 4.0, 60% of the compound is undissociated, whereas at pH 6.0, only 1.5% is undissociated.
* This restricts the use of benzoic acid and its sodium salts to high-acid products such as apple cider, soft drinks, tomato catsup, and salad dressings. In fact, high acidity of these foods is generally sufficient to prevent growth of bacteria but not that of certain molds and yeasts. As used in acidic foods, benzoate acts as an essential inhibitor for mold and yeast, although it is effective against bacteria.
* Used as fungistatic.

**Parabens**

* They are esters of para-hydroxylbenzoic acid.
* Most commonly used are heptyl, methyl and propyl parabens.
* Used in wide range of pH (up to pH 8.0).
* Mostly used as mold inhibitor, than yeast.

**Sorbate**s

* Sorbic acid → C5H7COOH
* Sorbic acid and its Ca+, Na+, or K+ salts are employed as food preservative.
* Sorbate at pH 4.0, 86% of the compound is undissociated, whereas at pH 6.0, only 2% is undissociated.
* Effective in acidic foods like poultry meat, fresh fish, perishable fruits, cheese, salad dressing, beverages, fruit juices and bakery.
* Like benzoates, sorbates are also effective against →molds and yeasts.
* Used as fungistatic.

 **Propionates**

* + Propionic acid→ C2H4COOH
* Propionic acid and its Ca+, Na+, or K+ salts are used as food preservative.
* Propionate at pH 4.0, 88% of the compound is undissociated, whereas at pH 6.0, only 6.7% is undissociated.
* Effective in low acid foods like breads, cakes and certain cheeses→ primarily used as mold inhibitor.
* Used as fungistatic.

**Sulfur dioxide and sulfites**

* Used as preservative and antioxidant.
* Antioxidant is a molecule that inhibits the oxidation of other molecules.
* React with various food constituents such as nucleotides, sugars and disulfide bonds.
* Effective as → bacteriostatic → against:
	+ - Lactic acid bacteria, *Acetobacter*, *C. botulinum* and some species of *salmonella*.

**Nitrites and nitrates**

* Sodium nitrate (NaNO3) and sodium nitrite (NaNO2) are used as curing agent for meat, because they:
	+ - Stabilize red meat color.
		- Inhibit some spoilage and food poisoning microorganisms.
		- Contribute to flavor development.
* It should be recalled that many bacteria are capable of utilizing nitrate as an electron acceptor and in the process effect its reduction to nitrite. The nitrite (NO2) is by far the more important of the two in preserved meats. Nitrite is highly reactive and is capable of serving as a reducing and an oxidizing agent.
* In acidic environment, NO2 ionizes to yield nitrous acid (3HONO), which further decomposes to yield nitric oxide (NO) and results color fixation in cured meats. Ascorbate or erythrobate acts also to reduce NO2 to NO.
* Under reducing condition, nitric oxide (NO) reacts with myoglobin to produce the desirable red pigment in meat called nitrosomyoglobin.
	+ The most important of nitrate (NO3) inhibits *C. botulinum,* but generally ineffective against **Enterobacteriaceae**, including the *Salmonella* species, and against the lactic acid bacteria.
	+ It is also effective against other clostridia, including *C. sporogenes* and *C. perfringens*.
	+ It is effective against *S. aureus* at high concentrations and, again, the effectiveness increases as the pH is lowered.
* Nitrite (NO2) inhibits *C. botulinum* by interfering with iron–sulfur enzymes such as ferredoxin and thus preventing the synthesis of adenosine triphosphate (ATP) from pyruvate.

**Hydrogen peroxide (H2O2)**

* This compound is a weak acid that is formed to some extent by all aerobic organisms, and it is enzymatically degraded by the enzyme catalase: **2H2O2** → **2H2O2- + O2.**
* The antimicrobial activity of H2O2 is used for food preservation.

**Salt (NaCl) and sugars**

* The early food uses of salt were for the purpose of preserving meats. This was based on the fact that at high concentrations, salt exerts drying effect on both food and microorganisms.
* The inhibitory effect of salt is not pH dependent as some other chemical preservatives, rather depends on the response of microorganisms’ to osmotic effect of salt concentrations.
* Microorganisms have different response to salt:
	+ - Yeasts and molds → less susceptible than → bacteria.
* Microorganisms that can grow in the presence of high concentrations of salt are referred to as halophiles; those that can withstand but not grow in high concentrations are referred to as halodurics.
* Sugars, such as sucrose, exert their preserving effect in essentially the same manner as salt. One of the main differences lies in relative to concentrations. It generally requires about six times more sucrose than NaCl to affect the same degree of inhibition. The most common uses of sugars as preserving agents are in the making of fruit preserves, candies, and condensed milk.
* Microorganisms differ in their response to concentrations of sugars:
* Yeasts and molds being less susceptible than bacteria.
* Microorganisms that are able to grow in high concentrations of sugars are called osmophiles; those that can withstand but not grow in high concentrations of sugars are referred to as osmodurics.

**2.6.6. Radiation**

* Use of non ionizing radiation (UV) and ionizing radiation such as (x-rays, gamma rays) to preserve food.
* Ultraviolet (UV) radiation is used to sterilize surfaces of food-handling equipment, but it does not penetrate foods.
* The major method used for radiation sterilization of food is gamma irradiation, because it can penetrate foods and used to extend the shelf life of meat, sea foods, vegetables and fruits. Gamma irradiation is a new method of preservation and kills microorganisms by using of magnetic rays which damage the genetic material of microorganisms. Such electromagnetic radiation has excellent penetrating power and must be used with moist foods because the radiation produces peroxides from water in the microbial cells, resulting in oxidation of sensitive cellular constituents.
* This process is known as **radappertization,** named after Nicholas Appert.
* Among the more interesting radiation-resistant bacteria that have been studied is *Deinococcus radiodurans.* This bacterium has a complex cell wall structure and tetrad-forming growth patterns. It also has an extraordinary capacity to withstand high doses of radiation, although the mechanism for its resistance is not understood.

**Mechanisms of antimicrobial action by radiation:**

* When an object (food or microorganism) is exposed to high-energy gamma-rays (10-1 to 10-2 nm), the energy is absorbed by thousands of atoms and molecules in a fraction of a second, which strips electrons from them. This produces negative and positive ion pairs. The released electrons can be highly energized and thus can remove electrons from other atoms and convert them into ions. This energization and ionization can adversely affect the normal characteristics of biological systems.
* Ionizing radiation produces both direct and indirect effects on microorganisms. The direct effect is produced from the removal of electrons from the DNA, thereby damaging them. The indirect effect is produced from the ionization of water molecules present in the cell. The hydrogen and hydroxyl radicals formed in this process are highly reactive and cause oxidation, reduction, and the breakdown of C–C bonds of other molecules, including DNA. Studies have shown that both single- and double strand breaks in DNA at the sugar–phosphate backbone can be produced by the hydroxyl radical. In addition, the radicals can change the bases, such as thymine to dihydroxy dihydrothymine. The consequence of these damages is the inability of microorganisms to replicate DNA and reproduce, resulting in death.

**2.6.7. Microbial Product–Based Inhibition**

The production of antimicrobial substances particularly by LAB, inhibit the growth of food spoilage and pathogenic microorganisms. As a result, the interest in the application of LAB and their metabolites in the prevention of food spoilage and extension of the shelf-life of foods have been increased during the last decade. Preservation of food using LAB and their antimicrobial metabolites termed as bioprotection or biopreservation to differentiate it from artificial (chemical) preservation. The main purpose of biopreservation is to extend the storage life as well as to enhance the food safety using LAB.

The antagonistic potential of LAB also involve in the production of bacteriocins which are active against food spoilage and pathogenic microorganisms. Bacteriocins are proteins secreted by certain strains of LAB. Some LAB produce bacteriocins with broad spectra of inhibition. Thus, there is increasing interest in the application of **bacteriocins** for the preservation of foods. Generally, they have the ability of inhibition or even a bacteriolytic effect on target species.

Bacteriocins have bactericidal effect against closely related bacteria, which bind to specific sites on the cell, and affect cell membrane integrity and function. The most approved and commonly used bacteriocin is **nisin**. It is nontoxic to humans and affects mainly gram-positive bacteria*.* Nisin can be used particularly in low-acid foods to improve inactivation of *Clostridium* *botulinum* during the canning process or to inhibit germination of any surviving spores.

In general, LAB and their antimicrobial metabolites have potential as natural substitute for chemical preservatives to control the growth of spoilage and pathogenic microorganisms in foods. This can be an alternative to satisfy the increasing consumers’ demands for safe, fresh-tasting, ready-to-eat, minimally-processed foods, richer in organoleptic and nutritional properties.

**Table 2.2:** Basic approaches to food preservation



**2.7. Food toxins and their sources**

* Foodborne diseases can be classified into **three** major groups namely food infection, food intoxication and food toxicoinfection.
* **Food infection :**
* It is the type of foodborne disease caused by consumption of food contaminated with pathogenic microorganisms.
* The infection is caused by the pathogenic microorganisms themselves.
* These can be fungal, bacterial and viral.
* **Food intoxication:**
* It is foodborne disease caused due to the consumption of pre-made microbial toxins in food or food containing microbial toxins.
* The infection is caused due to the toxins present in the food.
* **Food toxicoinfection**:
* It is foodborne disease caused due to the consumption of contaminated food with microorganisms where the microorganisms survive inside the host or die, and then releasing harmful toxins to produce disease symptoms.
* **Toxins** are poisonous substances that are produced by certain microorganisms. They are often the primary factor contributing to the pathogenic properties of the producing microbes.
* The ability of microorganisms to produce toxins is called **toxigenicity**.
* The term **toxemia** refers to the presence of toxins in the blood.
* Toxins that can transport by the blood or lymph can cause serious, and sometimes fatal, diseases. Some toxins produce fever, cardiovascular disturbances, diarrhea, and toxic shock syndrome.
* Toxins can also inhibit protein synthesis, destroy blood cells and blood vessels, and disrupt the nervous system by causing spasms. There are different sources of toxins. Some of them are listed below.

**2.7.1. Bacterial toxins**

* Of the 220 known bacterial toxins, nearly 40% cause disease by damaging eukaryotic cell membranes.
* Bacterial toxins are general two types, based on their position relative to the bacterial cell: **exotoxins** and **endotoxins**.
1. **Exotoxins**
* Exotoxins are produced inside some bacteria as part of their growth and metabolism and are secreted by the bacterium into the surrounding medium or released following lysis.
* Exotoxins are proteins, and many are enzymes that catalyze only specific biochemical reactions. Most exotoxins have enzymatic nature; as a result even small amounts are quite harmful because they can act over and over again.
* Exotoxins are mainly produced by gram-positive bacteria, but can be produced by gram-negative bacteria. The genes for most exotoxins are carried on bacterial plasmids or phages.
* Since exotoxins are soluble in body fluids, they can easily diffuse into the blood or lymph and are rapidly transported throughout the body.
* Exotoxins work by destroying particular parts of the host's cells or by inhibiting certain metabolic functions. They are highly specific in their effects on body tissues. Exotoxins are among the most lethal substances known. For instance, only 1mg of the botulinum exotoxins is enough to kill 1 million of mice. However, only a few bacterial species produce such potent exotoxins.
* Diseases caused by bacteria that produce exotoxins are often caused by minute amounts of exotoxins, not by the bacteria themselves. Exotoxins produce specific signs and symptoms of the disease. Thus, exotoxins are disease-specific. For example, **botulism** is usually due to the ingesting of exotoxins with food, not a bacterial infection. Likewise, staphylococcal food poisoning is intoxication*,* not a bacterial infection.
* The host body produces antibodies called **antitoxins** that provide immunity to exotoxins.
* When exotoxins are inactivated by heat or by formaldehyde, iodine, or other chemicals, they no longer cause the disease but can still stimulate the body to produce antitoxins. Thus, exotoxins that have altered by heat and chemicals are called **toxoids**. When toxoids are injected into the host body, they stimulate the immunity to produce antitoxins. As a result, toxoids are used as vaccines. For instance, diphtheria and tetanus can be prevented by toxoid vaccination.

**Naming of Exotoxins:**

* Exotoxins are named on the basis of several characteristics. One is the type of host cell that is attacked. For example, *neurotoxins* attack nerve cells, *cardiotoxins* attack heart cells, *hepatotoxins* attack liver cells, *leukotoxins* attack leukocytes, *enterotoxins* attack the lining of the gastrointestinal tract, and *cytotoxins* attack a wide variety of cells. Some exotoxins are named for the diseases with which they are associated.

**Types of Exotoxins:**

* Exotoxins are divided into **three** types based on their structure and function: (1) A-B toxins, (2) membrane-disrupting or pore forming toxins, and (3) super antigens.
1. **A-B Toxins:**
* A-B toxins are the first toxins to be studied intensively and are so named because they consist of two parts designated as A and B, both of which are polypeptides. The A part is the active (enzyme) component, and the B part is the binding component.
* They bind to the host cell on specific receptor and are translocated into the cell.
* They become active and modify some proteins or other components of the host cell.
* Most exotoxins are A-B toxins.
1. **Membrane-disrupting (pore forming)** **toxins:**
* Membrane-disrupting toxins cause lysis of host cells by disrupting their plasma membranes. Somedo this by forming protein channels in the plasma membrane; others disrupt the phospholipids portion of the membrane. The cell-lysis by exotoxins of *Staphylococcus aureus* is an example of an exotoxin that forms protein channels, whereas that of *Clostridium perfringenes* is an example of an exotoxin that disrupts the phospholipids. Membrane-disrupting toxins contribute to virulence factor by killing host cells, especially phagocytes, and by aiding the escape of bacteria from sacs within phagocytes (phagosomes) into the host cell's cytoplasm.
* Membrane-disrupting toxins that kill phagocytic of leukocytes (white blood cells*)* are called **leukocidins.** They act by forming protein channels. Leukocidins are also active against macrophages, phagocytes present in tissues. Most leukocidins are produced by staphylococci and streptococci. The damage to phagocytes decreases host resistance.
* Membrane-disrupting toxins that destroy phagocytic of erythrocytes (red blood cells), also by forming protein channels, are called **hemolysins**. Important producers of hemolysins include staphylococci and streptococci. Hemolysins produced by streptococci are called streptolysins. One kind of streptolysin is called *streptolysins* O *(*SLO*),* so named because it is inactivated by atmospheric oxygen. Another kind of streptolysin is called *streptolysin* S *(*SLS*),* because it is stable in the presence of atmospheric oxygen.
1. **Superantigens:**
* They bind to the host cell surface, but they are not translocated into the host cell.
* Generally, they modulate the immune response.
* Superantigens are antigens that provoke a veryintense immune response. Through a series of interactions with various cells of the immune system*,* superantigens non-specifically stimulate the proliferation of immune cells called T cells. These cellsare types of white blood cellsthat act against foreign organisms and regulate the activation and proliferation of other cellsof the immune system.
* In response to superantigens, T cells are stimulated to release enormous amounts of chemicals called ***cytokines*.** *Cytokines* are small protein molecules produced by various body cells*,* especially T cells that regulate immune responses and mediate cell-to-cellcommunication. The excessively high levels of cytokines released by T cells enter the bloodstream and give rise to a number of symptoms, including fever, nausea, vomiting, diarrhea, and sometimes shock and even death. Bacterial superantigens include the staphylococcal toxins that cause food poisoning and toxic shock syndrome.

**The brief description of exotoxins by some representative exotoxins:**

* + 1. **Diphtheria Toxin**: *Corynebacterium diphtheriae* produces the *diphtheria toxin* only when it is infected by a lysogenic phage carrying the *tox* gene. This cytotoxin inhibits protein synthesis in eukaryotic cells*.* It does this using an A-B toxin mechanism, which contains two polypeptides: A (active) and B (binding).
		2. **Erythrogenic Toxins**: *Streptococcus pyogenes* has the genetic material to synthesize three types of cytotoxins, designated as A, B and C. These *erythrogenic toxins* are superantigens that damage the plasma membranes of blood capillaries under the skin and produce a red skin rash. Scarlet fever caused by erythrogenic toxins of *S.* *pyogenes*, is named as rash.
		3. **Botulinum Toxin**:Botulinum toxinsare produced by *Clostridium botulinum,* which cause botulism*.* Although toxin production is associated with the germination of endospores and the growth of vegetative cells, little of the toxin appears in the medium until it is released by lysis late in growth. Botulinum toxin is an A-B **neurotoxin**; it acts at the neuromuscular junction (the junction between nerve cells and muscle cells) and prevents the transmission of impulses from the nerve cell to the muscle. The toxin accomplishes this by binding to nerve cells and inhibiting the release of a neurotransmitter called **acetylcholine**. As a result, botulinum toxin causes paralysis in which muscle tone is lacking (flaccid paralysis). Botulinum toxins are extremely potent.

C. *botulinum* produces several different types of botulinum toxin, and each possesses a different potency. There are seven different types of toxin ( A, B, C, D, E, F, G) out of which A, B, E, F and G cause disease in humans and the others cause disease in hens and cattle. All these toxins are heat labile. The symptoms associated with consumption of the toxin include blurred vision, difficulty in swallowing, and breathing and speaking, dryness of the mouth and paralysis of different involuntary muscles.

* + 1. **Tetanus Toxin:**This is produced by *Clostridium tetani,* which is called *tetanus neurotoxin*, also known as *tetanospasmin.* This A-B *neurotoxin* reaches the central nervous system and binds to nerve cells that control the contraction and relaxation of various skeletal muscles. These nerve cells normally send inhibiting impulses that prevent random contractions and terminate completed contractions. The binding of *tetanospasmin* blocks this relaxation pathway. The result is uncontrollable muscle contractions, producing the convulsive symptoms (spasmodic contractions) of tetanus, or "**lockjaw**."
		2. **Vibrio Enterotoxin:** *Vibrio cholerae* produces an A-B *enterotoxin* called *cholera toxin.* Subunit B binds to epithelial cells and subunit A causes cells to secrete large amounts of fluids and electrolytes (ions). This leads to severe diarrhea that may be accompanied by vomiting. This enterotoxin is heat-labile*,* because it is sensitive to heat.
		3. **Staphylococcal Enterotoxin:** *Staphylococcus aureus* produces superantigen toxins that affect the intestines in the same way as *Vibrio* enterotoxin. Strains of *S. aureus* produce six different enterotoxins (A, B, C, D, E and F). These enterotoxins are heat labile that can cause gastroenteritis. Primary symptoms associated with consumption of these toxins include salivation, nausea, diarrhea, abdominal cramps and vomiting. Secondary symptoms include sweating, chills and headache. A strain of *S*. *aureus* also produces a superantigen that results in the symptoms associated with toxic shock syndrome. It also produces scalded skin syndrome.



 **Fig. 2.5:** Mechanism of action of exotoxin on the host cell

1. **Endotoxins**
* Endotoxins differ from exotoxins in several ways. Endotoxins are part of the outer portion of the cell wall of gram-negative bacteria. Gram-negative bacteria have an outer membrane surrounding the peptidoglycan layer of the cell wall. This outer membrane consists of lipoproteins, phospholipids, and lipopolysaccharides (LPSs). The lipid portion of LPS, called **lipid A**, is the endotoxin. Thus, endotoxins are lipopolysaccharides, whereas exotoxins are proteins.
* Endotoxins are released when gram-negative bacteria die and their cell walls undergo lysis or breaks, thus liberating the endotoxin. Antibiotics used to treat diseases caused by gram-negative bacteria can lyse the bacterial cells; this reaction releases endotoxin and may lead to an immediate worsening of the symptoms.
* Endotoxins exert their effects by stimulating macrophages to release cytokines in very high concentrations. At these levels, cytokines are toxic. All endotoxins produce the same signs and symptoms, regardless of the species of microorganism, although not to the same degree. These include chills, fever, weakness, general pains, and in some cases, shock and even death. Endotoxins can also induce miscarriage.
* Another consequence of endotoxins is the activation of blood-clotting proteins, causing the formation of small blood clots. These blood clots obstruct capillaries, and the resulting decreased blood supply induces the death of tissues. This condition is referred to as *disseminated intravascular coagulation (DIC).*
* The fever (pyrogenic response) caused by endotoxins is occurred as follows. When gram-negative bacteria are ingested by macrophages, the bacteria are degraded in vacuoles and the LPSs of the bacterial cell wall are released that contain endotoxins. These endotoxins induce macrophages to produce cytokines called **interleukin-1 (IL-1)**, formerly called *endogenous pyrogen*. The **interleukin-1** is carried via the blood to the hypothalamus, a temperature control center in the brain. The **interleukin-1** induces the hypothalamus to release lipids called prostaglandins, which reset the thermostat in the hypothalamus at a higher temperature. The result is a fever. Bacterial cell death caused by lysis or antibiotics can also produce fever by this mechanism. Both aspirin and acetaminophen reduce fever by inhibiting the synthesis of prostaglandins.
* Shock refers to any life-threatening decrease in blood pressure. Shock caused by bacteria is called septic shock. Gram-negative bacteria cause *endotoxic shock.* Like fever, the shock produced by endotoxins is related to the secretion of a cytokine by macrophages. Phagocytosis of gram-negative bacteria causes the phagocytes to secrete tumor necrosis factor (TNF), sometimes called *cachectin.* TNF binds to many tissues in the body and alters their metabolism in a number of ways. One effect of TNF is damage to blood capillaries; their permeability is increased, and they lose large amounts of fluid. The result is a drop in blood pressure that results septic shock.
* Endotoxins do not promote the formation of effective antibodies.
* Medical equipment that has been sterilized may still contain endotoxins, because they are heat stable.
* Microorganisms that produce endotoxins include:
	+ - *Salmonella typhi*
		- *Proteus* spp*.*
		- *Pseudomonas* spp.
		- *Neisseria* spp.

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 **Fig. 2.6**: Mechanism of action of endotoxin on the host cell to produce fever

**Table 2.3**: General characteristics of endotoxin and exotoxin

|  |  |
| --- | --- |
| **Exotoxins** | **Endotoxins** |
| Produced by both G+ve and G-ve bacteria  | Produced only by G-ve bacteria |
| Released from cells (Extracellular) | Integral part of the cell wall (part of outer membrane) |
| Protein | Lipid A of lipopolysaccharide |
| High potency | Low potency |
| Usually heat labile  | Heat stable  |
| Specific receptors on host target cells | Diverse range of host cells and systems affected  |
| Specific effects in host  | Diverse range of effects in host |
| Toxoids can be made by treating with formalin or heat or other chemicals | Toxoids cannot be made |

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 **Fig. 2.7:** Exotoxins versus Endotoxins

**2.7.2. Fungal toxins (mycotoxins)**

* Mycotoxins are produced by fungi as secondary metabolites. The primary metabolites of fungi as well as for other organisms are those compounds that are essential for growth. Secondary metabolites are formed during the end of the exponential growth phase and have no apparent significance to the producing organism relative to growth or metabolism.
* In general, they are formed when large pools of primary metabolic precursors such as amino acids, acetate, pyruvate, and so on, accumulate. The synthesis of mycotoxins represents one way the fungus has of reducing the pool of metabolic precursors that it no longer requires for metabolism.
* There are different types of mycotoxins produced by different fungi. Some of them are listed below.

**i. Aflatoxins**

* Aflatoxins are the most widely studied of all mycotoxins. Knowledge of their existence dates from 1960, when more than 100,000 Turkey poults died in England after eating peanut meal imported from Africa and South America. From the poisonous feed were isolated a mould *Aspergillus flavus,* and a toxin produced by this organism that was designated aflatoxin *{Aspergillus* *flavus* toxin—A-fla-toxin). Studies on the nature of the toxic substances revealed the following four main components.



 **Fig. 2.8:** Components of aflatoxin

* Aflatoxin B1 (AFB1) is produced by all aflatoxin positive strains, and it is the most potent of all. AFM1 is a hydroxylated product of AFB1 and appears in milk, urine, and faeces as a metabolic product. AFL, AFLH1, AFQ1, and AFP1 are all derived fromAFB1.
* AFB2 is the 2,3-dehydro form ofAFB1, and AFG2 is the 2,3-dihydro form of AFG1.
* The toxicity of the six most potent aflatoxins decreases in the following order: B1 > M1 > G1 > B2 > M2 > G2.
* When viewed under ultraviolet (UV) light, six of the toxins fluoresce as noted:
	+ - B1 and B2—blue
		- G1—green
		- G2—green-blue
		- M1—blue-violet
		- M2—violet
* They are polyketide secondary metabolites whose carbon skeleton comes from acetate and malonate.
* Aflatoxins have been found in a wide variety of foods, including milk, beer, cocoa, corn, raisins, soybean meal, and fresh beef. In general, they produced in *Aspergillus flavus* infected grains and peanut products.
* Aflatoxins are carcinogenic to humans.
* They cause kidney and liver cancer.

**ii. Alternaria Toxins**

* Several species of *Alternaria* (including *A. citri, A. alternata, A. solani,* and *A. tenuissima)* produce toxic substances that have been foundin apples, tomatoes, blueberries, grains, and otherfoods. The toxins produced includealternariol, alternariol monomethyl ether,altenuene, tenuazonic acid, and altertoxin-1.

**iii. Citrinin**

* Citrinin is produced by *Penicillium citrinum, P. vindication,* and other fungi.It has been recovered from polished rice, moldy bread, country-cured hams, wheat, oats, rye, andother similar products. Under long-wave UVlight, it fluoresces lemon yellow. It is a knowncarcinogen.
* While citrinin-producing organisms are found on cocoa and coffee, this mycotoxin as well as others is not found to the extent of growth. The apparent reason is the inhibition of citrinin in *P. citrinum* by caffeine. The inhibition of citrinin appears to be rather specific, since only a small decrease in growth of the organisms occurs.



 **Fig. 2.9:** Structure of Citrinin

**iv. Ochratoxins**

* The ochratoxins consist of a group of at least seven structurally related secondary metabolites of which ochratoxin A (OA) is the best known and the most toxic. Ochratoxin B (OB) is dechlorinated OA and along with ochratoxin C (OC), it may not occur naturally. OA is produced by a large number of storage fungi, including. *Aspergillus* *ochraceus, A. alliaceus, A. ostianus,* *A. mellus,* and other species of aspergilli. Among penicillia that produce OA are *P. viridicatum,* *P. cyclopium, P. variable,* and others.
* OA is heat stable.
* It is both hepatotoxic and nephrotoxic.
* Ochratoxins have been found in corn, dried beans, cocoa beans, soybeans, oats, barley, citrus fruits, moldy tobacco, hams, peanuts, coffee, and other similar products.



 **Fig. 2.10:** Structure of Citrinin

**v. Ergot toxin/ alkaloid**

* It is produced by *Claviceps purpurea,* a fungus causing infections on different grain crops. It is very common in rye and rarely in wheat and barely. The toxin can restrict blood flow in the limbs, which leads to loss of limbs due to the development of gangrene.
* The disease caused by this species is called ergotism.
* It may also cause hallucinogenic symptoms, producing bizarre behavior.



 **Fig. 2.11:** Structure of Ergotamine

**2.7.3 Algal Toxins**

* Several algae are capable of producing very toxic compounds that can accumulate in food chain and can affect birds and mammals.
	1. **Saxitoxin**
* Its name was obtained from Alaskan butter Clam (Saxidomas giganteus) from which the toxin was isolated, even though; it is produced by a dinoflagellate called *Gonyaulax catenella*.
* Causes a condition called paralytic shell fish poisoning (PSP)
* The toxin blocks nerve impulse transmission and cause tingling and numbness of fingertips and lips; incoherent speech and respiratory paralysis.
* Highly potent toxin which is generally heat sensitive.
	1. **Brevitoxin**
* Produced by a dinoflagellate called *Ptychodiscus brevis*.
* Cause neurotoxic shell fish poisoning (NSP) which is less common than paralytic shell fish poisoning.
* This toxin also affects the proper functioning of the nervous system.
	1. **Dinophysis toxin**
* Produced by another dinoflagellate known as *Dinophysis fortii*.
* The toxins are lipophilic toxins.
* The type of poisoning caused is diarrheal shell fish poisoning (DSP)
* Major symptoms associated with the toxin include diarrhea, abdominal pain, nausea and vomiting.