Advances in BIOTECHNOLOGY

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Advances in Biotechnology

Chapter 1

Extraction of Fungal Chitosan and its Advanced Application

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1. Definition and Chemical Structure

Biopolymer is a term commonly used for polymers which are synthesized by living organisms [1]. Biopolymers originate from natural sources and are biologically renewable, biodegradable and biocompatible. Chitin and chitosan are the biopolymers that have received much research interests due to their numerous potential applications in agriculture, food industry, biomedicine, paper making and textile industry. Chitin is a polysaccharide, made of N-acetyl-D-glucosamine units connected by β (1→4) linkage (**Figure 1A**). When the acetyl-D-glucosamine units in chitin lose acetyl groups, the molecule is called chitosan (**Figure 1B**) [2].

Chitosan is a natural co-polymers of chitin, composed by units of 2-amino-2-desoxi-Dglycopyranose and of 2-acetamide-2-desoxi-D-glycopyranose interconnected by glycosidic bonds β-1,4 in variable proportions. The first type of units is frequently present in chitosan. This polymer is naturally found in the cell wall of fungi, mainly in the Mucorales order [3,4]. Chitosan is formed by the chitin deacetylation, and the group N-acetyl can be suffer several degrees of deacetylation. Chitosan is characterized according to its deacetylation level and

molar mass, once such features may influence the degradability and in the polysaccharide hydrolysis [2,5]. According to the medium acetylation level (AL), chitosan may be obtained with physical-chemical properties differentiated regarding the solubility parameters, pKa and viscosity [5,6,7]. It is difficult to obtain chitosan with high deacetylation level as due long process of isolation, and the degradation of the polymer also increases [5,7].

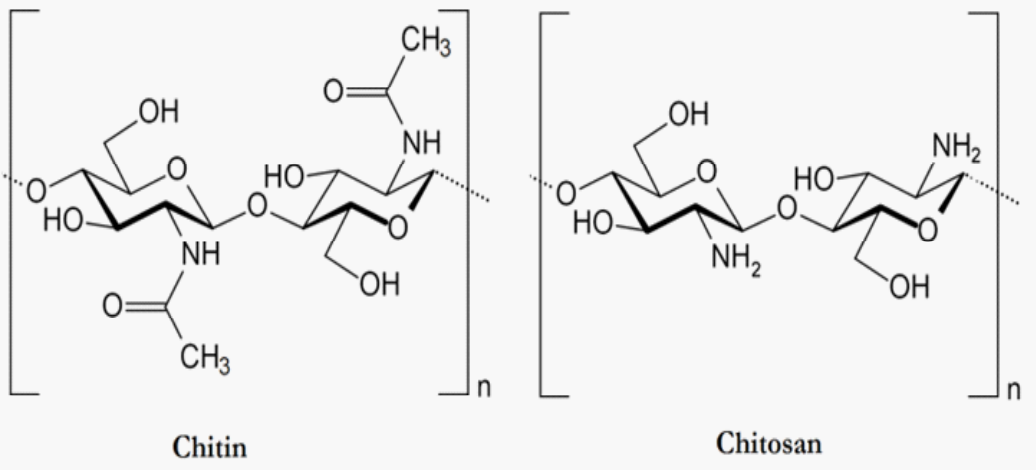

Figure 1: Chemical structure of chitin and chitosan

2. Occurrence and Biological Functions in Nature

Chitin is a characteristic compound found in fungi and some animals. In animals, chitin mainly exists in the shells of crustaceans and mollusks, in the backbone of squids and in the cuticle of insects. Long chitin molecules are associated with proteins by covalent bonds and together they form a complex structural network. On crustacean's shells, calcium carbonate deposits into the network contributing to strength of the shells and protection of the organisms [2]. In fungi, chitin exists in the cell wall of spores and hyphae. It is associated with glucan molecules in form of microfibrils, which are embedded in an amorphous matrix and provide the framework in cell wall morphology [3]. Chitosan is not native to animal sources, but a small number of fungi, such as *Mucor, Absidia* and *Rhizopus* species have chitosan as one of the structural components in the cell wall [3].

The amount of chitin in animal and fungi is specific to species, age and environmental conditions where the organism exists. Chitin content in the dry shells of crabs, lobsters and shrimps ranges from 14 to 27 % [4], while in the fungal cell wall it varies from 2 to 42 %, the lowest value corresponding to yeasts, and the highest values to Euascomycetes [3].

2.1. Properties of chitosan

Chitosan is a weak base insoluble in water but soluble in dilute aqueous solutions of various acids, the most widely used is acetic acid [8]. The acid solubility is explained by the protonation of the free amino group, characteristic in the chitosan *in natura*, which change to NH_2 to NH_3^+, whereas in alkaline condition, the hydro solubility is due to the formation of

carboxylate, from the introduced carboxylic group [9,10]. The possibility to obtain a variety of polymer derivatives with differences solubility, thermal stability, reactivity with other substances and specificity regarding the binding site, providing several biological applications of the chitosan [11]. Some applications of the chitosan, it is highligh it's the use in the pharmaceutical industry, more specifically related to dental clinic [12]

3. Production of Chitosan from Fungal Sources

Production of chitin and chitosan from fungal mycelium has recently received increased attention due to significant advantages. For example, while crustacean waste supplies are limited by seasons and sites of fishing industry, fungal mycelium can be obtained by convenient fermentation process that does not have geographic or seasonal limitations [10]; fungal mycelia have lower level of inorganic materials compared to crustacean wastes, and thus no demineralization treatment is required during the processing [11]; crustacean chitin and chitosan may vary in the physico-chemical properties, while fungal chitin and chitosan have relatively consistent properties because of the controlled fermentation conditions [12]; fungal chitin and chitosan are apparently more effective in inducing the plant immune response and are potentially more suitable for agricultural applications [13].

Many fungal species, including *Absidia glauca, Absidia coerulea, Aspergillus niger, Mucor rouxii, Gongronella butleri, Phycomyces blakesleeanus, Absidia blakesleeana, Rhizopus oryzae, Trichoderma reesei* and *Lentinus edodes* have been investigated for the production of chitin and chitosan [10-12,14-19]. Among all investigated species, the most commonly researched one is *M. rouxii* [10,14,15] and quantities of chitin and chitosan in its mycelia can reach 35% of cell wall dry weight [16].

Fungi are usually harvested at their late exponential growth phase to obtain the maximum yield for chitin and chitosan. Although fungi can be grown on solid media, cultivation for chitin and chitosan isolation is usually carried out in the yeast peptone glucose broth (YPG), potato dextrose broth (PDB) or molasses salt medium (MSM), the performance of different media and didnot find significant difference in the yield and physico-chemical properties of chitosan and chitin obtained [14].

Extraction process from fungal sources is similar to industrially utilized except that no demineralization treatment is required due to low mineral content in fungal mycelia [16]. Generally, the extraction procedure consists of three steps:

(1) alkaline treatment to remove protein and alkali soluble polysaccharides;

(2) acid reflux to separate chitin and chitosan

(3) precipitation of chitosan under alkaline conditions.

Removal of proteins by alkaline treatment is commonly performed with 1N NaOH at 95 °C from 1 to 6 h or at 121 °C from 0.25 h to 1 h [17]. Separation of chitosan by acid treatment is usually carried out by 2 to 10 % acetic or hydrochloric acid at 95 °C for 3 to 14 h. For example, Synowiechi et al. [16] used 2 % NaOH at 90 °C during 2 h for alkali treatment and 10 % acetic acid at 60 °C during 6 h for acid reflux during extraction of chitin and chitosan from *M. rouxii.* Hu et al. [18] adopted autoclaving at 121°C in both alkaline and acid treatments of *Absidia glauca mycelia*. However, the temperature and time of acid treatment had to be reduced to 25 °C and 1 h to avoid the depolymerization of chitosan during extraction from zygomycetes strains [19].

Most of the studies in this field concentrate on the fermentation processes to produce fungal mycelia for chitin and chitosan extraction [10-12,14-19]. Relatively few studies have focused on the fungal waste from industrial fermentations or mushroom industry [17]. However, considering the amount of waste that accumulates during processing, citric acid industry and mushroom industry, specifically from *Agaricus bisporus* growing practices, can provide plenty of raw materials for fungal chitin and chitosan production.

Citric acid is the most widely used organic acid in food, beverage and pharmaceutical industries. The industrial production is based on *A. niger* submerged fermentation. The current world requirements for citric acid are estimated to be 400,000 tons per year [20]. Taking into account that 20 % dry mycelium waste is produced under industrial fermentation conditions, approximately 80,000 tons of *A. niger* mycelium waste accumulates every year [21]. Managing this waste presents an extra expense for the producers and alternative solutions for mycelium disposal have been evaluated. One of the potential outputs for the spent mycelia is in feed supplements. However, this type of feed seems to be difficult to compete with the other low price feeds.

White common mushroom, *Agaricus bisporus*, is the most consumed mushroom in the U.S. In last several years the production has been relatively constant and sales totaled 382 million kilograms in the 2002/03 season [22]. The waste accumulated during mushroom production and harvest consists mainly of stalks and mushrooms of irregular dimensions and shape. Depending on the size of the mushroom farm, the amount of waste ranges between 5 and 20% of the production volume. This waste material results in approximately 50,000 metric tons per year that currently has no application (personal communication).

4. Purification of Chitosan

4.1. Chemical methods

The obtained chitosan has to be purified to make it suitable for the pharmaceutical use. The purification process was designed in three steps [23]:

1) Removal of insolubles with filteration

2) Reprecipitation of chitiosan with 1 N NaOH

3) Demetallisation of retrieved chitosan

4.1.1. Removal of insolubles with filtration

One mg/ml chitosan acetic acid 1% (v/v) solution is prepared by a magnetic stirrer until an homogenous solution is obtained. The insolubles were removed by filteration through Whatman filter paper 22μm.

4.1.2. Reprecipitation of chitosan with 1N NaOH

Chitosan was precipitated from filtered chitosan solution by titration with 1 N NaOH until pH value of 8.5. The chitosan obtained is washed several times with distilled water by centrifuging at 8,000 to10,000 xg. All the above steps were carriedin the presence of reducing agent Dithiothreitol, (DTT) in order to provide more consistency and reproducibil-ity between chitosan batches for biomedical applications (any other hydroxides other than NaOH are reactive which would another step in purification if such materials are used).

4.1.3. Demetallisation of retrieved chitosan

Reprecipitation precedes demetallisation by the addition of 1 ml of 10% w/v Aqueous solution of sodium dodecyl sulfate (SDS) and stirring for 30 min for dissolving the protein left over finally. After leaving the solution stirring at room temperature overnight, 3.3 ml of 5% w/v ethylenediaminetetraa-cetic acid (EDTA) was added and stirred at room temperature for 2 additional hours for precipitation of heavy metals with EDTA. The water insoluble chitosan precipitate was collected by centrifugation at 5000xg for 30 min using REMI and washed several times with distilled water by resuspending and re-centrifugation for 30 min. the residue obtained is dried in hot air oven at 60 gently to prevent physical damage in the chain structure. The obtained dried chitosan is stored in the dessicator.

4.2. Biological methods

An alternative way to solve chemical extraction problems is to use biological methods. The use of proteases for deproteinisation of crustacean shells would avoid alkali treatment. Besides the application of exoenzymes, proteolytic bacteria were used for deproteinisation of demineralised shells [24]. This approach allows obtaining a liquid fraction rich in proteins, minerals and astaxanthin and a solid chitin fraction. The liquid fraction can be used either as a protein-mineral supplement for human consumption or as an animal feed [25]. Deproteinisation processes have been reported for chitin production mainly from shrimp waste using mechanical [26], enzymatic [27,28] and microbial processes involving species like *Lactobacillus,*

Pseudomonas aeruginosa K-187 and *Bacillus subtilis* [29]. Biological demineralisation has also been reported for chitin production from crustacean shells; enzymatically, using for instance alcalase, or by microbial process involving species like *L. pentosus* 402 or by a natural probiotic (milk curd). In these biological processes, demineralisation and deproteinisation occur mainly simultaneously but incompletely [18,24,29].

4.2.1. Use of lactic acid bacteria for chitin recovery

Fermentation has been applied to fish for many years and represents a low-level (artisanal) and affordable (neither capital nor energy intensive) technology [25]. It consists in the ensilation of crustacean shells and a low-cost in situ production of lactic acid from by-products such as whey, lignocellulose and starch. Lactic acid production by lactic acid bacteria induced a liquefaction of the semi-solid waste and led to a low pH and activation of proteases [28]. The protein-rich liquid could be separated from the chitin, which remained in the sediment [24]. This method might offer a commercial route for the recovery of chitin [26].

Lactic acid is formed from the breakdown of glucose, creating the low p^H, which improves the ensilation that suppresses the growth of spoilage microorganisms. Lactic acid reacts with the calcium carbonate component in the chitin fraction, leading to the formation of calcium lactate, which precipitates and can be removed by washing. The resulting organic salts from the demineralisation process could be used as de- and anti-icing agents and/or preservatives [26]. Deproteinisation of the biowaste and simultaneous liquefaction of the shrimp proteins occurs mainly by proteolytic enzymes produced by the added *Lactobacillus*, by gut bacteria present in the intestinal system of the shrimp, or by proteases present in the biowaste [25]. It results in a fairly clean liquid fraction with a high content of soluble peptides and free amino acids [26].

Lactic acid fermentation combined with chemical treatments has been studied as an alternative to chemical extraction of chitin, reducing the amount of alkali and acid required [27]. It was considered as a pretreatment of shrimp waste followed by demineralisation and deproteinisation using low concentrations of HCl (0.5 M) and NaOH (0.4 M).

4.2.2. Use of non-lactic acid bacteria for chitin recovery

Non-lactic acid bacteria have also been tested for chitin recovery. Fermentation of shrimp (*Metapenaeopsis dobsoni*) shell in jaggery broth using *Bacillus subtilis* for the production of chitin and chitosan showed that the level of acid produced as well as the proteolytic activity of *B . subtilis* allowed shell demineralisation and deproteinisation [29]. About 84 % of the protein and 72 % of minerals were removed from the shrimp shell after fermentation Pseudomonas aeruginosa K-187 strain isolated from the soil of northern Taiwan is a producer of protease and chitinase/lysozymes when cultured in a medium containing shrimp and crab shell wastes

as the sole carbon sources [30]. It was shown that *P. aeruginosa* K-187 is capable of shell waste deproteinisation in either solid-state, liquid- -solid or liquid fermentation. Higher deproteinisation yield was recorded in solid-state fermentation, 82 % after 5 days, showing that *P. aeruginosa* K-187 is more efficient than the proteolytic bacterium *P. maltophilia*, known to be highly efficient in the deproteinisation of prawn shell waste. The use of protease produced by *P. aeruginosa* K-187 was therefore promising in deproteinisation of crustacean wastes [29].

4.3. Physicochemical properties and analysis

Properly processed, highly purified chitin and chitosan are white and odorless. Their chemical structures are similar to those of cellulose. The only difference is that the 2-hydroxy group of the cellulose has been replaced with an acetamide or amino group in chitin or chitosan, respectively [28]. Therefore, the physicochemical properties and research methodology for all three biopolymers are presumably similar. For example, chitin and chitosan are insoluble in the common organic and inorganic solvents, but soluble in salt organic mixtures of LiCl-N,N-DMAc, which is a common solvent for cellulose [30].

4.4. Applications of chitin and chitosan

Natural and non-toxic biopolymers chitin and chitosan are now widely produced commercially from crab and shrimp shell waste. During the past few decades, chitin and chitosan have attracted significant interest in view of a wide range of proposed novel applications [19]. Their unique properties, biodegradability, biocompatibility and non-toxicity make them useful for a wide range of applications Chitin is mainly used as the raw material to produce chitin-derived products, such as chitosans, oligosaccharides, and glucosamine [1]. There are now over 2000 concrete applications, and the field of nutrition is the largest user of chitosan with 1000 tonnes consumed in 2000. The worldwide industrial production of these derivatives in year 2000 is estimated to be above 10 000 tonnes [18].

4.4.1. Antimicrobial activity

It has been shown that chitosan posses strong antimicrobial activity against both gram-positive and gram-negative bacteria, including the foodborne pathogens, such as *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus,* and *Listeria monocytogenes* [31-33].

In its free polymer form, chitosan exhibits antifungal activity against *Alternaria alternata, Rhizopus oryzae, Aspergillus niger, Phomopsis asparagi,* and *Rhizopus stolonifer*. The antifungal activity of chitosan depends on its concentration, molecular weight, degree of substitution, and the type of functional groups added to the chitosan, as well as the type of fungus [32]. Whilst derivatives of the polymer can be created to target specific pathogens, chitosan shows natural antifungal activity without the need for chemical modification.

Two theories have been proposed for the antimicrobial mechanism of chitosan. Based on one, interaction between positively charged chitosan molecules and negatively charged microbial cell membranes results in the disruption of the cytoplasmic membrane and, ultimately, leakage of intracellular constituents [32,33]. By the other theory, chitosan oligosaccharides easily permeate into the nucleus of eukaryotic cell and interfere with the transcription of RNA and the synthesis of proteins [34]. However, chitosans with high molecular weight (above 100 kDa) generally express stronger antibacterial activity than chitosan oligomers [35].

Recent studies on chitosan depolymerisation have drawn considerable attention, as the products obtained are more water-soluble. Beneficial properties of chitosan and its oligosaccharides include: antitumour; neuroprotective; antifungal and antibacterial and anti-inflammatory [16,31,33].

The antimicrobial activity of chitin, chitosan, and their derivatives against different groups of microorganisms, such as bacteria, yeast, and fungi, has received considerable attention in recent years. Two main mechanisms have been suggested as the cause of the inhibition of microbial cells by chitosan [34]. The interaction with anionic groups on the cell surface, due to its polycationic nature, causes the formation of an impermeable layer around the cell, which prevents the transport of essential solutes. It has been demonstrated by electron microscopy that the site of action is the outer membrane of gram negative bacteria [35]. The permeabilizing effect has been observed at slightly acidic a condition in which chitosan is protonated, but this permeabilizing effect of chitosan is reversible [34]. Chitosan has been confirmed to possess a broad spectrum of antimicrobial activities [35]. However, the low solubility of chitosan at neutral pH limits its application. In this study H_2O_2 was taken to degrade the chitosan into water soluble chitosan. Several studies prove that an increase in the positive charge of chitosan makes it bind to bacterial cell walls more strongly [36]. The molecular weight is the main factor affecting the antibacterial activity of chitosan, from the results obtained. In contrast, some authors have not found a clear relationship between the degree of deacetylation and antimicrobial activity [37]. These authors suggest that the antimicrobial activity of chitosan is dependent on both the chitosan and the microorganism used [38-40,41]. studied the antimicrobial activity of hetero-chitosans with different degrees of deacetylation and Molecular weight against three Gram negative bacteria and five Gram-positive bacteria and found that the 75% deacetylated chitosan showed more effective antimicrobial activity compared with that of 90% and 50% deacetylated chitosan [39,40].

These important properties of chitosan are believed to have many commercial applications of high economic interests [19]. The antifungal and antibacterial activities of chitosan can be employed in production of biofertilizers and biopesticides of economical benefits [20]. Likewise the radical scavenging or the anti-oxidant activity of chitosan is of great interest in food industries and its possible use as natural additives has lead to a great interest in replac-

ing synthetic additives [41]. The use of the antimicrobial activity of chitosan has been used for development of antimicrobial films intended for use in packaging materials for foods, medical supplies and so on, or as laminated coating on items for which surface colonization is undesirable. Chitosan used as coating on fruits and vegetables is almost as effective as the fungicide TBZ at preventing spoilage during storage at proper conditions. Chitosan activity as anti-coagulant is useful in biomedical applications [42] like wound dressing, surgical sutures and for other treatments like reducing oxidative stress in live cells [23], Antitumor activity anti-inflammatory, effect HIV-1 inhibitors, antihypertensive, Hypoglycemic and hypolipidemic effect [40-43] etc. still research is going on. Many studies have been conducted to explore the many possibilities of utilizing the various properties of chitosan and research is still going on these aspects. Chitosan as a commercial chemical has promising range of applications [44].

4.4.1.1. Antimicrobial activity and applications in food preservation

It has been shown that chitosan posses strong antimicrobial activity against both gram-positive and gram-negative bacteria, including the foodborne pathogens, such as Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, and Listeria monocytogenes [44-47]. Two theories have been proposed for the antimicrobial mechanism of chitosan. Based on one, interaction between positively charged chitosan molecules and negatively charged microbial cell membranes results in the disruption of the cytoplasmic membrane and, ultimately, leakage of intracellular constituents [46]. By the other theory, chitosan oligosaccharides easily permeate into the nucleus of eukaryotic cell and interfere with the transcription of RNA and the synthesis of proteins [47]. However, chitosans with high molecular weight (above 100 kDa) generally express stronger antibacterial activity than chitosan oligomers [45].

4.4.2. Wastewater treatment with chitin and chitosan

Chitin and chitosan can be used for the adsorption or fixation of heavy metals [47] and dyes. Chitosan is a polycation polymer effective in coagulation, flocculation and dehydration of activated sludge, and hence used in wastewater treatment [16,18]. Another recent application is immobilization of microorganisms or sludge in chitosan matrices for wastewater treatment in extreme environmental conditions (extreme pH, presence of organic solvents), allowing the reuse of cells and hence their implementation in continuous process.

4.4.3. Applications of chitin and chitosan in food

Only limited attention has been paid to food application of these versatile biopolymers [48]. They offer a wide range of unique applications, which are non-exhaustively listed in **Table 4**. The use of chitosan in the food industry is related to its functional properties, and nutritional and physiological activities. Chitosan exhibits water-, fat- and dye-binding capacity, as well as emulsifying properties [49]; it was shown to be useful in the preparation of stable

emulsions without any other surfactant [45]. It has been used as a dietary supplement due to some interesting properties.

4.4.4. Biomedical application of chitin and chitosan

Chitin and chitosan show excellent biological properties such as non-toxicity, which is illustrated by a dose limit per day of 17 g/kg [16], biodegradation in the human body, biocompatibility, and immunological, antibacterial, wound-healing and haemostatic activity, in cell culture, tissue engineering and drug delivery [50,51], since it is highly biocompatible and biodegradable in physiological environment [20,46]. Chitin is also used as an excipient and drug carrier in film, gel or powder form for applications involving mucoadhesivity.

4.5. Anti-infammatory effects

Inflammation is a physiological body immune response against pathogens, toxic chemicals or physical injury. While acute inflammation is a short-term normal response that usually causes tissue repair by recruitment of leukocytes to the damaged region, chronic inflammation is a long-term pathological response involving induction of own tissue damage by matrix metalloproteinases (MMPs) [52,53].

Although the anti-inflammatory effects of chitin and its derivatives have been rarely reported, in recent years data has been accumulating. First of all, it was found that chitin is a size-dependent regulator of inflammation [54]. While both intermediate-sized chitin and small chitin stimulates TNF production in murine peritoneal macrophages, large chitin fragments are inert, Furthermore, it was found that chitin stimulates the expression of TLR2, dectin-1, the mannose receptor and inflammatory cytokines, differentially activated NF-κB and spleen tyrosine kinase. Chitosan was confirmed to partially inhibit the secretion of both IL-8 and TNF-α from mast cells, demonstrating that water-soluble chitosan has the potential to reduce the allergic inflammatory response [55]. Since mast cells are necessary for allergic reactions and have been implicated in a number of neuroinflammatory diseases, chitosan nutraceuticals may help to prevent or alleviate some of these complications. Chitosan oligosaccharide may possess an anti-inflammatory effect via the inhibition of TNF-α in the LPS-stimulated inflammation. These functions of chitosan to exert anti-inflammatory effect could be unilized in the nutraceutical industry as well as in functional foods for prevention and alleviation of inflammatory diseases. In addition, it was reported that chitosan promotes phagocytosis and production of osteopontin and leukotriene B by polymorphonuclear leukocytes, production of interleukin-1, transforming growth factor b1 and platelet-derived growth factor by macrophages, and production of interleukin-8 by fibroblasts, enhancing immune responses [56].

4.6. Anticancer effects

In recent years, it was revealed that the tumor inhibitory effect of COS is probably related to their induction of lymphocyte cytokines through increasing T-cell proliferation. Basically, the antitumor mechanism of COS is enhanced by acquired immunity via accelerating T-cell differentiation to increase cytotoxicity and maintain T-cell activity [57]. The antitumor effects of various low-molecular weight chitosans, such as water-soluble 21- or 46-kDa molecules with low viscosity, produced by enzymatic hydrolysis of over 650-kDa chitosan, which displayed decreased tumor growth and final tumor weight in sarcoma 180-bearing mice due to increase of natural killer cell activity [58,59]. The results indicate the low-molecular-weight water-soluble chitosans and oligochitosans might be useful in preventing tumor growth, partly through enhancing cytotoxic activity against tumors as an immunomodulator [60].

4.7. Drug delivery system

To provide anticancer chemotherapy, chitosan is attracting increasing attention as drug and gene carriers due to its excellent biocompatibility, biodegradability, and nontoxicity [61]. Chitosan has an important role in delivery of drugs, with the potential to improve drug absorption and stabilize drug components to increase drug targeting. In addition, as a potential gene deliverer, chitosan can protect DNA and increase the expression period of genes. Chitin or chitosan derivatives, which were conjugated with some kinds of anticancer agents, can execute better anticancer effects with a decrease of side effects and gradual release of free drug in the cancer tissues. Furthermore, chitosan nanoparticles were synthesized and applied for in vivo antitumor activity [62]. On the other hand, for ocular drug delivery, liposomes coated with low-molecular weight chitosan may be potentially applicable to clinic uses [63].

Nanoparticles enable chitosan to elicit dose-dependent tumor-weight inhibition with highly impressive antitumor efficacy in vivo. The doses and particle quantum size have a great effect on their efficacy as drug carriers. In particular, with small particle size and positive surface charge, the complex could exhibit higher antitumor activity than other chitosan derivatives [64]. Smaller sized particles seem to enhance efficacy of the particle-based drug delivery systems. Basically, chitosan nanoparticles are produced with a mean particle size ranging from 40 to 100 nm and a positive surface charge of about 50 mV [65]. To introduce these products into in vitro cell culture systems, they should be filtered by a membrane with diameter of 0.45 μm and autoclaved. In in vivo animal models, different administration routes of chitosan nanoparticles, such as intravenous (i.v.) or intraperitoneal injection (i.p.) and oral administration (p.o.), could exhibit little difference in antitumor activities. However, because nanoparticulate systems have been developed to improve the blood circulating time and tumor targeting efficacy of vincristine, administration of chitosan nanoparticles i.v. can contribute in vivo efficacy to antitumor activities followed by a prolonged blood half-life of drugs [66].

4.8. other applications of chitin and chitosan derivatives

Chitin and chitosan derivatives may effectively reduce soil-borne diseases. In addition, chitin exhibits several functions, including retention of nutrients in the soil, and contributes to the nitrogen cycle [14,67-69]. Chitin and chitosan have a versatile application potential in agriculture. In addition, they have found various other applications [15]. Chitin can also be transformed into saccharides under certain conditions . It can also be used as a slowly degrading substrate in microbial fuel cells [69,70,].

5. Chitosan Nanoparticles

Chitosan is soluble in acidic conditions - in solution the free amino groups on its polymeric chains can protonate, giving it a positive charge. Chitosan nanoparticles are biocompatible, relatively non-toxic, biodegradable, and cationic in nature [71,72]. Chitosan nanoparticles can be formed by incorporating a polyanion such as tripolyphosphate (TPP) into a chitosan solution under constant stirring [73].

These nanoparticles can then be used for drug delivery and gene therapy applications. Due to its poor solubility at pH more than 6.5, a number of chemically modified chitosan derivatives with improved water solubility can be used as well [74,75]. Ionic gelation is the most commonly used method for synthesising chitosan nanoparticles [6]. In this method, chitosan precursors are cross-linked using sodium tripolyphosphate (TPP). The method typically yields large sized (100–300 nm) particles with a high degree of polydispersity. Even though ionic gelation is a widely used method and factors governing the size and dispersivity of chitosan nanoparticles (such as the concentration of reactants, temperature, pH, and the level of deacetylation) are well known [7] our basic understanding of the process at mechanistic level is poor. In the ionic gelation process, TPP cross links randomly oriented chitosan molecules, which, in turn, are connected to other similarly cross-linked moieties. Such intra- and intermolecular cross-linking is rather uncontrolled and leads to polydispersity in the synthesized preparation [76].

5.1. Applications of chitosan nanoparticles

The applications of chitosan nanoparticles are [74,75]:

- As antibacterial agents, gene delivery vectors and carriers for protein release and drugs
- Used as a potential adjuvant for vaccines such as influenza, hepatitis B and piglet paratyphoid vaccine
- Used as a novel nasal delivery system for vaccines. These nanoparticles improve antigen uptake by mucosal lymphoid tissues and induce strong immune responses against antigens.

- Chitosan has also been proved to prevent infection in wounds and quicken the wound-healing process by enhancing the growth of skin cells.

- Chitosan nanoparticles can be used for preservative purposes while packaging foods and in dentistry to eliminate caries.

- It can also be used as an additive in antimicrobial textiles for producing clothes for healthcare and other professionals.

- Chitosan nanoparticles show effective antimicrobial activity against Staphylococcus saprophyticus and Escherichia coli.

- These materials can also be used as a wound-healing material for the prevention of opportunistic infection and for enabling wound healing.

- The nanoparticles have also been proven to show skin regenerative properties when materials were tested on skin cell fibrocblasts and keratinocytes in the laboratory, paving the way to anti-aging skin care products.

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Chapter 2

Isolation and Separation of Phenolics using HPLC Tool: A Consolidate Survey from the Plant System

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Abstract

HPLC is a versatile tool for separation of phenolics from the plant systems. Many studies are conducted for separation of phenolics using HPLC tool. This chapter summarized the work done in this area using various solvents, plant parts and assay condition in tabulated form.

1. General Introduction

In recent times, one of the key interests in food science and technology is the extraction, identification, and characterization of novel functional ingredients of natural origin. These ingredients are used as natural preservatives against food degradation, health promotion activities and value addition. Plants produce an amazing diversity of low molecular weight compounds. Although the structures of close to 50,000 have already been elucidated [1]. There are probably hundreds of thousands of such compounds. Only a few of these are part of 'primary' metabolic pathways (those common to all organisms). The rest are termed 'secondary' metabolites [2].

Amongst this diverse pool of metabolites, polyphenols are aromatic hydroxylated compounds, commonly found in vegetables, fruits and many food sources that form a significant portion of our diet, and which are among the most potent and therapeutically useful bioactive substances. The plant phenolics play important role in many physiological functions like, protein synthesis, nutrient uptake and oxidative enzyme (peroxidases) activities [3]. Photosynthe-

sis and structural components. In addition, they also provide defense against microbial attacks and by making food unpalatable to herbivorous predators [4]. Thus, phenolics are overall important in many growth and development activities of the plants.

Besides the importance for the plant itself, such metabolites determine the nutritional quality of food, colour, taste, smell, antioxidative, anticarcinogenic, antihypertension, anti-inflammatory, antimicrobial, immunostimulating, and cholesterol-lowering properties [5]. The health benefits of fruit and vegetables are mainly from the phytochemicals and a range of polyphenolics [6]. Significant antioxidant, antitumor, antiviral and antibiotic activities are frequently reported for plant phenols. They have often been identified as active principles of numerous folk herbals. In recent years, the regular intake of fruits and vegetables has been highly recommended, because the plant phenols and polyphenols they contain are thought to play important roles in long term health benefits and reduction in the risk of chronic and degenerative diseases.

2. Synthesis and Structure

Plant secondary metabolites have been fertile area of chemical investigation for many years, driving the development of both analytical chemistry and of new synthetic reactions and methodologies. The subject is multi-disciplinary with chemists, biochemists and plant scientists all contributing to our current understanding [7]. High concentrations of secondary metabolites might result in a more resistant plant. Their production is thought to be costly and reduces plant growth and reproduction [8]. Therefore, defense metabolites can be divided in to constitutive substances, also called prohibitins or phytoanticipins and induced metabolites formed in response to an infection involving de novo enzyme synthesis, known as phytoalexins [9]. Phytoanticipins are high energy and carbon consuming and exhibit fitness cost under natural conditions [10], but recognized as the first line of chemical defense that potential pathogens have to overcome. In contrast, phytoalexin production may take two or three days, as by definition first the enzyme system needs to be synthesized [11].

Chemical investigation of plant secondary metabolites remains a fertile area of research from multidisciplinary angles with chemists, biochemists and botanists. Isolation, identification biochemical pathways and contribution of these metabolites in the physiology of plants have enormously enriched the volume of data in last few decades. Based on their biosynthetic origins, plant secondary metabolites are divided into four major groups: (i) terpenoids (ii) N-containing alkaloids (iii) sulfur containing compounds and (iv) phenolics (**Table1**). Phenolics are reported as most widely studied compounds amongst them.

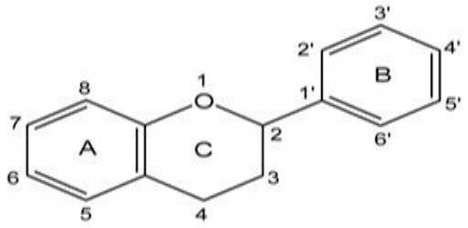
Plant phenolics are synthesized from carbohydrates via shikimate pathway. This is commonly present in plants and microbes as biosynthetic route to aromatic acids. Phenolics are characterized by having at least one aromatic ring with one or more hydroxyl groups at-

tached. In excess of 8000 phenolic structures have been reported and they are widely dispersed throughout the plant kingdom [12]. Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. Based on arrangement of their carbon atoms and the number they are commonly found as conjugated to sugars and organic acids. In general, phenolics are distributed into two groups the flavonoids and the non-flavonoids.

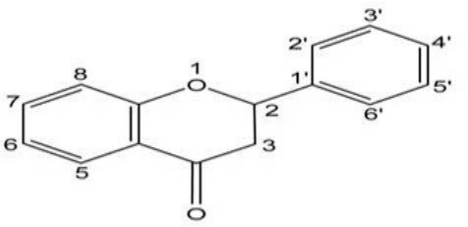
2.1. Flavonoids

Flavonoids are polyphenolic compounds comprising fifteen carbons, with two aromatic rings connected by a three-carbon bridge. They are the most numerous of the phenolics and are found throughout the plant kingdom [13]. They are present in high concentrations in the epidermis of leaves and the skin of fruits and have important and varied roles as secondary metabolites. In plants, flavonoids are involved in such diverse processes as UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance [14]. The main subclasses of flavonoids are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins. Other flavonoid groups, which quantitatively are in comparison minor components of the diet, are dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones and aurones.

2.1.1. Basic structure of flavonoid

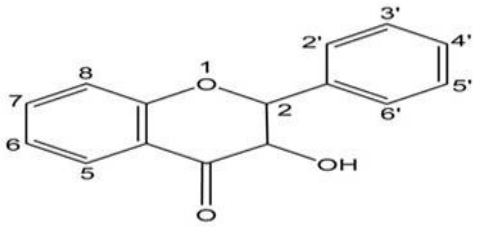


(a) Flavones
(eg. Apigenin, Luteolin, Chrysin)



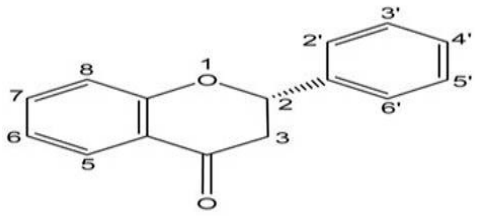
Position	5	7	3’	4’
Apigenin	OH	OH	-	OH
Luteolin	OH	OH	OH	OH
Chrysin	OH	OH	-	-

(b) Flavonols
(eg. Quercetin, Kaempferol, Galangin)



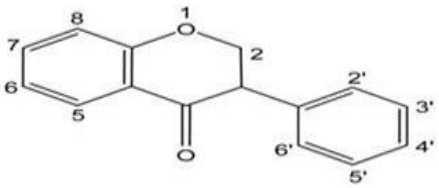
Position	5	7	3’	4’	5’
Quercetin	OH	OH	OH	OH	-
Kaempferol	OH	OH	-	OH	-
Galangin	OH	OH	-	-	-

(c) Flavanone
(eg. Naringenin, Hesperetin)



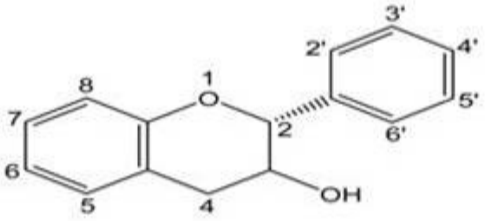
Position	5	7	3’	4’
Naringenin	OH	OH	-	OH
Hesperetin	OH	OH	OH	OCH_3

(d) Isoflavones
(eg. Ganistein, Daidzein)



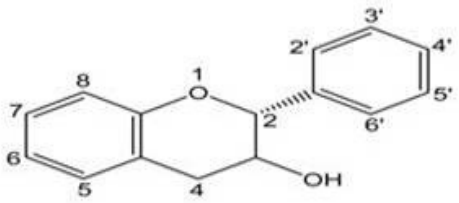
Position	5	7	4’
Ganistein	OH	OH	OH
Daidzein	-	OH	OH

(e) Flavan-3-ol
(eg. (+)- Catechin, (-)-Epicatechin, (-)-Epigallocatechin)



Position	3	5	7	3’	4’	5’
(+)-Catechin	β OH	OH	OH	OH	OH	-
(-)Epicatechin	α OH	OH	OH	OH	OH	-
(-)Epigallocat-echin	α OH	OH	OH	OH	OH	OH

(f) Flavanol
(eg.Taxifolin)



Position	5	7	3’	4’
Taxifolin	OH	OH	OH	OH

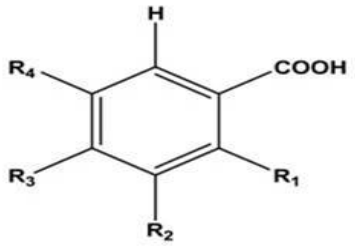
2.2 Non-flavonoids

The main non-flavonoids of dietary significance are the C6–C1 phenolic acids, most notably gallic acid, which is the precursor of hydrolysable tannins, the C6–C3 hydroxycinammates and their conjugated derivatives, and the polyphenolic C6–C2–C6 stilbenes [5].

2.2.1. Phenolic acids

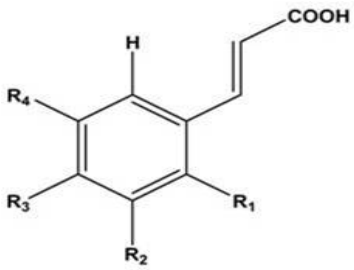
Phenolic acids are also known as hydroxybenzoates, the principal component being gallic acid. The name derives from the French word galle, which means a swelling in the tissue of a plant after an attack by parasitic insects. The swelling is from a build up of carbohydrate and other nutrients that support the growth of the insect larvae. It has been reported that the phenolic composition of the gall consists of up to 70% gallic acid esters [15].

(a) Hydroxybenzoic Acids



Position	R1	R2	R3	R4
Benzoic acid	H	H	H	H
Gallic acid	H	OH	OH	OH
Vanillinic acid	H	OCH_3	OH	H
Salicylic acid	OH	H	H	H

(b) Hydroxycinnamic Acids



Position	R1	R2	R3	R4
Cinnamic acid	H	H	H	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

Figure 2: Structures of the important naturally occurring phenolic acids (a) Hydroxybenzoic Acids (b) Hydroxycinnamic Acids

2.2.2. Stilbenes

Members of the stilbene family which have the C6–C2–C6 structure, like flavonoids, are polyphenolic compounds. Stilbenes are phytoalexins, compounds produced by plants in response to attack by fungal, bacterial and viral pathogens. Resveratrol is the most common stilbene [16].

The phenolics are present in all parts of the plant, however, quantity differ from one part to other and also with the age of the plant. Quantification data of the same species may also vary with ecophysiological conditions. Thus data on quantification of phenolics are often questioned [17] mainly due to diverse extraction and quantification procedure. Infect, determination of phenolics depends on analytic strategy of the selected sample the analytes and nature of the problem. In general, analysis of phenolics includes separation, identification and measurement using range of solvents and their combinations (**Table 2**). In majority of the methods separation is achieved by HPLC, although GC is used in some instances. HPLC is a versatile and widely used technique for the isolation of natural products. HPLC is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture mainly because it offers high performance over ambient pressure [18]. For phenolics, RP-HPLC (reverse phase) is most common mode of separation is explored with a C18 column and variable mobile phases (**Table 2**).

Currently, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the quality control of herbal plants. The resolving power of HPLC is ideally suited to the rapid processing of such multi component samples on both an analytical and preparative scale [19].

HPLC is a dynamic adsoption process and is a separation technique conducted in the liquid phase in which a sample is separated into its constituent components by distributing between the mobile phase and stationary phase. HPLC utilizes a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure [20] .

Reverse-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. It is estimated that over 65% of all HPLC separations are carried out in the reversed phase mode. The reasons for this include the simplicity, versatility and scope of the reverse-phase method as it is able to handle compounds of a diverse polarity and molecular mass e.g. to identify secondary plant metabolites [21].

In addition, the term used for mobile phases in reversed phase chromatography is “buffer”. However, there is little buffering capacity in the mobile phase solutions since they usually contain strong acids at low pH with large concentrations of organic solvents. Adequate buffering capacity should be maintained when working closer to physiological conditions [22].

In order to identify compound by HPLC a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. UV detectors are popular among all the detectors because they offer high sensitivity and also because majority of naturally occurring compounds encountered have some UV absorbance. Photodiode Array (PDA) and UV-VIS detectors at wavelengths 190-380 nm are normally used to identify the phenolics [21].

The high sensitivity of UV detection is bonus if a compound of interest is only present in small amounts within the sample. Besides UV, other detection methods are also being employed to detect phytochemical among which is the Diode Array Detector (DAD) coupled with Mass Spectrometer(MS) [23].

Liquid chromatography coupled with Mass Spectrometry (LC/MS) is also a powerful technique for the analysis of complex botanical extracts. It provides abundant information for structural elucidation of the compounds when tandem mass spectrometry (MS) is applied. Therefore, the combination of HPLC and MS provide better facilities for rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable [24]. HPLC combined with diode array detector (HPLC/DAD), electrochemical detection (HPLC-ED), mass-spectrometer (HPLC/MS) have been successfully employed in qualitative and quantitative determination of various types phytoconstituents including alkaloids, flavonoids, tannins, glycosides, triterpenes, sterols etc [25]. The processing of a crude source material to provide a sample suitable for HPLC analysis as well as the choice of solvent for sample reconstitution can have a significant bearing on the overall success of natural

product isolation [26]. The source material, e.g., dried powdered plant, will initially need to be treated in such a way as to ensure that the compound of interest is efficiently liberated into solution. In the case of dried plant material, an organic solvent (e.g., methanol, chloroform) may be used as the initial extracting and following a period of maceration, solid material is then removed by decanting off the extract by filtration [23]. The filtrate is then concentrated and injected into HPLC for separation. The usage of guard columns is necessary in the analysis of crude extract. Many natural product materials contain significant level of strongly binding components, such as chlorophyll and other endogenous materials that may in the long term compromise the performance of analytical columns. Therefore, the guard columns will significantly protect the lifespan of the analytical columns [22]. So, HPLC is a versatile, reproducible chromatographic technique for the estimation of secondary metabolites in the plants. It has wide applications in different fields in term of isolation, quantitative and qualitative estimation of active molecules. In Table-2 an overview of advanced extraction techniques to isolate and purify of plant based compounds, primarily by HPLC technique is summarized.

An antioxidant by definition is a substance that significantly delays or prevents oxidation of its oxidizable substrate when present at low concentrations compared to those of its substrate (Halliwell and Gutteridge 1989; Halliwell 1990). Packer et al. (1995) stated that many criteria must be considered when evaluating the antioxidant potential of a compound. Some of these concerning chemical and biochemical aspects are: specificity of free radical quenching, metal chelating activity, interaction with other antioxidants, and effects on gene expression [27].

Potential sources of antioxidant compounds have been searched in several types of plant materials such as vegetables, fruits, leaves, oilseeds, cereal crops, barks and roots, spices and herbs, and crude plant drugs. Free radical damages the structural and functional components of the cell such as lipid, protein, carbohydrates, DNA, and RNA. Banana peel contains high content of micronutrient compared to fruit pulp [28]. It attracts great attention because of their nutritional and antioxidant properties, especially the compounds, ascorbate, catechin, gallocatechin, and dopamine. Due to the importance of these compounds, it is necessary to understand its initial production and losses during fruit development, ripening, and maturation [29].

It is well established that phenolic compounds are commonly distributed in plant leaves, flowering tissues and woody parts such as stem and bark. The antioxidant potential of plant materials strongly correlates with their content of the phenolic compounds [30]. In plants, these antioxidant phenolics play a vital role for normal growth and protection against infection and injuries from internal and external sources [31,32].

Different parts of the same plant may synthesize and accumulate different compounds or different amounts of a particular compound due to their differential gene expression, which

in turn affects the antioxidant potential and other biological properties of the plant extracts produced [33,34]. Many studies have confirmed that the amounts and composition of phenolic and flavonoid compounds is diversified at the sub-cellular level and within plant tissues as well [35,36]. Plant phenolics, such as phenolic acids, stilbenes, tannins, lignans, and lignin, are especially common in leaves, flowering tissues, and woody parts such as stems and barks [37].

A universally define acceptable solvent, 80 % MeOH and 70 % EtOH are generally preferred solvents for phenolics extraction from plants [38]. The DPPH (2,2-Diphenyl-1-picrylhydrazyl radical) radical is widely utilized to evaluate the free radical scavenging capacity of antioxidants [39]. The DPPH is one of the few stable organic nitrogen radicals, and has a purple color. The radicals absorb at 517 nm. Antioxidant potential can be determined by monitoring the decrease in the absorbance. The result is reported as the amount of antioxidant utilized to decrease the initial DPPH concentration by 50%. The assay is simple and rapid; however, the interpretation is difficult when the test samples have maximum absorption in the range of UV-light that overlaps with DPPH at 517 nm [38].

The phenolic compounds known for its radical scavengers, therefore, it is worthwhile to determine the phenolic content in the plant chosen for the study [40]. Many available methods of quantification of total, mono and di phenolic content in food products or biological samples are based on the reaction of phenolic compounds with a colorimetric reagent, which allows measurement in the visible portion of the spectrum. The monohydroxy benzoic acids act as very weak antioxidants: owing to the electronegative potential of a single carboxyl group, only m-hydroxy bezoic acid has antioxidative potential. This activity increases considerably in the case of dihydroxy substituted benzoic acids, whose antioxidant response is dependent on the relative positions of the hydroxyl groups in the ring. Gallic acid (3,4,5-trihydroxy benzoic acid) is the most potent antioxidant of all hydroxybenzoic acids [41].

Due to the great variety and reactivity of phenolic compounds, the analysis is very challenging [42]. In the early days of high-performance liquid chromatography, it was stated that: “While LC gives accurate, specific results, it is slow relative to total phenol assay procedures, requires expensive equipments and specialized skills. Moreover, in many cases, the details provided by this method (i.e. relative concentrations of each isomer) are not needed”. Even though some of those claims are basically still valid, the introduction of enhanced resolution and increased automation has resulted in HPLC (also known as high-pressure liquid chromatography) becoming the most popular analysis method for plant phenolics [43] .

3. Conclusion

The most studied bioactivity of the phenols is their antioxidant status. The action of phenols as antioxidants is viewed in plants where phenols are oxidized in preference to other food constituents or cellular components and tissues. Thus, measurement of antioxidant potential of

a phenol or mixture of phenols has been applied. The need for profiling and identifying individual phenolic compounds has seen traditional methods replaced by high-performance chromatographic analyses. The limited volatility of many phenols has restricted the application of GC to their separation. Merken and Beecher (2000) [44] have presented a comprehensive review on the analytical chemistry of food flavonoids in which they present detailed tabulations of columns and mobile phases used in HPLC. The most common mode of separation exploits reversed-phase systems typically with a C18 column and various mobile phases.

Table 1: Number of Secondary Metabolites reported from higher plant (Satyawati and Gupta 1987)

Type of secondary metabolite	Approximate numbers
Nitrogen-containing Secondary metabolites	
Alkaloids	21000
Amines	100
Non-protein amino acids (NPAAS)	700
Cyanogenic glycoside	60
Glucosinolates	100
Alkamides	150
Lectins,peptides,polypeptide	2000
Secondary metabolites without nitrogen	
Monoterpenes including iridoids	2500
Sesquiterpenes	5000
Diterpenes	2500
Triterpenes, steroids, saponins	5000
Tetraterpenes	500
Flavonoids, tannins	5000
Phenylpropanoids, lignin, coumarins, lignans	2000
Polyacetylenes, fatty acid, waxes	1500
Anthraquinones and othes polyketides	750
Carbohydrates, organic acids	200

Table 2: Review on various parameters of phenolic compounds investigated in plants using HPLC analysis.1

Sr No.	Plant name	Plant family	Plant part	HPLC System				Use of the compound
				Column	Mobile phase	Compound extracted		
						Phenols	Flavanoids	
1	*Alpinia officinarum*	Zingiberaceae [46]	air dried leaves powder	A separon SIX C18 (5mm) RP-cartridge 15cm ´3 mm I.D.	methanol- 5mM diammonium hydrogenphosphate pH 7.3 (65:35)	Vinblastin		use in neoplastic diseases
2	*Betula pubescens*	Betulaceae; [47]	leaves	Spherisorb ODS-2 Col.(250×4.6mm i.d.5µm)	A.5% aq.Formic acid B.Acetonitrile	2 acylated compounds (1st time) 1. myricetin-3-O-α-L-(acetyl)-rhamnopyranoside 2. quecetin-3-O-L-(4-O-acetyl)-rhamnopyranoside Chlorogenic acid	Myricetin glycosides Quecetin Glycosides Kaempferol glycosides	Antioxidant activity
3	*Camellia sinensis* L.	Theaceae [48]	dry leaves	2 type column 1. Nova Pak C18-4mm column (3.9mm´15 cm) from waters (miliford MA)	Acetonitrile, ethyl acetate, methanol in combination	Catechins, caffeine		antioxidant, anti-mutagenic, anti-carcinogenic, hypochol-esterolemic activity
				2.Ultrapac Spherisorb ODS 2-3 mm column (4.6mm´10 cm) from LKB (Bromma, Sweden) UV detector	with 0.1% orthophossphoric acid 8.5:2:89.5,v/v/v phase			
4	*Melissa officinalis* L.	Lamiaceae [49]	dried leaves	Lichrocart 125-4 superspher RP 8-E, 4mm (Merck)	A.H_2O-H_3PO_4 85% (100:0.3)	Luteolin derivatives		
					B.MeCN-H2O-H3PO4 85% (80:20:0.3)	Rosmarinic acid		and functoinal gastrointestinal disorders
5	*solanum nigrum*	Solanaceae [50]	root,stem, leaves	ODS-col. 25´0.26 cm	1.CAN 2. 0.01M tris	solasonine, solamargine, solanine (glycoalkaloids)		pharmaceutical industry
6	*Schisandra chinensis Baill.*	Schisandraceae [51]	seeds	separon SGX C18 5mm (150 ´ 3 mm I.D)	methanol- deionised water (75:25)	lignin separation Gomisin A, Gomisin B		prevent liver injuries, lipid peroxidation
		Mangnoliaceae						stimulate liver regeneration
								inhibit hepato-carcinogenesis
7	*Lactuca sativa L.*	Asteraceae [52]	leaves	150´3 mm(5 mm) Luna C18 col. With 4 mm ´ 3 mm I.D. C18 ODS precol.	4 step linear gradient system used starting from 93% water (pH 3.2 by H_3PO_4) upto 75% CH_3CN	caffeic acid, chlorogenic acid isochlorogenic acid polyphenols		treatment of rhinitis, asthma, cough and pertussis
8	*Beet roots*	Amaranthaceae [53]	roots	1. a Li- chrospher 100RP-18 125 ´ 4 mm, 5mm with guard col. 4´ 4mm, 5mm 2. a zorbax SB C8 150´ 4.6 mm, 5 mm guard col.12.5 ´4.6 mm, 5mm	binary gradient mixture of 2. 30mM potassium phosphate buffer at pH 2.3 and acetonitrile	Folates (naturally occuring vitamin B)		health protecting roles

9	*Hamamelis virginiana L* Witch hazel.	Hamamelidaceae [54]	dry twing, bark, leaves	A kingsorb 5mm C18 (150 ´ 4.6mm)	A.0.1% (v/v) orthophosphoric acid in water B. 0.1% (v/v) orthophosphoric acid in methanol	Hamamelitanin catechins gallic acid		used as components of skincare products. in dermatological treatment of sunburn, irritated skin, atopic eczema. to promote wound healing via anti-inflammtory effects
10	*Centaurium erythraea*	Gentianaceae [55]	Micropropagate plant	hypersil ODS col. (250 ´ 4 mm 5mm hewlett packard	1.ACN 2.3% v/v acetic acid	secoiridoid glucosides gentiopioside, sweroside, swertiamarine		fungitoxic, antibacterial, choleretic, pancreatic, hepato-protective
11	*Alpinia officinarum Hance(AO)*	Zingiberaceae [56]		RP-col.(ZOR BAX, Eclipse SB- C18 5mm, 4.6 ´ 250 mm) C18 guard col.	methanol-water-phosphoric acid(60-38-2,v/v/v isocratically		Flavonoid Galangin 3-O-methayl galanin	anticlastogenic, anti-mutagenic, anti-oxidative, radical scavenging hypolipidemic agent due to its inhibition of pancreatic lipase
12	*Piper regnellii (Miq.) C.DC. Var*	Piperaceae [57]	dried root stem leaves	Metasil ODS col. 5mm 150mm ´4.6 mm	mixture of acetonitrile-water(60:40 v/v)	conocarpan (neolignan)		use for treatment of wounds, swellings, skin irritations
	Pallescens (C.DC.) Yunck				containing 2% acetic acid	eupomatenoid-5 eupomatenoid-6		
13	*Platycladus orientalis L.*	Cupressaceae [58]	leaves	Agilent Eclipse XDB-C18 Col. (3.5mm,12.5mm´4.6 mm I.D.)	methanol and CAN		flavanoids	antioxidant, antiallergic diuretic properties
	Franco						Quercitrin amentoflavone	use for treatment of gout, rheumatism, diarrhea
14	*Paeonia lactiflora* oriental medicinal plant	Paeoniaceae [59]	dry plant	Inertsil ODS-3 C18 (250 ×6mm) 5mm I.D C18 guard col.	acetonitrile- water (gradient HPLC method	Paeoniflorin, albiflorin		cleansing heat, cooling blood, invigoraing blood circulation
15	*Prunus x domestica* L.	Rosaceae [60]	fruit, leaf, leaf petiole	SGE Walkosil 11 5C18 RS column (150 ´ 4.6 i.d.5mm particles	50 mM NaC_{14} in 0.1% H_3Po_4	Ghrelin hormone		promotes food intake, weight gain and adiposity in rodents
	Marus alba	Moraceae		120 A Pore size		(in Parenchyma cells)		
16	*Vitex-agnus-castus*	Verbenaceae [61]	dry leaves	5mm ODS 2.4´250 mm at ambient temp.	A. water with 1% glacial acetic acid	Caffeic acid		Antioxidant activity
					B. water with 6% glacial acetic acid	Ferulic acid		
					C. water acetonitril(65:30 v/v) with 5% glacial acetic acid	Rutin, p-Coumaric acid		
17	*Origanum dictamnus*	Lamiaceae [61]	dry leaves	5mm ODS 2.4´250 mm at ambient temp.	A. water with 1% glacial acetic acid	Catechin		Antioxidant activity
					B. water with 6% glacial acetic acid			
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid			

18	*Teucrium polium*	Lamiaceae [61]	dry leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	Tyrosol		Antioxidant activity
					B. water with 6% glacial acetic acid	Caffeic acid		
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	Ferulic acid, luteolin		
19	*Lavandula vera*	Lamiaceae [61]	dry leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	p-hydroxybenzoic acid		Antioxidant activity
					B. water with 6% glacial acetic acid	Catechin		
					C. water acetonitril(65:30 v/v) with 5% glacial acetic acid	Vanillic acid Caffeic acid, Ferulic acid, Naringenin		
20	*Lippia triphylla*	Verbenaceae [61]	dry leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	Hydroxytyrosol		Antioxidant activity
					B. water with 6% glacial acetic acid	Caffeic acid		
					C. water acetonitril (5:30 v/v)with 5% glacial acetic acid	Ferulic acid, Apigenin		
	Greek aromatic olive plants							
21	*Capparis spinosa*	Capparaceae [61]	leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	Hydroxytyrosol	Quercetin	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	Caffeic acid	Rutin	
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	p-Coumaric acid, Vanillic acid, Ferulic acid, Gallic acid		
22	*Castanea vulgaris*	Cupuliferae [61]	leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gallic acid	quercetin	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	ferulic acid	rutin	
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid		naringenin	
23	*Geranium purpureum*	Geraniaceae [61]	leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gallic acid	quercetin	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	gentisic acid	rutin	
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	caffeic acid, p-Coumaric acid, vanillic acid, syringic acid, p-hydroxybenzoic acid		
24	*Nepeta cataria*	Labiateae [61]	herb	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gallic acid	luteolin	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	caffeic acid	eriodictyol	
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	ferulic acid		
25	*Origanum dictamnus*	Labiateae [61]	leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gallic acid	quercetin	significance for human diet and antimicrobial activity

					B. water with 6% glacial acetic acid	caffeic acid		
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	p-Coumaric acid, p-hydroxybenzoic acid		
26	*Spartium junceum*	Leguminosae [61]	flower	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gentisic acid	luteolin	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	caffeic acid	quercetin	
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	p-Coumaric acid, vanillic acid		
27	*Jasminum officinalis*	Oleaceae [61]	flower	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gallic acid	quercetin	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	p-Coumaric acid		
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid			
28	*Phytolacca americana*	Phytolaccaceae [61]	leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	caffeic acid	rutin	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	p-Coumaric acid		
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	vanillic acid, p-hydroxybenzoic acid, ferulic acid		
29	*Ruta graveolens*	Rutaceae [61]	leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid B. water with 6% glacial acetic acid	ferulic acid, p-hydroxybenzoic acid, gentisic acid, caffeic acid	Quercetin, rutin	significance for human diet and antimicrobial activity
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid			
30	*Styrax officinalis*	Styracaceae [61]	leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gallic acid	quercetin	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	gentisic acid	naringenin	
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	caffeic acid, p-Coumaric acid, vanillic acid, p-hydroxybenzoic acid, ferulic acid		
31	*Cuminum cymium*	Umbelliferae [61]	seeds	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gallic acid	rutin	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	caffeic acid	quercetin	
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	ferulic acid	naringenin	
32	*Foeniculum vulgare*	Umbelliferae [61]	herb	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gallic acid	rutin	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	caffeic acid	quercetin	
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	ferulic acid		

33	*Himulus hipulus*	Urticaceae [61]	leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gallic acid	eriodictyol	significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	p-Coumaric acid	quercetin	
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	p-hydroxybenzoic acid		
34	*Urtica dioica*	Urticaceae [61]	leaves	5mm ODS 2.4′250 mm at ambient temp.	A. water with 1% glacial acetic acid	gallic acid		significance for human diet and antimicrobial activity
					B. water with 6% glacial acetic acid	syringic acid		
					C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	ferulic acid		
35	*Canarium album* L.	Burseraceae [62]	fruit	RP-C18(250mm′4mm)	A.0.5% (v/v) acetic acid	Gallic acid	1st reported	used for the treatment of faucitis, stomatitis, hepatitis, toxicosis
	chinese olive fruit		budding to flower stage		A. methanol	methyl gallate, ethay gallate, ellagic acid, brevifolin carboxylic acid, sinapic acid, hyperin		
					B. water with 0.2% sulphuric acid	rosmarinic acid, vanillic acid, chlorogenic acid, gallic acid, cinnamic acid	Luteolin, coumarin, rutin	strong antioxidant activity, oil as antimicrobial agent
36	*Tobacco*	Solanaceae [63]	dried leaves	Hypersil C4 (4.6mm ′ 150 mm,5mm	acetonitrile and water	solenosol		cardiac stimulant lipid antioxidant antibiotic
37	*Echinacea pallida* (Nutt)	Asteraceae [64]	dry root/capsules	1.as above 2. chromolith performance RP-C18 (100mm′4.6mm guard col. RP-C18 (5mm′4.6mm)	1.water 2.ACN	Polyacetylenes polyenes		antifungal, antibacterial compound inhibitor of no. of enzymes eg. Cholesterol, acytransferase
38	*Borago officinalis L.*	Boraginaceae [65]	leaves	TOSO HAAS Semi-micro ODS-80 TS col. (5mm,2mm′ 25 cm)	A. 2% acetic acid B. acetonitrile	rosmarinic acid		antioxidant and antiradical activity
39	*Alpinia zerumbet*	Zingiberaceae [66]	leaves, roots	RP-18 ZORBAX ODS col. 25 ′ 0.46 cm 5mm particle size	A.1% v/v acetic acid B. methanol/ acetonitrile/ acetic acid (95:4:1 v/v/v)	from leaves oil 1,8 cineol, methyl cinnamate	Rhizomes DDK, methyl cinnamate, dihydro-5,6-dehydrokawain	insecticidal antifungal activity
40	*Alpinia zerumbet*	Zingiberaceae [66]	flower, seeds	RP-18 ZORBAX ODS col. 25 ′ 0.46 cm		Flower	seeds	antioxidant activity
				5mm particle size	A.1% v/v acetic acid B. methanol/ acetonitrile/ acetic acid (95:4:1 v/v/v)	syringic acid, p-hydroxybenzoic acid, ferulic acid	p-hydroxybenzoic acid, vanillic acid, syringic acid	used for the treatment of cardiovascular hypertensions, antipasmodic agent
41	*Anisophyllea dichostyla R.Br.*	Rhizophoraceae [67]		C18-RP col. (250′4mm 5mm)	1.2% acetic acid 2.methanol	catechins ellagic acid derivatives epicatechin		medication against anorexia, fatigue and intestinal infection

42	*Hippophae rhamnoids*	Elaeagnanceae [68]	SB berriers, leaves	phenomenex c18,ODS-2,5mm, 250mm´4.6mm	A.2% acetic acid B.metanol	protocatechuic acid, p-hydroxybenzoic acid, cinnamic acid, vanillic acid, gallic acid,		antitumor, antiviral, antioxidant properties, medicinal and cosmetic applications
	Sea Buckthorn					caffeic acid, p-Coumaric acid, ferulic acid, salicyclic acid		
43	*Vitis vinifera* L. *(white)*	Vitaceae [69]	Grapes	C18 kromasil 300mm´4.6mm 5mm particle size	A.acetonitrile/ acetic acid/ water(35:2:63) B. 2% acetic acid	5 DHF (dihydroflavonols) 7Q (quercetin derivatives), 4 Kaempferol derivatives		produce high quality wine
44	Cymbopogon citrates *lemon grass*	Gramineae [70]	leaves	Spherisorb.S5 ODS-2 column (250 ´4.6mm I.D 5mm) guard column C18 (30 ´ 4 mm I.D 5mm)	A. 5% aqueous formic acid v/v B. methanol	O and C glycosyl flavones Apigenin, loteolin		anti inflammatory, diuretic activities Hypotensive, vasorelaxating
45	*Rosmarinus officinalis*	Labiatae [71]	Oil	5mm ODS 2.4´250 mm at ambient temp.	A. water with 1% glacial acetic acid B. water with 6% glacial acetic acid C. water acetonitril(65:30 v/v)with 5% glacial acetic acid	syringic acid ferulic acid		multiple biological effect such as antioxidant activity antimicrobial activity prevention of human pathologies
46	*Origanum dictamnus*	Labiatae [71]	oil	5mm ODS 2.4´250 mm at ambient temp.	A. water with 1% glacial acetic acid B. water with 6% glacial acetic acid C. water acetonitril(65:30 v/v)with 5% glacial acetic acid	caffeic acid	Naringenin, eriodictyol	multiple biological effect such as antioxidant activity antimicrobial activity prevention of human pathologies
47	*Origanum majorana*	Labiatae [71]	oil	5mm ODS 2.4´250 mm at ambient temp.	A. water with 1% glacial acetic acid B. water with 6% glacial acetic acid C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	high ferulic acid	Catechin, rutin, quercetin	multiple biological effect such as antioxidant activity antimicrobial activity prevention of human pathologies
48	*vitex-agnus-cactus*	Verbenaceae [71]	oil	5mm ODS 2.4´250 mm at ambient temp.	A. water with 1% glacial acetic acid B. water with 6% glacial acetic acid C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	P-hydroxybenzoic acid		multiple biological effect such as antioxidant activity prevention of human pathologies
49	*styrax officinalis*	Styracaceae [71]	oil	5mm ODS 2.4´250 mm at ambient temp.	A. water with 1% glacial acetic acid B. water with 6% glacial acetic acid C. water acetonitril (65:30 v/v) with 5% glacial acetic acid	vanillic acid		multiple biological effect such as antioxidant activity antimicrobial activity prevention of human pathologies

50	*Ephedra sinica stapfs*	Ephedraceae [72]		HS F5 col. (150mm ´ 4.6 mm I.D.5mm	Isocratic ammonium acetate (7mM) in acetonitrile-water (90:10v/v)	ephedrin alkaloids		for treatment of asthma, bronchial spams, as a stimulant and diaphoretic
	Ephedra vulgaris Rich.				ammonium acetate (7mM) in acetonitrile-water (90:10v/v)	Synephrine Norephedrine Norpseudoephedrine Ephedrine		Used as a as a stimulant and diaphoretics
51	*Eucommia ulmodies Oliver.*	Eucommiaceae [73]	dried leaves	C18 col.(150mm ´ 4.6 mm I.D.5mm)	1. methanol 2. 0.5% acetic acid	chlorogenic acid		antibacterial, antimutagenic, antioxidant, quality control
52	*Eugenia jambolana Lam.*	Myrtaceae [74]	bark	ODS RP C18 col.(250 ´4.6 mm, 5mm	1. 3% trifluoroacetic acid 2. acetonitrile-methanol (80:20 v/v)		Quercetin Kampeferol total flavonols	antioxidant, antiallergic, anti-artherogenic, anti-inflammatory, antimicrobial antihrombotic, cardio-protective, vasodilatory effect
53	*Acacia nilotica*	Fabaceae [74]	bark	ODS RP C18 col.(250 ´4.6 mm, 5mm	1. 3% trifluoroacetic acid 2. acetonitrile-methanol (80:20 v/v)		Myricetin Quercetin Kampeferol total flavonols	antimicrobial antihrombotic, cardio-protective, vasodilatory effect
54	*Azadirachta indica*	Meliaceae [74]	bark	ODS RP C18 col.(250 ´4.6 mm, 5mm	1. 3% trifluoroacetic acid 2. acetonitrile-methanol(80:20 v/v)		Quercetin Kampeferol total flavonols	antimicrobial antihrombotic, cardio-protective, vasodilatory effect
55	*Terminalia arjuna*	Combretaceae [74]	bark	ODS RP C18 col.(250 ´4.6 mm, 5mm	1. 3% trifluoroacetic acid 2. acetonitrile-methanol (80:20 v/v)		Quercetin total flavonols kampeferol	antimicrobial antihrombotic, cardio-protective, vasodilatory effect
56	*Moringa oleifera*	Moringaceae [74]	leaves, roots	ODS RP C18 col.(250 ´4.6 mm, 5mm	1. 3% trifluoroacetic acid 2. acetonitrile-methanol (80:20 v/v)		**Leaves** Myricetin Quercetin Kampeferol total flavonols	antimicrobial antihrombotic, cardio-protective, vasodilatory effect
							Roots Myricetin Kampeferol total flavonols	antimicrobial antihrombotic, cardio-protective, vasodilatory effect
57	*Ficus religiosa*	Moraceae [74]	fruit	ODS RP C18 col.(250 ´4.6 mm, 5mm	1. 3% trifluoroacetic acid 2. acetonitrile-methanol (80:20 v/v)		Myricetin Kampeferol Quercetin total flavonols	antimicrobial antihrombotic, cardio-protective, vasodilatory effect
58	*Aloe barbadensis*	Asphodelaceae [74]	leaves	ODS RP C18 col.(250 ´4.6 mm, 5mm	1. 3% trifluoroacetic acid 2. acetonitrile-methanol (80:20 v/v)		Myricetin Kampeferol Quercetin total flavonols	antimicrobial antihrombotic, cardio-protective, vasodilatory effect

59	*Pinguicula lusitanica* L. Pale butter wort carnivorous perennial plant	Lentibulariaceae [75]	invitro plantlets used	An alltima HP 3mm (150 ´ 4.6 mm I.D.)	A. 0.1% v/v formic acid B. acetonitrile	iridoid glucosides, Caffeoyl Phenylethanoid Glycosides a. globularin b. verbascoside		imp role in defense mechanism against herbivores. resistance to or protection from fungal and viral attacks
60	*Viburnum prunifolium*	Caprifoliaceae [76]		A Pinnacle ODS amine C18 (250mm´4.6mm, 5mm protected by	As above	iridoidic component		
	Black Haw			ODS amine C18 guard col. (10mm ´4 mm,5mm)		iridoid glucosides		
61	*Glycine max L.*	Fabaceae [77]	seed	RP-C18 col. (125mm ´ 4mm, Lichro) (ART,5mm Merck K GaA)	1. 0.1% acetic acid in water	9 type anthocyanin		Detoxification
	black soyabean				2. 0.1 % acetic acid in CAN	1. catechin-cyanidin-3-O-glucoside 2. delphinidin-3-O-galactoside 3. delphinidin-3-O-glucoside 4. cyanidin-3-O-Galactoside 5. cyanidin-3-O-glucoside		anti inflammatory
						6. petunidin-3-O-Glucoside 7. pelargonidin-3-O-glucoside 8. peonidin-3-O-glucoside 9.cyanidin		to improve blood process
62	*Cannabis sativa L.*	Cannabinaceae [78]		waters Xterra MS C18 analytical col. (5mm,250mm ´ 2.1mm)	mixture of methanol/ water containing 50mM of	Cannabinoids, D9 Tetrahydrocannabinol (THC), THC acid (THCA), cannabidiol (CBD)		psychoactive properties
				(5mm 10mm ´ 2.1mm guard col.	ammonium formate	CBD acid (CBDA), cannabigerol (CBG), CBG acid (CBGA), cannabinol (CBN), D8-tetrahydro cannabinol(8- THC)		
63	*Origanum majorana*	Lamiaceae [79]	dried aerial part	250mm´4.6mm, 4mm Hypersil ODS C18 column	A.Acetonitrile	trans-2 hydroxy cinnamic acid	Amentoflavone, apigenin, quercetin	used as fungicides and insecticides
			budding to flower stage		B.water with 0.2% sulphuric acid	rosmarinic acid, vanillic acid, chlorogenic acid, gallic acid, cinnamic acid	Luteolin, coumarin, rutin	strong antioxidant activity, oil as antimicrobial agent
64	*Microula sikkimensis*	microula benth family [80]	dried seeds	Eclipse XDB C8 col. (150mm ´4.6mm, 5mm)	A. 50% of acetonitrile	fatty acids		imp in treatment for cardiovascular and hepatic disease
	rare wild oil plant				B. 50% of acetonitrile containing 20mM/L	linolenic acid		
					ammonium formate buffer (pH 3.7)	linolic acid		
					C. mixture solution of acetonitrile and DMF	saturated and unsatutated acid		

					(acetonitrile/ DMF, 100:2 v/v)			
					(acetonitrile/ DMF, 100:30 v/v)			
65	*Rheum emodi*	Polygonaceae [81]	rhizomes	A Purospher- star RP-18e colu.	1.ACN-methanol (95:5 v/v)	Anthraquinone derivatives	1 to 3 6 month TC plant	antifungal, antimicrobial, cytotoxic, antioxidant activities
			tissue culture plant use	4.6mm i.d.´250 mm , 5mm	2. water- acetic acid (99.9-0.1 v/v)	1. emodin glycoside 2. chrysophanol glycoside 3. emodin 4.chrysophanol 5.physcion	9 month TC plant	
66	*Cordia americana*	Boraginaceae [82]	leaves	RP-C18 (5mm´100 mm:5mm)	A.water- acetonitrile: formic acid (90:10:0.1)			anti-inflammatory, wound healing activities
					B.Acetonitrile-formic acid (0.1%)			
67	Allium sativum	Liliaceae [83]	root, shoot, bulbs, leaves	C18 Nucleosil 100 ODS (5mm), analytical col.4.6mm ´ 150mm	methanol- water (50:50 v/v)	allicin		anti diabetic activity IN VITRO antimicrobial, anto-thrombotic, anticancer, antioxidant
	green garlic plant (immature)			C18 guard col.with 20ml loop.				IN VIVO cardiovascular disorders, arteriosclerosis
68	*Rheum emodi*	Polygonaceae [83]	rhizomes	A Purospher- star RP-18e colu.	1.ACN-methanol (95:5 v/v)	Anthraquinone derivatives	1 to 3 6 month TC plant	antifungal, antimicrobial, cytotoxic, antioxidant activities
			tissue culture plant use	4.6mm i.d.´250 mm , 5mm	2. water- acetic acid (99.9-0.1 v/v)	1. emodin glycoside 2. chrysophanol glycoside 3. emodin 4.chrysophanol 5.physcion	9 month TC plant	
69	*Aristolochia species*	Aristolochiales [84]		X Terra MS C18 (150 mm´ 2.1 mm, I.D.5mm	0.2% formic acid water and acetonitrile	Aristolochic acids (Aas)		anti inflammatory agents for arthritis, gout, rheumatism and dieresis
	1. Radix aristolochia					Aristoloctams(Als)		
	2. Caulis aristolochia anshurinensis							
	3.Fructus aristolochia							
70	*Banisteriopsis caapi.*	Malpighiaceae [85]	fresh leaves, stem	Gemini C18 110A° col.	for catechin analysis 1. water 2. acetonitrile	Harmine Harmaline tetrahydro harmine		responsible for mono-aminooxidase (Mao)A inhibitor

			large branch	Phenomax, 150mm ´4.6mm I.D.5mm	for alkaloid analysis 1.acetonitrile containing 0.1% acetic acid 2. 50mM ammonium acetate (pH 4.2)	proanthocynidines like epicatechin		procynidine produce antioxidant effect
71	*C. annuum* L.	Solanaceae [86]	Ripe paprika	C18 Phenomenex column (Torrance, CA, USA) Gemini series (250 ×4.6 mm i.d., 5 lm particle size)	A .(0.03 M phosphoric acid in water) and B. (MeOH)		Quercetin Luteolin Kaempferol	High antioxidant and anticancer activities
72	*Eucommia ulmoides* Oliv.	Eucommiaceae [87]	Dried leaves	reversed phase SunFire™ C18 (250 mm _ 4.6 mm i.d., 5 lm, Milford, MA, USA) column.	A (0.4% acetic acid in water) and B (acetonitrile),	1, geniposidic acid; compound 2, caffeic acid; compound 3; chlorogenic acid; compound 4, ferulic acid; compound 5, quercetin-3-O-sambubioside; compound 6, rutin; compound 7, isoquercitrin.		antioxidant activity (Cho et al., 2003; Yen & Hsieh, 1998), glycation inhibitory activity (Kim, Moon, Lee, & Choi, 2004) and anti-obesity activity
73	Artichoke (*Cynara scolymus* L.)	Asteraceae 88]	Fresh artichoke samples (hearts)	Agilent Zorbax C18 column (4.6 _ 150 mm, 1.8 lm)	A. Acidified water (0.5% acetic acid, v/v) and B. acetonitrile	3 hydroxybenzoic acids, 17 hydroxycinnamic acids, 4 lignans, 7 flavones, 2 flavonols, and 1 phenol derivative		antioxidative, anti-carcinogenic, antigenotoxic, cholesterol-lowering, hepato-protective, bileexpelling, diuretic, and anti-inflammatory, as well as antifungal, anti-HIV, and antibacterial
74	sarang semut (*Myrmecodia pendan*).	Rubiaceae [89]	powder	Luna 5U-C18 (2) 100A column (250 mm ×4.5 mm, 5 _m) plus Jasco, quaternary gradient pump (pu-2089) plus Jasco	A. deionized water and 1% acetic acid B. methanol (HPLC grade) and 1% acetic acid		kaempferol, luteoline, rutine, quercetine and apigenin)	antioxidant activities (Tian et al., 2009), metal chelation (Heim et al., 2002; Seyoum et al., 2006) and anti-proliferative, anti-carcinogenic, antibacterial, anti-inflammatory, antialergic, and antiviral effects
75	*Convolvulus pluricaulis* Shankhpushpi	Convolvulaceae [90]	leaves	Phenomenex C18 column (250 mm × 4.6 mm, 5 µm) (California, USA)	a isocratic mixture of methanol and water containing 0.1% v/v formic acid in the ration of 30: 70.	scopoletin		to treat chronic bronchitis and asthma.
76	*Glycyrrhiza glabra* Linn.	Fabaceae [91]	roots	C-18 reverse phase column (250 x 4.6 mm internal diameter, particle size 5 µm, Luna 5 µm C-18),	methanol: water (70:30 v/v)	important metals like Ca, K, Fe and Mg		Antimicrobial activity
77	water watercress (*Nasturtium officinale*–	Brassicaceae [92]	Dried material	RP-C18 column (4.6 mm × 250 mm) packed with 5-µm diameter particles	methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0% of acetic acid	Rutin, chlorogenic, and caffeic acids		Antioxidant activity

78	rose hip (Rosa L.) *Rosa canina, Rosa dumalis, Rosa gallica, Rosa dumalis* subsp.boissieri and *Rosa hirtissima*	Rosaceae [93]	Fruits	C18 (250 × 4.6 mm I.D.)	(A) water/acetic acid (98:2) and (B) water/ acetonitrile/acetic acid(78:20:2).	gallic acid, 4-hydroxy benzoic acid, caftaric acid, 2,5-dihidroxy benzoic acid, chlorogenic acid, t-caffeic acid, p-coumaric acid and ferrulic acid	methyl gallat, (þ)-catechin and (_)-epicatechin	Strong antioxidant activities
79	Emblica officinalis	Phyllanthaceae [94]	fruit	A reversed-phase column, Zorbax SB RP C-18 (250mm_4.6mm_5 mm pore size),	0.1% orthophosphoric acid in water (v=v) and acetonitrile	vitamin C (ascorbic acid), phenolic acids (gallic acid and ellagic acid), hydroxycinnamic acid (chlorogenic acid)	myricetin, quercetin, and kaempferol	Use for cancer, cardio-vascular disorders, ageing, diabetes, and Hypertension
80	Swertia chirayita *S. minor, S. densifolia, S. lawii, S. corymbosa and S. angustifolia var.* *pulchella*	Gentianaceae [95]	Powder of whole dry plant	C18e (5 mm) column (250–4.6 mm).	methanol and water (90:10)	BA, betulinic acid; OA, oleanolic acid; UA, ursolic acid		chronic fever, malaria, anaemia, bronchial asthma, liver disorders, hepatitis, gastritis, constipation, dyspepsia
81	*Annona muricata*	Annonaceae [96]	Dried leaf powder	a Waters Symmetry® C18 column (5 mm, 4.6×50 mm) with Waters Sentry TM universal guard column (5 mm, 4.6×20 mm)	A (50 mM sodium phosphate in 10 % methanol; pH 3.3) and B (70 % methanol)	Cinnamic acids (-) –Epicatechin gallate Coumarid acid Anthraquinones Isoferulic acid	Quercetin Luteolin	anti-spasmodoic, hypotensive
82	*Schinopsis brasiliensis* Engl.,	Anacardiaceae [97]	Stem bark	a Phenomenex Gemini NX C18 column (250 × 4.6 mm, 5 _m).	0.05% orthophosphoric acid: methanol	Gallic acid		uses for the treatment of diar-rhea and coughs, and can also be used as an antiseptic and analgesic
83	*Ziziphus joazeiro*	Rhamnaceae [98]	leaves	HPLC–DAD) a Phenomenex C18 column (4.6 mm _ 250 mm) packed with 5-lm diameter particles	A.water containing 1% formic acid and B.acetonitrile	gallic acid, caffeic acid, ellagic acid, catechin and epicatechin	quercetin, isoquercetin, quercitin, kaempferol and rutin	antifungal (Cruz et al., 2007), gastro-protective (Romão, Costa, Terra, Boriollo, & Soares, 2010) and anti-microbial properties (Silva et al., 2011).
84	*Corylus maxima* Mill.	Betulaceae [99]	leaves	a Zorbax SB C18 col-umn (150 mm × 3.0 mm, 3.5 _m;	0.2% (v/v) acetic acid, methanol	myricetin-3-O-rhamnoside and quercetin-3-O-rhamnoside – and two diarylheptanoids – oregonin and hirsutenone		Antioxidant activity
85	*Paronychia argentea* Lam.,	Caryophyllaceae [100]	Aerial part	HPLC-UV/DAD conditions and HPLC–ESI-MSn conditions an Ascentis C18column (250 mm × 4.6 mm I.D.,5 µm,	HPLC-UV/DAD conditions (A) 0.1 M HCOOH in H2O and (B) ACN		isorhamnetin-3-O-dihexoside, quercetin-3-O-glucoside, quercetin methylether-O-hexoside, quercetin, jaceosidin and isorhamnetin (1st time)	Antioxidant activity
86	Libyan herb species, *viz Sage, Thymus,* *Rosemary, Chamomile, Artemisia*	Lamiaceae [101]		C18 reversed-phase analytical column, 5 µm particle size, with dimension 250 × 4.6 mm	A.Buffer solution B.methnol	Rutin Ascorbic acid		antibacterial, anti inflammatory, antitumor, antiallergic, antiviral and antiprotozoal.

87	*Rheum spiciforme* & *Rheum webbianum*	Polygonaceae [102]	Root and rhizomes	C18 column (250 mm× 4.6 mm; Sunfire)	A.methanol B.2% acetic acid	Emodin Aloe Emodin Rhein		anti-cancer and anti-oxidant activities.
88	*Limonium brasiliense* (Boiss.) Kuntze,	Plumbaginaceae [103]	rhizome	an Agilent Zorbax C-18 (250 mm × 4.6 mm) 5 _m column	A.water: concentrated phosphoric acid (100:0.2, v/v,) and B.acetonitrile: concentrated phosphoric acid (100:0.2, v/v,)	GC, gallocathechin; EGC, pigallocatechin.		Anticancer and antioxidant activity
89	*H. perforatum* (St. John's wort), *L. angustifolia* (lavender), *M. sylvestris* (tall mallow), *M. officinalis* (lemon balm), *S. officinalis* (sage) and *R. officinalis* (rosemary)	Lamiaceae [104]	leaves	LiChrospher_ 100, RP-18 (250 _ 4 mm, 5 lm) column,	A (methanol), B (acetonitrile) and C (0.3% trichloroacetic acid in water)	Rosmarinic acid		Antioxidant activity
90	*Phoenix dactylifera* L.)	Arecaceae [105]	Date fruits	an Atlantis C18 column (150 * 4.6 mm, 5 lm particle size)	0.1% (v/v) formic acid in water (eluent A) and acetonitrile (eluent B).	Rutin, Sinapic acid, Ferulic acid, Coumaric acid, Syringic acid, Caffeic acid, Vanillic acid, Catechin, Gallic acid		Antibacterial and cytotoxic activity
91	Kumquat (*Citrus japonica* var. margarita)	Rutaceae [106]	fruit	RP-C18 column (250 mm _ 4.6 mm, 5 lm,	A. deionized water B. acetonitrile		C-glycosides 30,50-di-C-b -glucopyrano sylphloretin (DGPP), acacetin 8-C-neohesperidoside (margaritene), acacetin 6-C-neohesperidoside (isomargaritene), apigenin 8-C-neohesperidoside, and Oglycosides, such as acacetin 7-O-neohesperidoside (fortunellin), isosakuranetin 7-O-neohesperidoside (poncirin) and apigenin 7-O-neohesperidoside (rhoifolin).	Antioxidant activity
92	*Raphanus sativus* L. var. caudatus Alef	Brassicaceae [107]		Reverse Phase-C18 column (5 lm particle size, 250 × 4.6 mm)	isocratic 5% THF-95% water	Sulforaphene Sulforaphane		Anticancer activity
93	*A. barbadensis*	Asphodelaceae [108]		An Optimapak C18column (250 × 4.6 mm, 5 µm, RStech, Seoul, Korea)	A. 0.1% phosphoric acid solution and B. 100% ace-tonitrile	3: aloin.		sorethroats and diarrhea
	Catechu	Leguminosae		As above		1: (+)-catechin, 2: (−)-epicatechin,		
	Uncaria gambir	Rubiaceae		As above		1: (+)-catechin, 2: (−)-epicatechin,		

94	Salvia cadmica	Lamiaceae [109]		Eclipse XDB C-18 reversed-phase column (250 mm × 4.6 mm length, 5 _m particle size	methanol	(1. Gallic acid, 2.Protocatechuic acid, 3. (+)-Catechin, 4. p-Hydroxybenzoic acid, 5. Chlorogenic acid, 6. Caffeic acid,7. (−)-Epicatechin, 8. Syringic acid, 9. Vanillin, 10. p-Coumaric acid, 11. Ferulic acid, 12. Sinapinic acid, 13. Benzoic acid, 14. o-Coumaric acid, 15. Rutin, 16. Hesperidin, 17.Rosmarinic acid, 18. Eriodictyol, 19. trans-Cinnamic acid, 20. Quercetin, 21. Luteolin, 22. Kaempferol, 23. Apigenin		
95	*Rheum emodi*	Polygonaceae [102]		a C18 column (250 mm x 4.6 mm; Sunfire)	A. methanol and B. 2% acetic acid	Aloe-emodin, emodin and rhein		antiviral, antimicrobial and hepato-protective activities
96	*Zanthoxylum canthopodium*	Rutaceae [110]	leaves	AcclaimTM120 C 18 column (5 µm particle size, 250 x 4.6 mm)	acetonitrile and 1% aq. the acetic acid	ascorbic acid, free phenolic acids such as gallic acid, methyl gallate, caffeic acid, syringic acid, ferulic acid, para (*p*)-coumaric acid, sinapic acid	(catechin, rutin, quercetin, myricetin, apigenin and kaempferol),	Antioxidant activity
97	*Ornithogalum species* *Ornithogalum virens*, *Ornithogalum thyrsoides, Ornithogalum dubium,*	Asparagaceae [111]	bulb	Acclaim TM 120 C18 (25 cm x 4.6 mm, 5 µm)	1% aq. acetic acid (Solvent A) and acetonitrile (Solvent B),	gallic acid, caffeic acid, p-coumaric acid, syringic acid, sinapic acid, ferulic acid,	catechin, rutin, apigenin, quercetin, myricetin, and kaempherol	useful in treatments of stomach upsets like gastric ulcers, peptic ulcers, duodenal ulcers, acidity, etc. showed anticancer, antimicrobial, cytotoxic and antioxidant properties
98	Salvia L. species namely *S. brachyantha* (Bordz.) Pobed, *S. aethiopis* L., and *S. microstegia* Boiss. and Bal.	Lamiaceae [112]	Plant powder	C18 reversed-phase Inertsil ODS-4 (150 mm _ 4.6 mm, 3 _m, GL Sciences, Tokyo, Japan) analytical column	A. water, 5 mM ammonium formate and 0.1% formic acid B. methanol, 5 mM ammonium formate, and 0.1% formic acid	apigenin, luteolin, p-coumaric acid, and chlorogenic acid.	quercetin, myricetin, and kaempherol	anticancer, antimicrobial, antioxidant properties
99	*Coffea arabica*	Rubiaceae [113]	leaves	HPLC-UV C18, reverse-phase (5 µ), Gemini column (250 × 4 mm I.D.; Phenomenex,	A. 2 % acetic acid in water B. acetonitrile	Isomangiferin . Mangiferin		health-promoting phenolic compounds.
100	*Zanthoxylum naranjillo* and *Z. tingoassuiba*	Rutaceae [114]	Leaves and stems	a Shimadzu Shim-pack CLC-Phenyl (particle diameter 5 µm, 250×4.60mm) column equipped with a pre-column and on a Phenomenex Onyx monolithic C18 (100×4.60mm) column equipped with a pre-column	A. methanol/ water (þ0.2%formic acid) B. 5 to 100% methanol	sesamin		anti-inflammatory, analgesic, and antimalarial action
101	*Equisetum arvense* L.,	Equisetaceae [115]	Strile stem	Kintex 5u RP C18 ìg, 4.6 mm internal diameter × 250 mm	(A) 0.05% formic acid (HCOOH) and (B) 0.05% formic acid-acetonitrile (CH3CN),(50:50 v/v)	Synapin acid, caffeic acid, gallic acid, vanillic acid, ferulic acid, syringic acid, p-coumaric acid	Epicatechin, catechin,quercetin,rutin,naringenin,myricetin, luetolin	anemia, inflammation, diabetes, ulcers, cancer, convulsions, anxiety and depressive disorders
102	*Dipsacus sativus* (Linn.) Honck.	Dipsacus [116]	Dried leaves	a Waters column C18 (250 mm, 4.6 mm, 5 µm)	methanol and acetic acid in water 15:85 (v/v)	Isovitexin, Saponarin		treatment of cardio-vascular disease

103	*Salvia fruticosa* Mill.	Lamiaceae [117]	Plant powder	a reverse phase NOVA-PAK C18 column at ambient temperature (20°C).	methanol and phosphate buffer (43 : 57)		luteolin and rutin,	antioxidant and anti-inflammatory activities

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Chapter 3

Advances in Microbial Genomics in the Post-Genomics Era

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Abstract

In the pre-genomic era, microbes have been used for hundreds of years due to their applications in products such as bread, beer and wine. The use of these microbes in biotechnology is only possible when scientists know the mystery about this tiny creature. In the post-genomic era, thousands of whole genome sequences along with advanced analysis tools, techniques and technologies have been developed for the exploration of hidden potentials in these microorganisms. In this chapter, we summarize the timeline and advancements in microbial genomics made in the post-genomic era. Microbial evolution through 16S rRNA, bacterial genome sequencing boost by Next-generation and third generation sequencing technologies has also been discussed. Comparative genomics approaches to identify industrial microbes, pathogenic, non-pathogenic, rare and uncultivated microbes have also been described. Pangenome analyses for exploring the genome diversity and plasticity. Finally, reverse vaccinology and subtractive genomics approaches have been discussed in the context of its potentials to identify putative vaccine and drug targets.

Keywords: Post-genomics era; Comparative genomics; Phylogenomics; 16s rRNA; Next-generation sequencing; Pathogenomics; Computational tools; Reverse Vaccinology

1. Introduction

Microbes originated around four billion years ago when the earth was hotter and the environment was anoxic. These old inhabitants of the globe are considered as the foundation of the biosphere in both environmental and evolutionary perspectives. These omnipotent crea-

tures occupy 60% of the earth's biomass. They make their own status by using their high adaptability powers. They are found in extreme environments such as hot springs, marshy places, molten lavas, and Antarctica regions where no other living organism can survive. Moreover, they have huge industrial, medical, forensics and environmental applications. Therefore, after realizing their importance microbiologists tried to explore microbes for their own benefits. However, that was not an easy task. Scientists spend years to perform the morphological and molecular characterization of microbes. Pre-genomic era was difficult because of difficult and costly sequencing techniques. Fortunately, advancements in genomics has now revolutionized every aspect of microbiology. Now after twenty years of first bacterial genome sequencing, it is necessary to find out what we did and what we have to do in this post-genomic era. Pre-genomics era started from the quest of sequence and about finding phylogenetic relationships among microbes and other organisms. The era ended in 1995 when first free-living microbe *Haemophilus influenza* was sequenced by using Whole-genome shotgun sequencing technology. However, the post-genomic era is going to extend over several generations and we will get the fruit of hard work of pre-genomic era in the post genomic era [1].

We have presented a brief history of different events that occurred in last two decades in the chronological order as shown in **Figure 1**. This timeline highlights the progress of sequencing in twenty-two years. From 1995 to 2017, development of advanced sequence technologies such as Next-generation sequencing (NGS) has greatly influenced the microbial genomics. In the past, laborious microbiology and molecular techniques were used for classification and characterization of microbes but now bioinformatics is an alternative to those microbiology and molecular techniques. This approach used to dig out the information about antibiotic resistance, microbial diversity, and to understand microbial communities and their genetic make-up [2].

Due to the advancement of computational approaches, there is huge data in the form of sequences available in different databases like UniProt, NCBI, and GOLD, etc. that is obtained from thousands of environmental microbes, pathogenic bacteria, and other industrially important bacteria. The total number of genome sequences available at NCBI are shown in **Figure 2.**

Now, annotation and analyses of these sequences are quite difficult for microbial bioinformaticians as compared to producing sequence data. They require more advanced and sophisticated data handling pipelines to analyze and interpret genomic or proteomic data. A general way of analyzing data requires commands run on programmes like Ubuntu or Linux operating systems [2]. For quick microbial genome annotation, differently advanced pipelines include RAST, PATRIC, command like software PROKKA, MicroScope etc. are used. Moreover, for metagenomics analysis MG-RAST, EBI metagenomics and Prokaryotic Genome Annotation Pipeline has been developed by NCBI which is capable of analyzing >2000 prokaryotic ge-

nome per day [3]. There is only 13-15% of available data of prokaryotes in public databases. There is still a need to discover new environmental microbes to explore more about these tiny creatures' secrets [4]. However, microbes are not easy to culture in the lab because of numerous factors e.g. temperature, fastidious growth, oxygen requirements etc. therefore only less than 1% can be cultured. It was difficult to explore those un-cultured microbes. However, due to advancement in sequencing technology and computational methods, microbial genomes can be obtained directly from environmental samples and sequenced. By using these techniques, we got 8000 genomes that get us closer to the comprehensive genomic representation of the microbial world [5]. There are two categories of post-genomic studies of microbes that include: (a) Direct sequence analyses studies based upon analysis of the genomic sequence information (b) Indirect sequence analysis require only some part of genomic sequence information. Direct sequence analyses enable us to analyze bacteria at the genomic level and help in the determination of small differences like single nucleotide polymorphisms (SNPs) [6].

Timeline of microbial genomics in post-genomic era

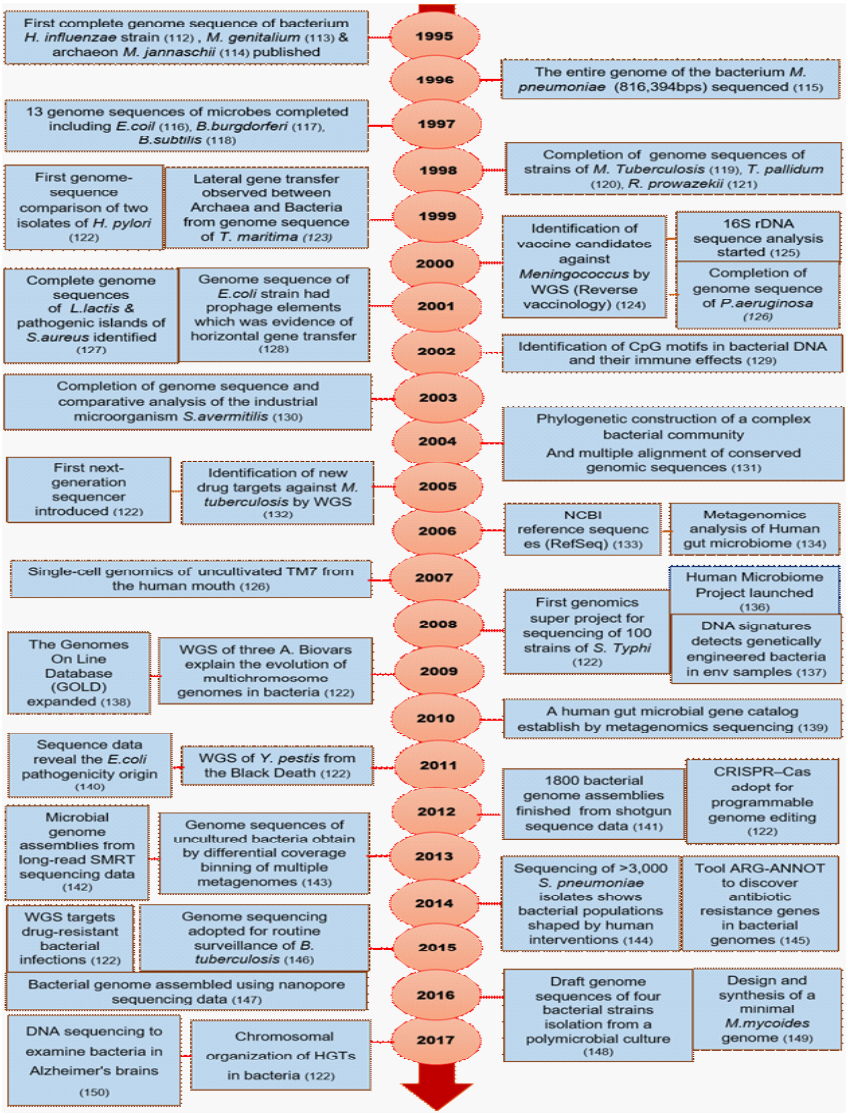

Figure 1: Microbial genomics over the decades: This timeline shows advancements in microbial genome sequencing in chronological order. The concept of the sequencing of microbes started in the nineties (pre- genomic era). In 1995, nonpathogenic *H. Influenza* sequencing by Craig Venter and his team was responsible for the inauguration of post-genomic era. Advanced genome sequencing technologies like Next Generation and Third Generation sequencing boost the microbial DNA sequencing.

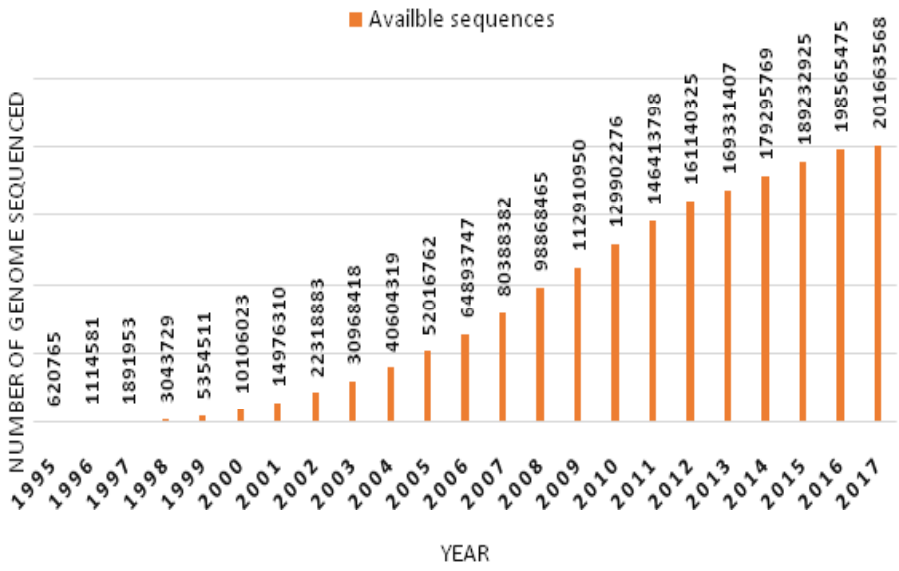

Figure 2*:* Available genome sequences in GenBank (NCBI) increased with the invention of new sequencing technologies (Source: www.ncbi.com).

2. Advancements in Sequencing Technologies in Post-Genomics Era

2.1. DNA sequencing

Determining the order of amino acid residues in polynucleotide chains revealed the information about hereditary material and biochemical properties that led to exploration of bacterial communities, their evolution and interaction with each other [7,8]. A milestone of DNA sequencing is shown in **Figure 3**.

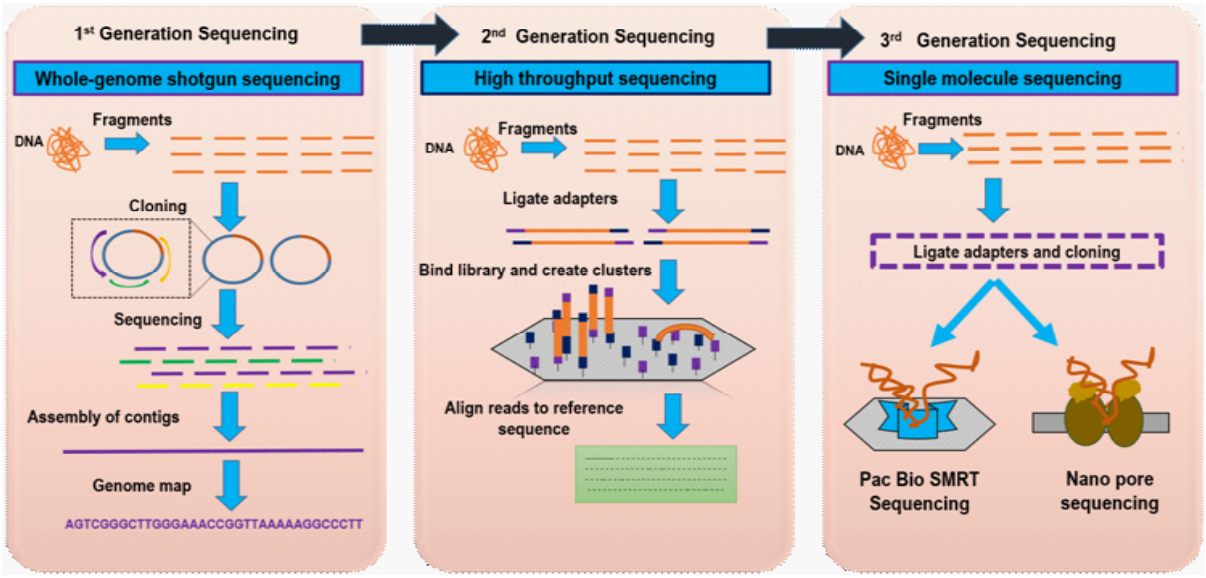

Figure 3: Advancements in microbial genome sequencing technologies in post-genomic era: Whole genome shotgun sequencing requires laborious sample preparation. High throughput sequencing gives high accuracy but short read lengths while single molecule sequencing gives low accuracy with long read lengths.

2.1.1. Whole genome shotgun sequencing

In 1995, Craig venter and co-workers at TIGR, presented the whole genome sequence for *Haemophilus influenza* [9] and *Mycoplasma* [10]. In this method, genomic DNA is subjected to random fragmentation and libraries are produced in *E.coli*. These clones are sequen

ced and computationally compared with sequence reads and the matching sequences are assembled [11]. DNA sequencing had some pitfalls; since amplified templates are produced in a single step, certain DNA stretches may skip replication well in *E.coli* [12].

2.1.2. High throughput sequencing or next generation sequencing

Earlier sequencing methods created draft genomes with approximately $50,000 cost. With the advancement in sequencing technology it has reached $1 cost which has revolutionized the microbial genomics [13]. Discovery of restriction enzymes by Hamilton smith and co-workers proved to be a significant event without which Next- generation DNA sequencing would not have been possible. DNA strand to be sequenced are cleaved with RE's to provide specific ends that function as initiating points for sequencing [11]. In 2000's Next-Generation Sequencing was introduced with 100-fold throughput using 454-pyrosequencing approach. Afterward, Illumina and ABI SOLiD were introduced. High-throughput sequencing or Next-generation sequencing can sequence multiple DNA molecules in parallel due to which millions of DNA molecules can be sequenced at a time and at low cost. Next-generation sequencing produces short read length which leads to the taxonomic classification of microbes [14]. The principle behind these technologies is a detection of emission light from the sequenced DNA while Ion torrent was introduced later that detects hydrogen ion [15]. Thus high-throughput sequencing technologies enable us to determine cellular genomics, the transcriptomic signature of various diseases and novel variants responsible for many diseases [16]. HTS provide insights into the genetic and phenotypic diversifications among closely related bacterial infection like *Mycobacterium abscessus, Pseudomonas aeruginosa, Staphylococcus aureus, Mycobacterium tuberculosis* etc [13].

Different commercially available sequencing platforms include; Illumina's platforms, Ion Torrent, 454 and Pacific Biosciences Real Time Sequencer. Illumina platforms have HiSeq2000 and MiSeq that perform an ultra-high-throughput analysis. These machines were tested against 24 host-associated and free-living microbial communities. HiSeq2000 allow large DNA parallel sequencing at low cost, while MiSeq is convenient for smaller projects [17]. Loman NJ and his team compared three benchtop high throughput-sequencing instruments included 454 GS, MiSeq (Illumina) and Ion Torrent PGM [18] by sequencing of *Escherichia coli O104:H4* to know their efficacy. These sequencers can generate bacterial genome sequence data, can identify and characterize bacterial pathogens. They reported that MiSeq had the highest throughput run as compared to Ion Torrent PGM and 454 GS [18]. Another study conducted to characterize *Helicobacter pylori* genome revealed that Illumina Nextera XT sequencing machine produced more accurate multi-locus sequence type in less time and cost as compared to MiSeq and Ion Torrent [19]. In clinical settings, high throughput sequencing technologies are widely used and also used to determine microbial community diversity in food industry during douche-koji making fermentation and in 62 Irish artisanal kinds of cheese

[20,21].

2.1.3. Advanced genomics with single-molecule real-time (SMRT) sequencing

To overcome second generation sequencing problems that included short read length (30-450bases), errors due to short read lengths, and laborious sample preparation methods; a newer system was introduced by Pac Bio's that is SMRT sequencing after 2007 [15]. Single molecule (SMRT) sequencing is a third- generation sequencing technique, which enables real-time observation of base sequences from individual strands of DNA or RNA [22,23]. Second generation sequencing provides a longer sequenced read length, flexibility, lower cost and higher throughput. In SMRT technology, the polymerase enzyme is affixed at the bottom of Zero-Mode Waveguide (ZMW) nano-holes. Polymerase incorporates fluorescently labeled bases to DNA template and makes immobilized complex at bottom of well. Detectors detect emitted lights as fluorescents base combines with the template [15]. Single-molecule real-time (SMRT) DNA sequencing allows detection of chemical modifications. For example, methylation was detected in *E.coli* [24].

2.1.4. Oxford nanopore sequencing

Nanopores sequencers are also based on single molecule concept but it detect bases without labels, produces long reads, relatively fast and with low GC bias errors. The principle of this technology is tunneling of molecules (polymer) through a pore that separates two sections. This allows identification of specific molecules. Oxford nano-pore has the MinION system that is real-time analyzer of DNA or RNA [25].

Next Generation Sequencing (NGS) Tools

Software mostly commonly use in Next generation sequencing are listed below in **Table 1**.

Table 1: NGS Tools

Sr. NO.	Tool	Function	Web Link	Reference
1.	**mrsFAST**	Map short reads, SNP-aware Mapping,	http://mrsfast.sourceforge.net.	[26]
2.	**ContextMap**	Is RNA sequence mapping algorithm, identification of indels	http://www.bio.ifi.lmu.de/ContextMap	[27]
3.	**SOAPsplice**	Detects splice junction sites from RNA-seq	http://soap.genomics.org.cn/soapsplice.html	[28]
4.	**Bowtie2**	support ultra-fast and memory efficient sequence alignment of local, gapped and paired end modes	https://sourceforge.net/projects/bowtie-bio/files/latest/download?source=files	[29]
5.	**NextGenMap (NGM)**	Read mapping program, memory efficient	http://cibiv.github.io/NextGenMap/	[30]

3. Genome Overview and Browsers

Thousands of genomes are sequenced so far but the follow-up knowledge is still very limited. Structural genomics plays a vital role in understanding the molecular genetics by providing insights into genomic DNA functional stretches [31]. The collection of all genetic material from species is termed as pangenome and could estimate with bioinformatics tools. Data could be visualize and analyze via various online genome browsers [32]. Genome browsers are visualization programs from which researchers can search, retrieve and analyze genomic sequences efficiently and conveniently [33]. Web-based Genome browsers are classified as 'Species-specific genome browser' and 'general genome browsers'. Species-specific genome browsers work on one specific organism while the general genome browsers deal with multiple species. Different genome browsers have different retrieval systems. For example, Ensembl employ BioMart system [34], UCSC system employs table browser [35].

Table 2: List of web-based general microbial genome browsers

Sr. No.	Browser	Description	Web Link
1.	**NCBI**	Provides free access to books of biomedical sciences, microbes	https://www.ncbi.nlm.nih.gov/
2.	**Ensembl**	Genome browser for bacteria, fungi, protists, metazoan, vertebrates, annotate genes, predict regulatory functions and multiple alignment	http://www.ensembl.org/
3.	**Genome Projector**	Hundreds of bacterial genomes with circular and linear maps	http://www.g-language.org/g3/
4.	**UCSC**	Graphical web-based browser, gene annotation and expression, integrates bacterial and archaeal specific tracks	http://archaea.ucsc.edu
5.	**(Integrated Microbial Genome) IMG**	Visualization software tool, Distribute data to public, provide the facility of panning, focus zooming and jump zooming	http://bioviz.org/igb

Table 3: List of web-based microbial species-specific genome browsers

Sr. No.	Browser	Species	Web Link
1.	**Saccharomyces cerevisiae Genome Database(SGD)**	*Saccharomyces cerevisiae*	https://www.yeastgenome.org/
2.	**Paramecium Database (ParameciumDB)**	*Paramecium tetraurelia*	http://paramecium.cgm.cnrs-gif.fr/cgi-bin/gbrowse2/
3.	**DictyBase**	*Dictyosteliumdiscoideum*	http://dictybase.org/db/cgi-bin/ggb/gbrowse/
4.	**CyanoBase**	*Cyanobacteria*	http://genome.kazusa.or.jp/cyanobase
5.	**The Legionella Genome Browser (LGB)**	*Legionella pneumophila*	http://genolist.pasteur.fr/LegioList/
6.	**The Enterobacter Genome Browser**	*Enterobacters*	engene.leibniz-fli.de/
7.	**The Xanthomonas Genome Browser (XGB)**	*Xanthomonas*	xgb.leibniz-fli.de/

3.1. Functionalities and features

High-throughput sequencing and high-performance computing provided with enormous genomic data and web-based genome browsers freely distribute this immense volume of data to researchers. These genome browsers accumulate entire data from different platforms and present it graphically [36]. Images, graphs, cycles, pathways, maps etc are drawn to aggregate the data to present information in less complicated manner to overcome the burden of servers [37].

3.2. Data retrieval and analysis

Data Retrieval and analysis are one of the principle attributes of genome browsers. Different browsers apply different approaches for data retrieval. For example, UCSC present the data in tabular form and ABrowse project apply BioMart system [34].

IGB employ MACS to analyze the results obtained from ChIP-Seq [38]. Genome browsers integrate with other platforms in order to provide better results. Genome browsers provide a platform where researchers collaborate to share their ongoing researches, discoveries and discuss their projects [39].

4. Advanced Computational Tools for Microbial Genomics in Post-Genomic Era

Table 4: Computational tools and their functions

Sr. No	Tool	Function	Web Link	Ref
1.	**BLAST**	Infer evolutionary and functional relationships	http://blast.ncbi.nlm.nih.gov	[40]
2.	**KEGG**	An integrated database resource, provides genomic, chemical and systemic information	http://www.kegg.jp	[41]
3.	**WebACT**	Database provide sequence comparisons between all prokaryotic genomes	webact.org/WebACT/home	[42]
4.	**MUMmer**	Provide ultra-fast alignment of genomes	tar -xvzf MUMmer3.0.tar.gz	[43]
5.	**BASys**	(Bacterial Annotation System)Provides automated bacterial genomic sequencing	http://wishart.biology.ualberta.ca/basys	[44]
6.	**Microbial Genome Viewer (MGV)**	Generate linear and wheel maps for data obtained from annotation and transcriptomic	http://www.cmbi.kun.nl/MGV	[45]
7.	**GeneWiz**	Predict linear or circular genome atlas, by genetic and physical properties of genome, one can make the diagram	http://www.cbs.dtu.dk/services/gwBrowser/	[46]
8.	**GeneMark**	Gene prediction in bacteria, metagenomes, metatranscriptomes, and archaea	http://opal.biology.gatech.edu/GeneMark/	[47]
9.	**(CGV)**	Circular Genome Viewer (CGV) generate static and graphical maps of Circular DNA, providing facilities of zoom in, labeled features and hyperlinks	http://stothard.afns.ualberta.ca/cgview_server/	[48]

10.	**SignalP**	Infer the presence and location of signal peptide cleavage site in nucleotide sequences among different organisms	http://www.cbs.dtu.dk/services/SignalP/	[49]
11.	**Prokka**	Provides genome annotation for bacteria, archaea and viruses	http://www.bioinformatics.net.au/software.prokka.shtml	[50]
12.	**LAST-TRAIN**	Accuracy of sequence alignment improved by inferring better score parameters and re-align	http://last.cbrc.jp/	[51]
13.	**Harvest suite (parsnp, gingr)**	Core genome alignment and visualization tool	HarvestOSX64v1.1.2.tar.gz	[52]
14.	**ClonalFrameML**	Infers recombination in bacterial genome	https://github.com/xavierdidelot/ClonalFrameML	[53]
15.	**POGO-DB**	Provides microbial genomic comparison and visualization tool	http://pogo.ece.drexel.edu	[54]
16.	**JSpeciesWs**	Identifies similarity b/w two genomes, measures average nucleotide identity, analyze correlation indexes of tetra-nucleotide signatures	http://jspecies.ribohost.com/jspeciesws.	[55]
17.	**(SRST2)**	Short Sequence Typing for Bacterial Pathogens (SRST2) detects genes, alleles and MLST from whole genome sequencing data	http://katholt.github.io/srst2/	[56]
18.	**GUBBINS**	Genealogies Unbiased By recomBinations In Nucleotide Sequences Identifies loci containing base substitution and generate phylogenetic tree based on point mutations	Sanger-pathogens.github.io/gubbins/	[57]
19.	**SpeciesFinder**	Predicts the species of a bacterium from complete or partial pre-assembled genomes	http://cge.cbs.dtu.dk/services/SpeciesFinder	[58]
20.	**Velvet**	Genome assembler, for short read sequences, remove errors and generate unique contigs	https://www.ebi.ac.uk/~zerbino/velvet/	[59]
21.	**FgenesB**	Bacterial Operon and gene prediction	http://linux1.softberry.com/	[60]
22.	**SPARTA**	SPARTA (Simple Program for Automated reference-based bacterial RNA-seq Transcriptome Analysis) analyzes differential gene expression, perform quality analysis of the data sets	sparta.readthedocs.org	[61]
23.	**OrthoANI**	OrthoANI(Orthologous Average Nucleotide Identity) measures overall similarity between two genome sequences	http://www.ezbiocloud.net/sw/oat.	[62]
24.	**Oufti**	Quantitative analysis of bacterial count and fluorescent signals	http://www.oufti.org/download/	[63]
25.	**Orione**	Conduct NGS data analysis and annotation by quality control of reads and their trimming	http://orione.crs4.it	[64]
26.	**VacSol**	Scrutinize the whole bacterial pathogen proteome to identify a vaccine candidate proteins	https://sourceforge.net/projects/vacsol/	[65]

5. Microbial Phylogeny and Evolution

Early life on earth was most probably consisted of RNA. According to endosymbiotic theory, archaea was the ancestor and they engulfed mitochondria from gram-negative bacteria or chloroplast from cyanobacteria that lead to the evolution of eukaryotes [66]. Phylogenetic analyses were necessary to explore the microbial diversity, their ecological or niche adaptation, pathogenic potential of unknown microbes, their ability to produce different types of natural products like enzymes etc. The term “Phylogeny” is derived from two Greek words Phylon meaning “clan or race” and genesis meaning “origin”. Therefore, it is the study of the evolutionary history of the organism [67].

Researchers used many approaches for classification of microbes. In 1759, Linnaeus tried to classify all living things and developed the binomial system (Genus species). He divided the world into Animal, Vegetable, and Mineral and put all the microscopic life in one genus i.e. Chaos. In the 1980’s, neo-Darwinian evolutionary theory explained the evolution of plants and animals over the last 560 million years but did not discuss the evolution of microorganisms. Therefore biological scientists from last two decades aimed to build a universal phylogeny [68]. Whittaker in 1969 gave five-kingdom system based on modes of nutrition like photosynthesis, adsorption, and ingestion. The five-kingdom system included Plants, Animals, Fungi, Protists, and Bacteria. However, it did not describe the origin of species. Therefore, microbiologists tried to classified microorganisms on the basis of their morphological, molecular, physiological and metabolic characters. Carl Woese and his coworkers in the 1970s proposed the “Universal tree of life” including Archaea, bacteria, Eucarya (**figure 4**) using 16s rRNA molecular approach for phylogenetic analysis. Phylogenetic analysis increased due to rapid advancements in biology and computational field, which led to the availability of huge genomic data about microbes [69].

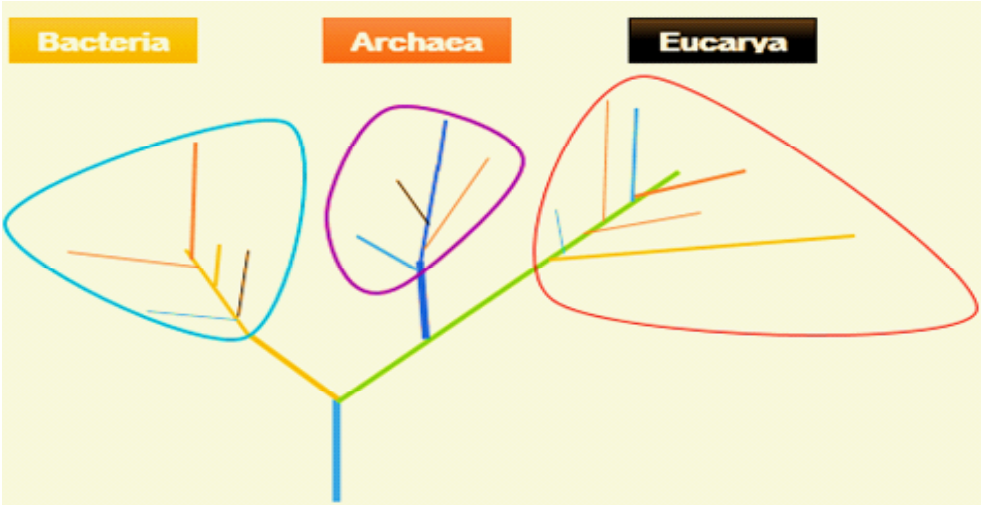

Figure 4: "The Universal Tree of Life" by Carl Woese and co-workers

5.1. Different approaches to construct phylogenetic tree in post-genomic era

The phylogenetic relationship can be determined using morphological (cell size, shape etc.), physiological, molecular (based on genetic material) and comparative genomic approaches. These methods include analyzing the shared gene content, gene order, construction of different phylogenetic trees etc. Due to the limited morphological and physiological characters, along with substantial number of variations among closely related taxa, scientists preferred molecular data. Initially, phylogenetic molecular markers included DNA sequences located on chromosomes and ribosomal RNA gene sequences [69]. Different bacterial genome sequenced after 1995 centered on sequenced data. Based on 16s rRNA sequencing proteobacteria were classified. Proteobacteria are considered as the largest taxonomic group because they comprise 50% of all cultured bacteria. Based on its branching in 16sRNA trees they are divided into five classes; alpha (covers 12% proteobacteria), beta (8%), and gamma (26%), while delta and epsilon covers other 4% [70].

Molecular markers 16s rRNA and rpoB genes (rplB, pyrG, fusA, leuS and rpoB) are compared for Actinobacteria, Bacteroides, Proteobacteria, and Cyanobacteria. Results revealed that rpoB markers were good in detecting minor groups among microbial assemblages [71]. A bulk of sequences allowed scientists to use comparative genomic approaches for phylogenetic study. Ludwig and Schleifer reconstructed the phylogeny of prokaryotes based on comparative sequence analysis of small subunit rRNAs [72]. Phylogenetic relationship of Streptococcus to other species was determined by using comparative genomic approaches. Moreover, these approaches were also used for identification and functional classification of homologous clusters, pan-genome analyses, population structure and virulence factors [73].

5.2. Reasons of evolution of microbes and horizontal gene transfers (HGTs)

Evolution of infectious species can also be determined using 16s rRNA sequences. Derrick and his Co found genus Leptospira pathogenic bacterium with the help of comparative genome analyses. They did pan-genome analyses, 16s rRNA gene sequencing, In-silico DNA-DNA hybridization, metabolic reconstruction and related gene clusters. They reported that Leptospira originated from noninfectious species and adapted different metabolic pathways that became the cause of infection. They also find out a unique signal responsive pathway, gene expressions and chemotaxis systems [74]. Different prokaryotic group's evolution is due to horizontal gene transfer (HGT). In HGT, microorganisms transfer genetic material from one species to other species. Mostly housekeeping genes are involved in HGT. It is an adaptation process and strongly influenced by environment. As earth's environment changed with the passage of time, microorganisms acquired more foreign genes to cope up environmental conditions [75].

5.3. Different phylogenetic molecular markers

Advancement in genomics has led to increasing number of full genomes and gene sequence data resulting in identification of various phylogenetic molecular markers other than 16s rRNA. These include elongation and initiation factors, large subunit rRNA, RNA polymerase, subunits of proton translocation ATPase, DNA gyrase, recA, aminoacyl tRNAsynthetases and so on. Most widely used molecular markers include nuclear ribosomal genes (18S rRNA in eukaryotes and the 16S rRNA in others and large subunit contains the 5S and 23S rRNAs) and more powerful markers in resolving species level phylogenies i.e. mitochondrial genes (cytochrome oxidase I and II (COI/II)), EF-1α, rpoA gene, lux Gene, Nuclear H3, recA, rpoB, rpoC1 etc. These markers can resolve phylogenetic relationship at deep levels of evolution [76]. Secondary structure can also be used for multiple sequence alignment. Le Q and co proposed QuanTest,a fully automated system for protein MSA [77]. However, these markers are more complex. In addition, phylogenetic trees derived from such markers may vary from one another. Therefore, phylogenetic trees of microbes derived from single gene i.e. small subunit rRNA is considered as universal [72].

5.4. Challenges and opportunities for phylogenetic tree reconstruction

Different molecular phylogenetic analysis predicted lateral gene transfer between closely related prokaryotes as well as distantly related prokaryotes. This lateral gene transfer became a hurdle in the understanding of exact evolutionary track of microorganisms. In addition, computing cost involved in the reconstruction of an evolutionary tree. Fortunately, with the advancement in the computational field this hurdle has been overcome. Advancement from16S rRNA genome sequencing to DNA sequencing platform has led to increased number of available sequence data for phylogenetic analysis. Thus, in the post genomic era, a large number of microbial sequences are available in public domains, continuous advancement in high throughput DNA sequencing techniques and the introduction of new phylogenetic inference methods has occurred. These three points provide a challenge and opportunity simultaneously to the researchers to study evolution, ecology, and taxonomy of microbes. One strategy to organize a large set of data in the form of hierarchical distance tree is by using single copy ribosomal protein marker distances. In this tree protein distance measures dissimilarity between the same kinds of markers and measures genomic distance average by ignoring the outlier. As a result, 60,000 organized genomes in a marker distance tree obtained, which result in >6000 species level clade and represented as 7597 taxonomic species. These findings will help the researchers to get pre calculated genomic group [78].

5.5. General steps for phylogenetic tree construction

There are four steps for phylogenetic tree construction of molecular sequences shown in **Figure 5.**

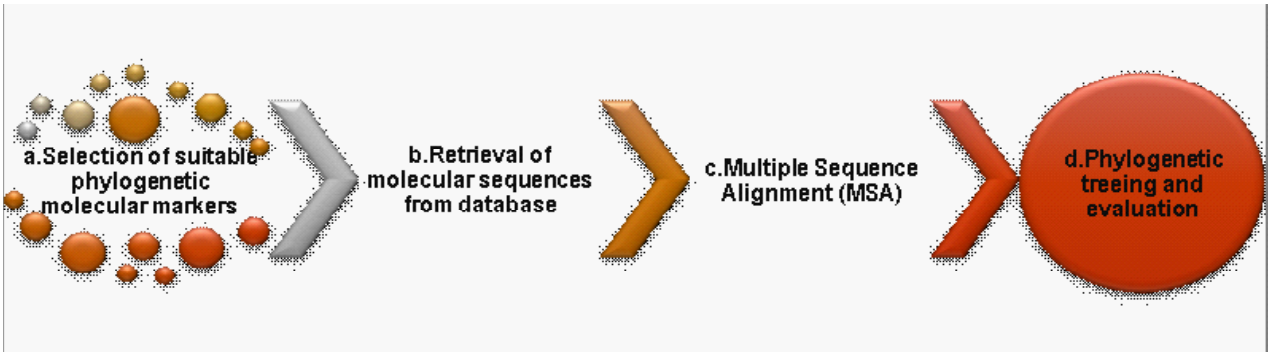

Figure 5: Four general steps of constructing phylogenetic tree

5.5.1. Selection of suitable phylogenetic markers

The phylogenetic marker is coding or non-coding DNA fragment (locus) used in phylogenetic reconstruction. These phylogenetic markers for microbes include nuclear encoded genes (like 16S rRNA, 5S rRNA, 28S rRNA), mitochondrial (cytochrome oxidase, mitochondrial 12S, cytochrome b, control region) and few chloroplast encoded genes (like rbcL, matK, rpl16) (67). Selection of suitable phylogenetic marker is crucial to study molecular evolution like duplications of genes, mutations, loss or gain of genes, genetic exchange such as recombination events, re-assortment, and horizontal or lateral gene transfer. For an ideal marker it should contain following characteristics:

(a) Single gene should be preferred over multiple genes e.g. use of mitochondrial and nuclear genes.

(b) Marker gene is aligned prior to phylogenetic tree construction; therefore, sequence alignment should be easy and without any ambiguous alignments.

(c) The substitution rate should be optimum to avoid saturation of multiple substitutions.

(d) Primers should be available for amplification of marker genes and universal primers be avoided since they may cause contamination in marker genes.

(e) Markers with too much variation in bases may not represent the true lineage [79].

5.5.2. Retrieval of molecular sequences from database

Molecular data can either be obtained from nucleotide or protein databases. This depends upon chosen organism/s.

5.5.3. Multiple Sequence Alignment (MSA)

Multiple Sequence Alignment (MSA) is for two or more than two molecular sequences. Purpose of MSA is to determine homology and evolutionary relationship between the under study sequences. Different types of alignment homology are obtained after multiple sequence alignment, shown in **Figure 6**.

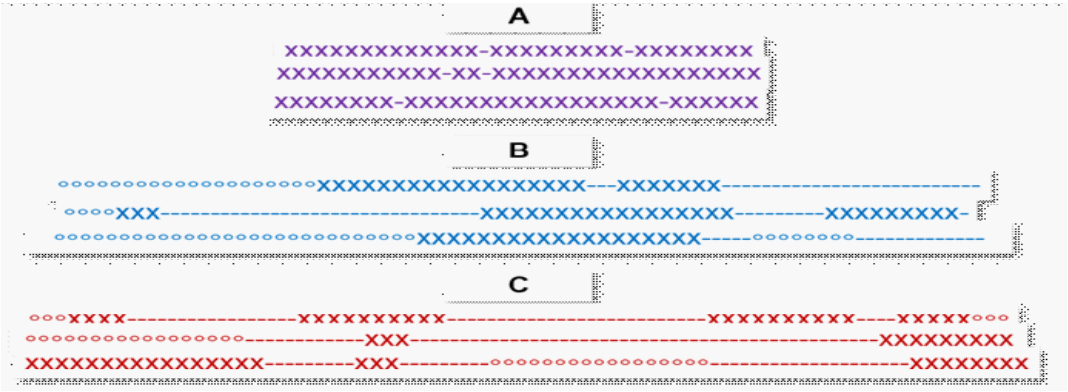

Figure 6: Different types of alignment homology. "x" represents an aligned amino acid residue, and “o” is an unalignable residue, ‘’--‘’ represents a gap. (A) Global sequence alignment (for comparing homologous genes) (B) Local sequence alignment (for finding homologous domains) (C) Long internal gaps.

There are different computer programs for multiple sequence alignment that are listed in **Table 5**.

Table 5: Computational tools for Multiple Sequence Alignment

Sr.No.	Tool	Year	Web link	Ref
1.	**T-Coffee**	2000 & new version in 2008	http://tcoffee.crg.cat/	[80]
2.	**MUSCLE**	2004	https://www.ebi.ac.uk/Tools/msa/muscle/	[81]
3.	**Kalign**	2005	https://www.ebi.ac.uk/Tools/msa/kalign/	[82]
4.	**ClustalW**	2007	http://www.clustal.org/	[83]
5.	**FAMSA**	2016	http://sun.aei.polsl.pl/REFRESH/famsa.	[84]
6.	**MAFFT**	2017	http://mafft.cbrc.jp/alignment/server/large.html	[85]
7.	**HAlign-II**	2017	http://lab.malab.cn/soft/halign/	[86]

5.5.4. Phylogenetic tree construction and evaluation

A phylogenetic tree is a graphical representation of the evolutionary relationships among genes or organisms. Phylogenetic tree is constructed when homologous residues aligned. Different methods or algorithms used to develop phylogenetic tree are distance based method, maximum parsimony, maximum likelihood and Bayesian models. Distance-based method does not use sequences directly while other three methods use sequence information, therefore, known as character-based methods shown in **Figure 7** [67,87].

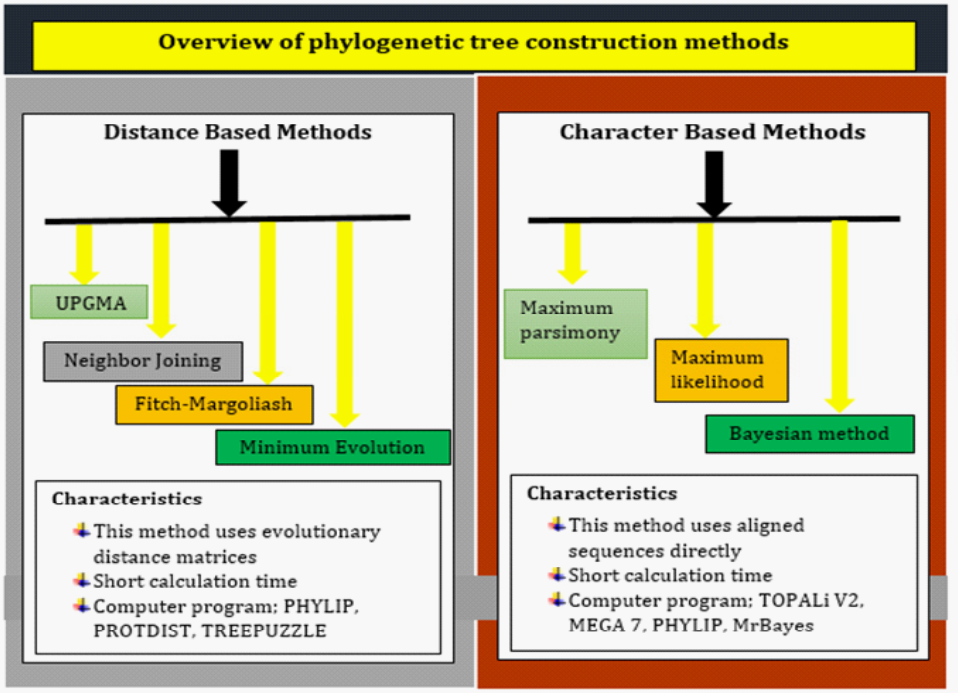

Figure 7: Different Phylogenetic Tree Construction Methods; UPGMA (Unweighted Pair Group Method with Arithmetic) proposed in 1958 by Sokal and Michener, Neighbor-Joining by Saitou and Nei (1987), Maximum parsimony by Henning (1966),maximum likelihood method by Felsenstein (1981).

5.5.5. Phylogenetic tree evaluation

Phylogenetic tree evaluation is necessary for the validity of tree and its shape. The phylogenetic tree represents species phylogeny if species under study are evolved from common ancestor. Branch length in the tree represents evolutionary distance that is tentatively correlated with evolutionary time. Therefore, branch length determines rate of evolution, gene duplication, and speciation events. Moreover, percentage of each external branch is calculated by bootstrapping method. If branch point scores or bootstrapping values is higher (approximately 90% or greater) then it predicts accurate tree. About 500-1000 times bootstrapping is required for reliable results. Other different statistical tests like Jackknifing, Kishino-Hasegawa test, Bayesian analysis and Shimodaira-Hasegawa employed to check the reliability and to confirm which tree is better. The Bayesian analysis is very fast and involves thousands of steps of resampling the results [66]. In an evolutionary tree, there are operational taxonomic units (OTUs) defined as the set of OTUs joined by the same ancestor or parental node [88]. Single 16S rRNA used to differentiate operational taxonomic units (OTUs)(89). How to interpret an evolutionary tree is shown in **Figure 8**.

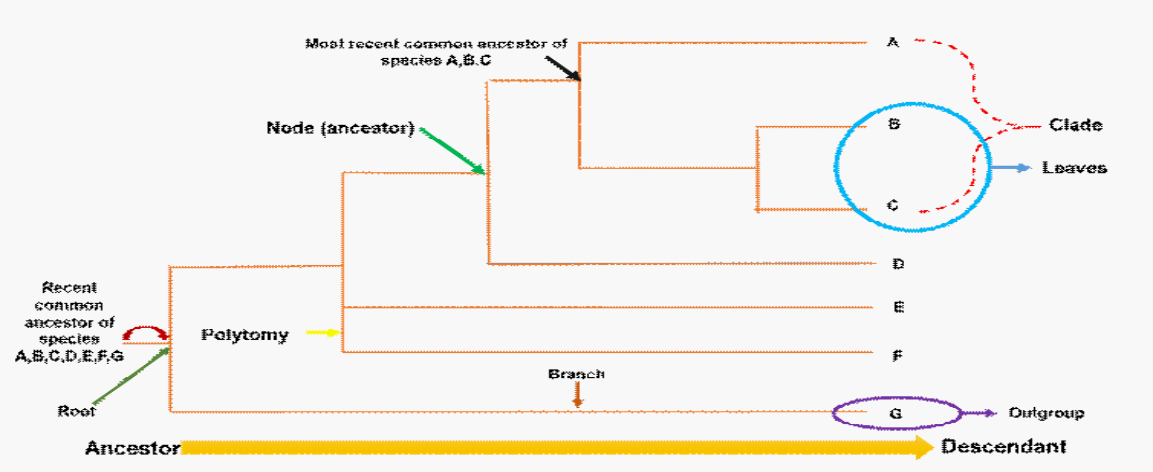

Figure 8: Interpretation of Evolutionary Tree

6. Comparative Genomics of Microbial Pathogens

Comparative genomics is a holistic approach that compares two or more than two genomes to identify the similarities and differences among the genomes and to study the biology of genomes. Comparative genome analysis can find out the different perspectives of organisms as shown in **Figure 9** [90].

In post-genomic era, comparative genomics has been widely used to distinguish pathogenic and non-pathogenic species; it helped identify virulence factors and genes involved in pathogenicity by sequence analyses [6,91]. More than 1800 bacterial genomes have been sequenced including *Escherichia coli* O157:H7, *Vibrio cholerae, Staphylococcus aureus, Streptococcus pneumoniae, Clostridium difficile* and *Mycobacterium tuberculosis* on which comparative genomics approaches can be applied [92].

Different applications of comparative genomics include gene identification, finding regulatory motifs, in the field of molecular medicine and molecular evolution, selecting model organisms, in clustering of regulatory sites, finding genomic islands, selection of industrially important organism and much more which still need to be explored [93]. These comparative genomes approaches used to differentiate between the multi-drug resistant pathogen *S. maltophilia* and the plant-associated strains *S. maltophilia* R551-3 and *S. rhizophila* DSM14405. *S. maltophilia* contained heat shock proteins and virulence factors that were absent in plant-associated strains [94]. Another disease leptospirosis is a globally widespread zoonotic disease with important health consequences for humans and domesticated animals. This genus *Leptospira* is divided into infectious species for mammals and non-infectious species. Comparative genomics studies revealed that infectious *Leptospira* contained novel virulence modifying proteins, CRISPR-Cas systems and different metabolic pathways like pathogen-specific porphyrin metabolism while non-infectious species did not have these adaptations [74].

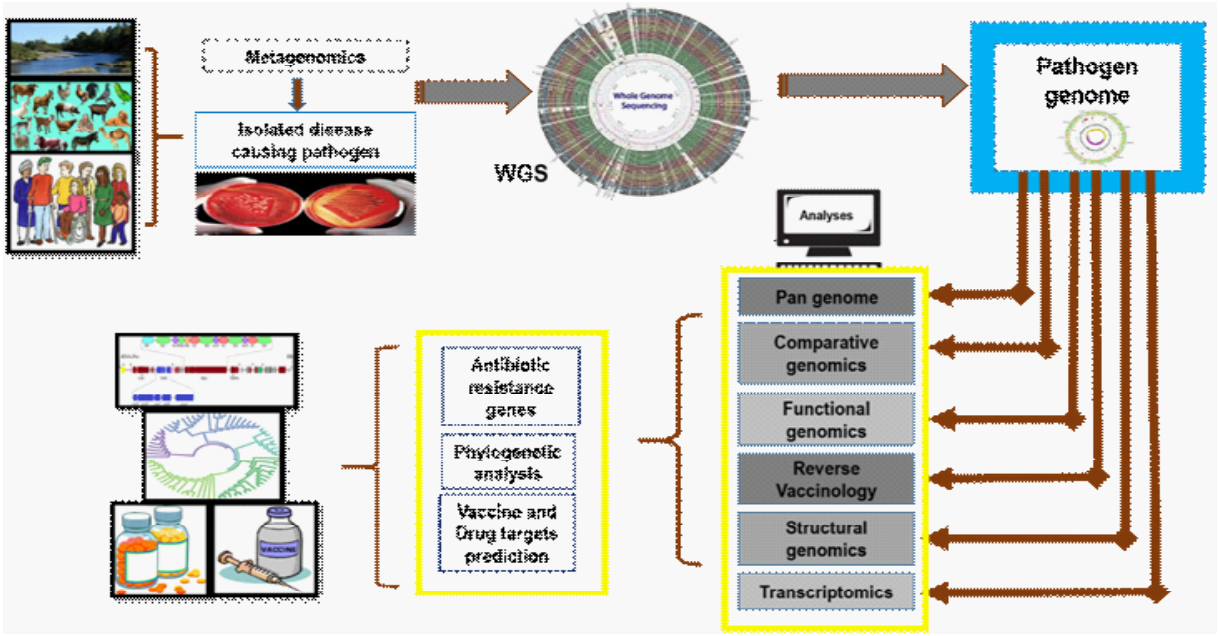

Figure 9: Overview of utilizing computational approaches for analysis of pathogen genome

6.1. Comparative genomic approaches

Comparative genomics considers many approaches for obtaining reliable results. Genome size is an important approach in comparative genomics. Genomic statistics include a number of coding regions, number of chromosomes, GC and AT contents, genome structure, and genome density. For example, genome size of soil-living bacteria has bigger than endosymbiotic bacteria. In addition, while transformation from free-living bacteria to pathogens they gain or lose number of genes. Comparative genomes studies consider these genomic statistics to find out the genomic differences and their reasons. These genomic statistics varies from species to species and even strains to strains [32]. In recent years, increasing number of available genomic information of multiple pathogenic and non-pathogenic bacterial species is also evident that genomic acquisition and reduction have an important role in evolution and pathogenecity. For example, human pathogens *Escherichia coli, Mycobacterium tuberculosis* and *Helicobacter pylori* cause diseases due to genome shifting [95].

Another important approach is finding homologous proteins (including orthologous and paralogous) that remains a challenge for researchers. For this purpose, protein sequences comparison is considered as the powerful tool. This comparison is based upon protein sequences of different species to trace back evolutionary history of many species. Computational tools BLAST, and other clustering tools k-means, affinity propagation, Markov clustering, FORCE, as well as transitivity clustering can be used for finding homologous estimation. In addition, identification of protein-protein interactions plays a vital role in determining biological processes within cells and characterizing those proteins that involved in pathogenicity. Different-proteome-wide common conserved protein-protein interactions (PPIs) for different pathogenic and non-pathogenic bacteria included *C. pseudotuberculosis, C. diphtheriae, C. ulcerans, M. tuberculosis, Y. pestis and E. coli* was determined [32].

6.2. Microbial pathogenomics

Pan-genome analysis of pathogen genome leads to identification of genome plasticity and pathogenic islands. The term pan-genome was first defined in 2005. Pan-genome consists of a core, dispensable and unique genomes. Core genes mostly have housekeeping and essential genes required for growth of bacteria. Dispensable genome carries foreign or modified gees obtained from horizontal genes transfer and these genes could be potential therapeutic targets. Unique genes are novel genes that only confined to particular strains or sometimes in species. These genes increase adaptability to host environment and increase virulence. Therefore comparative pan-genome study is important in studying antibiotic resistance, potential therapeutic targets, epidemiology and phylogenomics. Comparative genome along with pan-genome approach was used to investigate pathogenicity of seven *Campylobacter species*. Pan-genome results revealed 3933 core genome and 1,035 ubiquitous genes [96]. *Streptococcus* genus within phylum *Firmicutes* is among the most significant and diverse zoonotic pathogens. Considerable taxonomic approaches like DNA hybridization, 16S rRNA sequencing did not give the clear evolutionary implications of *Streptococci* species group. Therefore, comparative genomic approaches used to get a clear understanding of evolution of pathogenicity in *Streptococci.* Genome analysis revealed that pan-genome size increases with the addition of newly sequenced strains and core genome size decreases. Population structure analysis and phylogenetic analysis revealed two distinct lineages or clades formed within a species group. Virulence factors also evolved with species evolution [73].

6.3. Genome plasticity

Genome plasticity is the gain or loss of genes and gene rearrangements within specific strains of species for higher adaptability to a new environment. Genome plasticity comprised by several different mechanisms including gene arrangement, inversion, translocation, mutations, plasmid insertions from different organisms, and other insertions like transposons, insertion elements, bacteriophages and genomic islands. Genomic islands are large mobile elements that have cluster or bunch of genes that are directly or indirectly involved in bacterial pathogenicity (**Figure 10**).

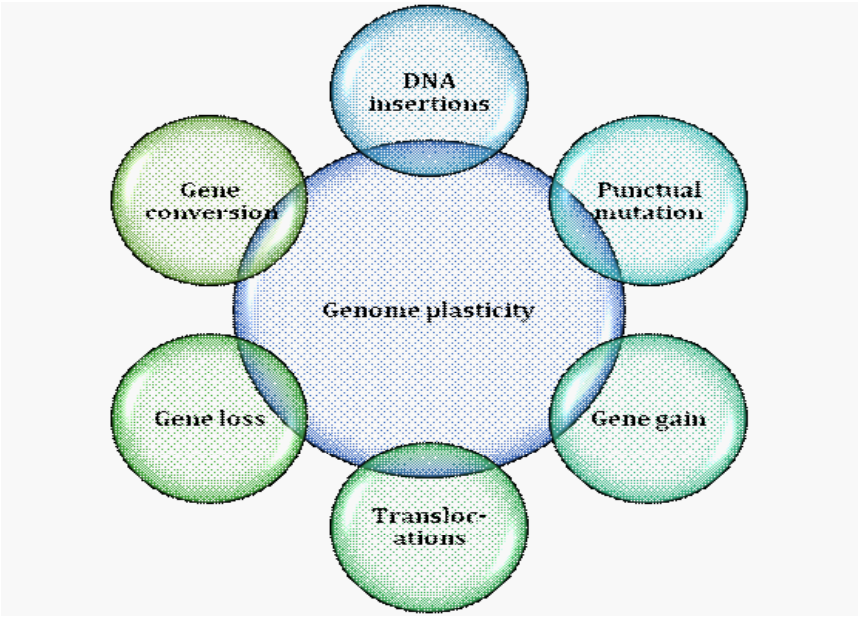

Figure 10: Mechanisms of genome plasticity

Whole genome sequence analysis of *Staphylococcus aureus* revealed mobile genetic elements that carry virulence and antibacterial genes. This horizontal gene transfer of mobile genetic elements mediates the evolution of methicillin resistance *Staph. aureus* [97]. In post-genomic era, researchers explore pathogenicity of microbes by genome comparison. Dao-feng and co predicted the pathogenic potential and international spread of *Staphylococcus argenteus* by genomic comparison analysis. The comparative genomic analysis (based on pan-core genome definition) performed among thirty *S. aureus* genomes, fifteen *Staphylococcus argenteus* and six *S. schweitzeri* genomes. Results revealed that all three species had rare core genome with interspecific recombination. Many virulence genes of *S. aureus, S. argenteus* and *S. schweitzeri* were homologous. Moreover, *S. argenteus* showed ambiguous biogeographical structure that was evidence of its international spread [98].

Pan-genome analysis can use for analysis of minor mutations like single nucleotide polymorphisms (SNPs) that are responsible for any kind of virulence. The pan-genome investigation of two *Mycobacterium tuberculosis* strains helped to identify SNPs, which led to the study of evolution and pathogenesis of these strains. Analysis showed that this species was highly clonal without any lateral gene transfer and these strains lost some genes that were present in other strains [99].

Comparative genomic analyses can be used for finding reasons of bacterial outbreaks in history. In Germany (May-June 2011) an outbreak caused by Shiga-toxin producing *E.coli* O104:H4 that infects more than 3000 people. Scientists tried to find out the reason of this virulence in *E.coli*. After comparative genomic analysis of different strains of pathogenic *E.coli*, they found that it belongs to rare serotype O104:H4. In addition, this strain belonged to enteroaggregative *E.coli* lineage that had acquired Shiga-toxin producing gene and antibiotic resistance gene (i.e. broad-spectrum beta-lactamase gene of CTX-M-15 class). They reported

the acquisition of stx2 prophage, gene encoding AAF/III fimbriae which was responsible for alternative adhesion mechanism [100]. *Shigellaflexneri* causes shigellosis that is a leading cause of bacillary dysentery in developing countries, especially in Asia. Infants under five are more susceptible to this disease. Based on O- antigen of outer membrane lipopolysaccharide there are 19 serotypes of *Shigellaflexneri.* Despite its disease causing ability, there was little knowledge about its virulence and genomic structure. Therefore, Pawan Parajuli, Marcin Adamski and Naresh K. Verma, 2017 used hybrid methods of long-read single-molecule real-time (SMRT) and short-read MiSeq (Illumina) sequencing technology to generate a high quality genome sequence of *S. flexneri* serotype 1c for the first time. Results revealed that Y394 chromosome of *S. flexneri* contained mobile genetic elements, IS elements and plasmids. These set of genes was actually responsible for bacterial evolution, diversification, adaptation, pathogen's virulence and antibiotic resistance of bacteria. From the detailed analysis, they also identified novel and highly modified O-antigen structure consisting of three different O-antigen modifying gene clusters that came by horizontal gene transfer from three different bacteriophages. These were the causes of pathogen's virulence and survival in host environment [48]. Pan-genome analysis of *Akkermansia muciniphila* was done for the first time. It is the inhabitant of the intestinal tract and plays a crucial role in human health. Whole genome sequencing and annotation done of 39 isolates. Results revealed the flexible pan-genome consisting of 5644 unique proteins. Comprehensive genomic analysis among human, mouse and pig microbiomes revealed transcontinental distribution of phylogroups of *A. muciniphila* across human gut microbiomes. Qualitative analysis showed its co-relation with anti-diabetic drug usage and body mass index. It also acquired antibiotic resistance genes by lateral gene transfer from symbiotic microbes [101]. Kono N, Tomita M and Arakawa K. Nobuki in 2017, developed the algorithm for reordering of the contigs based on experimental replication profiling (eRP) to facilitate the study of the complete genome sequences, genome rearrangements, and structural variations and to summarize the bacterial genome structure within a draft genome. They also suggested the appropriate timing for genomic sampling i.e. during exponential growth phase of bacteria to obtain information about contig position relative to terminus and replication origins [102].

7. Comparative Genomics for Industrial and Environmental Friendly Microbes

Comparative genomics is also useful for exploration of microbes that are involved in bioremediation and industry. Gang Zhou and his team-mates for the first time gave complete genome sequence of *Citrobacter werkmanii* with genome features and annotation. *Citrobacter werkmanii* BF-6 belongs to family *Enterobacteriaceae*. It has been used for bioremediation of heavy metals because it produced acid type phosphatase enzyme and can accumulate heavy metals due to biofilm formation. *C. werkmanii* BF-6 and *C. werkmanii* NRBC 105721 had closely related evolutionary relationship. They also found different genes involved in biofilm formation. The 12-biofilm producing genes and their location on chromosome BF-6 is illus-

trating below in **Figure 11** [103].

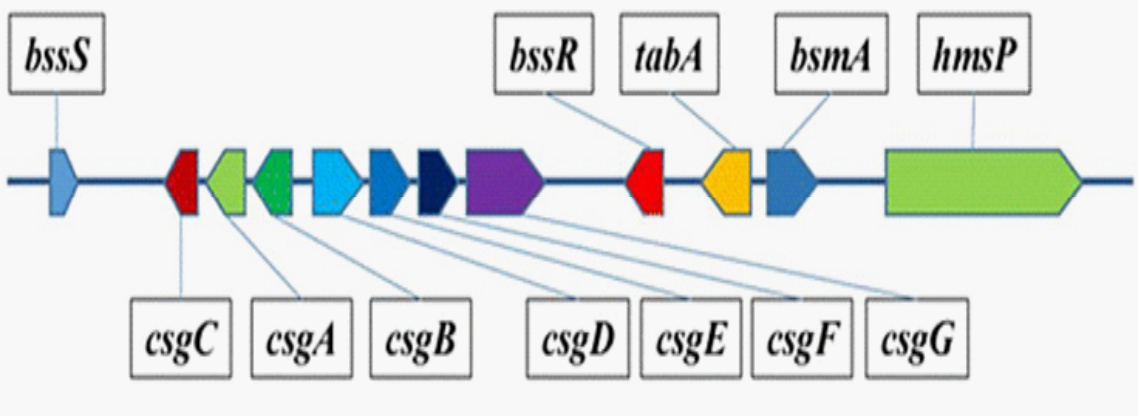

Figure 11: The relative position of biofilm producing genes on chromosome BF-6 by Citrobacterwerkmanii [103].

Industrially important species *Propionibacterium freudenreichii* (member actinobacterial group) genome was completely sequenced by using PacBio RS II sequencing platform. Genomes of 20 strains of P. freudenreichii were compared and results showed. Results showed two conjugative plasmids and three active lysogenic bacteriophages. It also helped in identification of different DNA modifications, which led to the characterization of restriction modification systems; that is CRISPR-Cas systems. The genomic difference observed in specific mucus binding and surface piliation among strains. These characteristics allowed them to grow at unfavorable conditions and help in the formation of biofilm [104].

In post genomic era, computational approaches integrated with "omics" included proteomics, genomics, and metabolomics for selection of drug and vaccine targets. For pathogenic bacteria, comparative and subtractive genomic approaches are being widely used. These identified targeted genes should be non-homologous to host. *Vibrio cholera* is a cholera-causing agent. By using a comparative genomic approach of *Vibrio cholera*, drug target Cholera endotoxin B subunit and membrane proteins like secG, secY, and secE were identified as potential vaccine targets [105].

8. Reverse Vaccinology to Identify Potential Vaccine and Drug Targets for Microbes

Development of vaccines with the help of computational approaches, utilizing genomic data, instead of culturing microbes, is termed as 'reverse vaccinology'. Vaccine development by conventional methods need culturing of pathogenic microbes and all biochemical, microbial and immunological techniques, and all this made it time consuming and laborious. Reverse vaccinology begins with the screening of pathogenic genome, which results in epitope prediction and epitope prediction is said to be the heart of reverse vaccinology [106]. Genomic sequencing discovery had paved the path for predicting the potential antigen candidates from complete genomic data. Predicted candidates are then used in vaccine preparation (**Figure 12**).

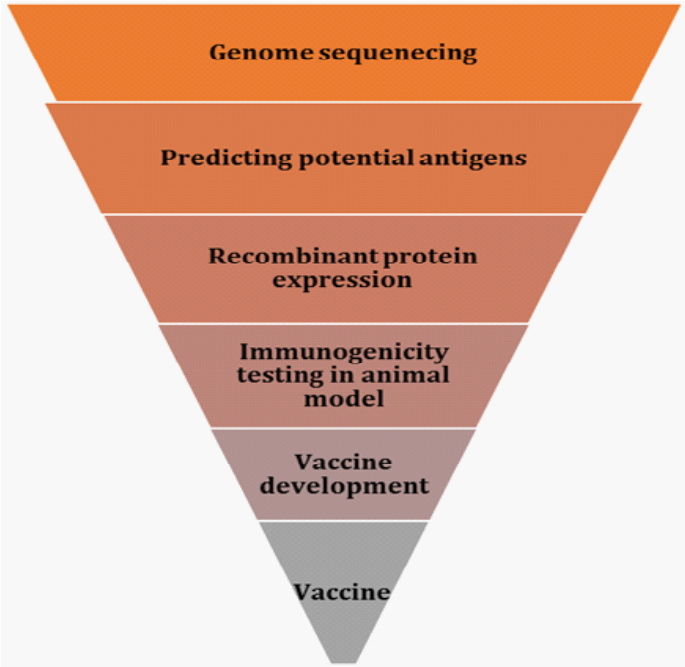

Figure 12: Steps involved in vaccine development by reverse vaccinology

Comparative genomics, metabolic pathways analysis, and additional drug prioritizing parameters were used to identify drug and vaccine targets against *Mycoplasma genitalium*, a pathogenic agent responsible for sexually transmitted diseases in human. Total 79 proteins were identified out of which 67 proteins were non-homologous essential proteins that could be potential drug and vaccine targets [107].

Ghosh S and co (2014) also identified drug and vaccine targets in *Staphylococcus aureus* by using comparative genomic approach. They identified 19 proteins as vaccine candidates and 34 proteins as drug targets [107].

Undoubtedly, vaccinologists have successfully eradicated life-threatening diseases. Still, there is a long way to go, to our surprise, there are only ~50 human vaccines out of which only 35-40 are licensed in the US and Europe [108]. The first vaccine developed using reverse vaccinology was against Serogroup *B meningococcus*, by *RinoRappuoli* [109]. They first screened the genome of *B meningococcus*, examined the genome for antigens. Expression of potential candidates was tested in *E. coli* and most potential candidates were applied in vaccine development. After massive efforts, this vaccine was approved safe and potent [110].

Different soft wares are involved in reverse vaccinology a few of them are listed below,

Programs identifying Open Reading Frames

Sr. No.	Software
1.	ORF-FINDER
2.	GLIMMER
3.	GS-FINDER

Programs identifying potential proteins

Sr. No.	Software
1.	ProDom
2.	Pfam
3.	PROSITE

8.1. Applications of reverse vaccinology

Reverse vaccinology (RV) is an efficient and cost-effective as compared to conventional vaccine development approaches. Software for reverse vaccinology includes VacSol, NERVE, VAXIGN, RANKPEP, Vaceed, PGAP. As eukaryotes possess enormous and complicated genome as compared to prokaryotes, therefore RV is more effective towards eukaryotic genome [111].

Bacterial diseases for which licensed vaccines have been developed using 'reverse vaccinology' approach are listed as follows (**Table 6**).

Table 6: Vaccines developed by using "Reverse Vaccinology" approach

Sr. No.	Bacteria	Disease	Vaccine	Trade name
1.	*Neisseria meningitides*	Meningococcal meningitis	Meningococcal Group B Vaccine	BEXSERO
2.	*Bacillus anthracis*	Anthrax	Anthrax Vaccine Adsorbed (AVA)	Biothrax
3.	*Vibrio cholera*	Cholera	Cholera Vaccine Live Oral	Vaxchora
4.	*Corynebacterium diphtheria*	Diphtheria	Diphtheria and Tetanus Toxoid Adsorbed	None
5.	*Yersinia pestis*	Plague	Plague vaccine	None
6.	*Streptococcus pneumoniae*	Pneumococcal	Pneumococcal Vaccine, Polyvalent	Pneumovax 23
7.	*Salmonella enterica*	Typhoid fever	Typhoid Vaccine Live Oral Ty21a	Vivotif

9. Future prospects

This study spells out that microbiology is turning into a data science; potent association of experimental and computational biologists can bring revolution in near future. Considering the present rate of advancements of technology in this discipline, is difficult to predict the future. Nevertheless, we will outline few improvements to be made. Undoubtedly, NGS require small amount of genetic material for analysis, but this is even lesser, for example in case of endangered species. In addition, improvements must be made to produce more and longer sequence reads, reduced sequence errors and algorithms for data analysis, this will surely result in improved transcriptomic and genomic data compilation. Future studies require focusing

on genome architecture and regulation as it is link with conservation biology. Cost effective sequencing technique is applied more frequently, generating more sequencing data and hence demands new infrastructures, analysis and data storage approaches and sharing databases. This revolution resulted in enhancements of bringing novel aims and objectives of genetic research in reach of molecular ecologists.

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Chapter 4

Advances in Biotechnology in the Post Genomics era

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1. Introduction

Pregenomic era comprised of efforts to sequence genome and now in the post genomic era where we have greater than 1000 genomes available, science is heading toward extracting valuable information from them. Sequencing has helped in revealing the hidden meaning of nucleotide and protein sequencing. Shifting from the trends of the pregenomic era to post genomic era resulted in enormous data. In this chapter, we have explored the impact of advancements in genomics on organisms ranging from viruses to plants with focus on their applications in Biotechnology. In particular, we have discussed the influence of rapidly available sequencing data in exploiting the viruses for our benefit, especially in vaccine development. In this regard, some Bioinformatics-based tools and software have been discussed. The Human Genome Project and its importance as an example and a motivation for other similar organism-specific large-scale sequencing projects has been highlighted. Finally, some aspects related to genomics-based Biotechnological aspects of plant sciences had been explored. We conclude that recent progress in genomics has brought about major breakthroughs in terms of applications of Biotechnology in different sectors such as vaccinology, proteomics, personalized medicine, as seen in **Figure 1**.

The journey began in 1976, when RNA of E.coli infecting bacteriophage, MS2, was sequenced completely [1]. Following this discovery, a DNA containing bacteriophage, PhiX174, was sequenced by Sanger and his team [2]. It was the first DNA based genome that was se-

quenced. PhiX174 was later used as a model organism in the ushering era of synthetic biology [3]. Sanger shot gun sequencing provided a platform to sequence genome with greater ease, but cost was a major constraint of this technique. In 1981, Cauliflower mosaic virus was sequenced and variation within the different strains were analyzed by using comparative genomics [4]. In 2004, complete genome sequencing of mimivirus blurred the distinction between bacteria and viruses [5]. Unlike bacteria, viruses do not contain rDNA to study phylogenetic relationship, so a clone based sequencing strategy was used to sequence and classify unculturable marine viruses [6]. The sequencing of these marine viruses gave insight into their role in biogeochemical cycles [7].

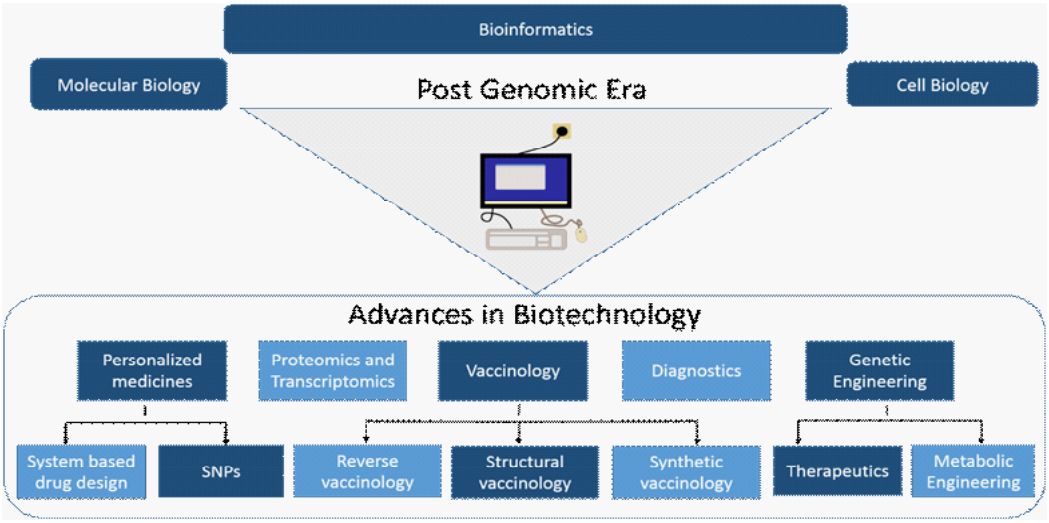

Figure 1: Advancements of Biotechnology in the postgenomic era in different sectors is illustrated.

2. Impact of Genomics in Virology

The development of Next Generation sequencing has brought about a revolution in the field of virology. Viral genomes, though rather small size, maintain their intellectual curiosity amongst scientists [8]. The emergence of pandemic viral infections such as H5N1 and H1N1 also necessitated the availability of whole genome sequence to gain an insight into the evolution and molecular epidemiology of these viruses [9]. This was particularly true since earlier phylogenetic analysis based on partial sequence had failed to comprehend the complex historical recombination events that are potentially responsible for pandemic emergence. NGS along with partitioning and barcoding has enabled the efficient sequencing of complete viral genomes leading to better understanding of the transmission and emergence of clinically important viruses [8].

The opportunity to sequence and compare multiple whole genomes has highlighted the crucial genetic differences between different viral isolates [9]. The current knowledge about sequencing has enabled researchers to analyze drug resistance in DNA and RNA viruses (Cytomegalovirus and Haemophilus influenza virus). High coverage sequencing (also termed as deep sequencing) helped to identify lesser drug resistant variants. However, whole genome sequencing of viruses can help us in understanding of better and potential drug resistant vari-

ants. Other than research purposes, sequencing analysis is equally important in clinical studies. For instance, highly active antiretroviral therapy in case of HIV has significantly improved the survival rate of HIV patients [10]. Apart from this, metagenomics analysis is also extensively used as a diagnostic tool. Herpes simplex virus was identified in the cerebrospinal fluid (CSF) of patients who were suspected to have viral meningoencephalitis. Pan viral screening is believed to aid in diagnostics of Central nervous system infections [11]. However, there is a need to develop a more rapid sequencing technology to share real time sequence information to guide healthcare sector for the control of outbreaks [8].

The rapid growth of viral genome sequences and their Bioinformatics analysis has brought about a revolution in viral genomics. The development has challenged the conventional classification and nomenclature of these organisms [12]. Genomics and Bioinformatics-based software and tools need to be developed to utilize the genome attributes such as phylogenomics and unique features in the strain's biology and also about the viral families. Therefore, the information derived from primary sequence data can be useful compared to the previous use of immunochemical methods that probed limited and often murky epitopes that are actually an indirect interpretation of the primary sequence data in the form of a tertiary sequence.

Viral sequencing data is being used in Forensic studies. Sexually transmitted viruses such as HIV (Human Immunodeficiency virus)were used to generate phylogenetic profiles of disease and link victim and assailant [13]. Some viruses such as HCV (Hepatitis C virus) [14], EBV (Epstein Bar virus) [15], and BKV (BK virus) [16] can prove to be significant in determining place of birth and locality of suspicious individuals.

Advances in Bioinformatics has enabled scientists to acquire a better understanding of the biology of pathogenic viruses. For example, viruses belonging to the Poxviridae family infect a variety of hosts and cause small pox disease in humans. Moreover, their natural occurrence and potential bioterrorism concerns has aroused an interest in the scientific community [12]. Ebola virus is also suspected to be a bioweapon [17]. A collection of genomes through recent advancements in genome sequencing has permitted the understanding of core genes (orthologous genes) that are present in all the members of the Poxviridae family. Faced with the challenges of analyzing simple and smaller genomes of viruses, a poxvirus-specific computational tool was developed by Hendrickson et al. to predict accurate gene sets [18]. This comparative approach highlighted the concept of reductive evolution in which loss of particular genes is thought to play an essential role in the speciation and restriction of emergent viruses to operate in particular environments. Eaton et al., explored the idea of core genes in the Iridoviridae family [19]. They concluded that genomes contain groups of repetitive sequences.A similar study was conducted in Nucleo-Cytoplasmic Large DNA Viruses (NCLDV) and orthologous genes were determined in 6 families using Comparative phylogenetics [20]. Thus, in the postgenomic era, numerous Bioinformatics tools have been developed for comparative

genome analyses which of course was dependent on the availability of genome sequences.

In pregenomic era Edward Jenner used a cow pox virus to induce immunity against smallpox viruses in the human, but understanding of mechanism of vaccines was limited at the time [21]. On the basis of further innovation and advancements in the field of Vaccinology, vaccines were categorized into first generation vaccines (having inactivated/killed lysate of pathogens), second generation vaccines (pure antigenic determinants of pathogen)and third generation vaccines or modern Vaccinology (that use genomics, transcriptomics and genome analysis to construct vaccine candidates) [22]. The approaches used in classical Vaccinology (1^{st} generation and 2^{nd} generation vaccines) were unable to fully overcome infections due to the diversity and complexity of microbial genomes. Poorly activated pathogen lysates may cause adverse effects, so there was a need to introduce novel strategy to develop universally applicable and safe vaccines.

3. Reverse Vaccinology

With the accessibility of complete genomic data of pathogenic microorganisms, an innovative approach known as “reverse vaccinology” has been designed for vaccine development. Computer-aided analyses can be conducted utilizing the genome sequence of a particular pathogen to predict the antigenic components for the development of a potential vaccine [23]. The advantages are multi-fold. There exists no requirement to grow and cultivate the microorganism. The entire procedure is done using computers without the requirement of laboratory apparatus such as pipettes, fermenters and so on. Pathogens requiring strict handling can be studied without any safety concerns. The framework takes into consideration all the proteins that are expressed (*invivo* or *invitro*) by a pathogen at a given time. Antigens used in conventional wet laboratory experiments are identified; moreover, novel antigens are discovered based on a completely different framework. In case of viruses, the mutation rate is higher so reverse vaccinology approach can provide data regarding putative antigenic vaccines that are conserved across all the strains in viral species. In case of Dengue virus 9000 viral sequences were analyzed to determine potential vaccine constructs that can elicit immunity against nearly all the strains of dengue virus [24]. Similar studies are conducted in Zika virus [25,26], human papilloma virus [27], Congo virus etc. [28,29]. More than 9500 reference sequences of viral genomes are available on NCBI. The reverse vaccinology approach provides new and yet unexplored insights into the mechanisms of immune intervention.

This top down strategy of the post genomic era has reduced the time and cost required for making vaccines. However, testing these vaccines in rodents and then in mammals is required before clinical trials. In contrast to classical vaccinology era the labor-intensive efforts are reduced [30,31]. Now whole genomes can be analyzed and only antigenic immunogenic, non-homologous to human and surface exposed vaccine constructs can be designed that can

elicit immune response in human body without any risk of allergy or autoimmunity [32]. The approach is illustrated in **Figure 2**.

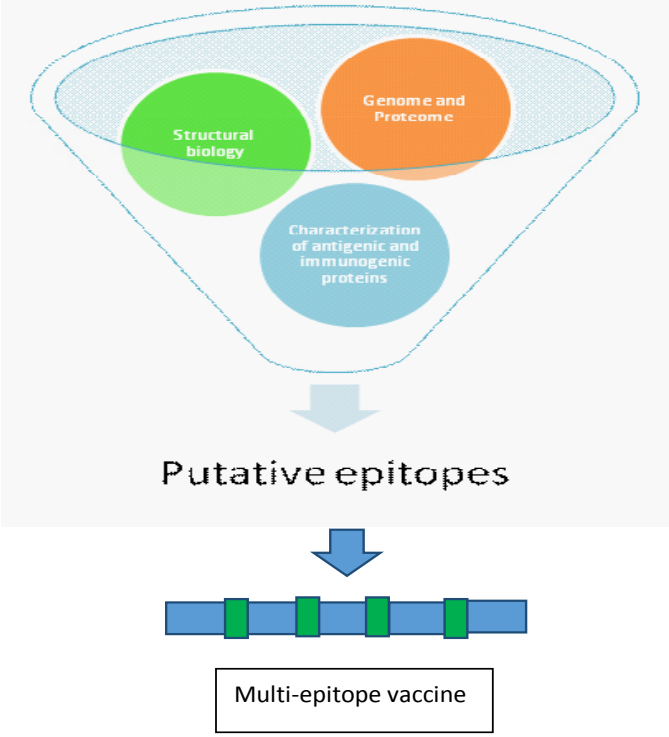

Figure 2: Reverse vaccinology. Genome and Proteome analysis can be used to predict epitopes. These epitopes are then characterized based on their antigenicity and immunogenicity [28]. Individual epitopes can be linked to make a multi-epitope vaccine [33].

The tools used in viral reversevaccinology and other in silico analysis are mentioned here-with:

Tool name	Use	Links
NetMHC(34,35) CTLPred(36) nHLAPred(37) Propred 1(38) Propred(39) RankPrep(40–42)	Prediction of MHC binding epitopes	http://www.cbs.dtu.dk/services/NetMHC/ http://crdd.osdd.net/raghava/ctlpred/ http://crdd.osdd.net/raghava/nhlapred/ http://crdd.osdd.net/raghava/propred1/ http://osddlinux.osdd.net/raghava/propred/ http://imed.med.ucm.es/Tools/rankpep
AVPpred(43)	Antiviral peptide prediction algorithm	http://crdd.osdd.net/servers/avppred/
HBVdb(44) GATU(45)	Annotation of viral genomes	http://hbvdb.ibcp.fr https://virology.uvic.ca/virology-ca-tools/gatu/
VaxiJen(46) ANTIGENpro (47)	Antigen prediction	http://www.jenner.ac.uk/VaxiJen
Allergen FP(48) ALGPred(49)	Allergenicity prediction	http://ddg-pharmfac.net/Allergen FP http://www.imtech.res.in/raghava/algpred/
SOLpro (50)	Solubility upon overexpression in E.coli	scratch.proteomics.ics.uci.edu/
siVirus(51)	Antiviral siRNA design software	http://sivirus.rnai.jp/
ViReMa(52)	Algorithm for detection of recombination junctions in viral Genomes	https://omictools.com/viral-recombination-mapper-tool

However, due to lack of adequate knowledge on aspects of immunological aspects of vaccine, good correlates of protective immunity are uncommon and is the major limitation of reverse vaccinology. Moreover, the approach is entirely protein-specific; other non-protein antigens such as polysaccharides and glycolipids are not covered in this method. Another drawback of reverse vaccinology is the genetic instability of some viruses. To circumvent this limitation, structure based reverse vaccinology and synthetic genomics can be applied for rational vaccine design. Structural vaccinology integrates data from structural biology, human immunology and bioinformatics to predict immunogenic and antigenic residues [53]. Crystal structure of Respiratory Synctial virus conjugated with fusion glycoprotein showed high neutralizing antibody titres using Structural vaccinology approaches [54]. However, in case of synthetic genomics, genomes can be artificially synthesized using genetic material. One of the recent examples of synthetic genomics was vaccine against avian influenza virus [55]. These vaccines can be manufactured rapidly and mimic natural viruses in their mode of action. Synthesized genomes and engineered antigens have improved the efficacy of vaccines, but understanding the pathogenesis of viruses is still of primary interest.

Some viruses have genome integration capabilities hence that they are actively used as viral vectors in gene therapy. These viral vectors are safe and effective [56]. Nevertheless, viral integration at certain sites may cause malignant transformation and altered gene expression. With the help of bioinformatics, a pipeline was recently designed to determine integration sites in NGS based viral vectors that could be used in gene therapy data. The tool is efficient and performs analysis by Agilent Sure Select through rapidly evolving targeted sequencing and PCR based linear amplification strategies. It is available at https://github.com/G100DKFZ/gene-is [57]. Some other tools that also determine viral integration sites are ViralFusionSeq [58] and Virus-Clip [59]. At the time of writing, GENE-IS is the first tool that gives information based on two sequencing strategies and has no specific constraints regarding input data.

4. The Human Genome Project and its Impact on Biotechnology

Work on the ENCODE (Encyclopedia of DNA Elements) project was made possible after the completion of the Human Genome Project [60]. The scientists working in the ENCODE project channelized their efforts to develop an understanding of the functional components of the human genome [61]. These efforts proved fruitful as they resulted in a huge amount of data regarding the regulatory networks that control the expression of human genes [62]. Computer aided pathway analysis has been used to locate protein and enzymes in their pathways and bioreactors, respectively. In 2005, computational analysis led to allocation of 622 enzymes in biological pathways and 2709 enzymes to bioreactors [63]. Nevertheless, more research is required to decipher the functions of low annotated human genes and large non-coding genomic regions that are transcribed [60].

The HGP has directly influenced advancements in the field of proteomics. Proteins as structural components, molecular machines, or signaling devices dictate the cell-specific functionality of the transcribed genome. The HGP has greatly aided the utilization of mass spectrometry, a crucial proteomics tool, by giving reference sequences and ultimately the predictions regarding the masses of all the tryptic peptides in the human proteome [64]. This is required for the mass-spectrometry based proteomics analysis. The Mass spectrometry (MS) has in turn been the driving force of novel applications like targeted proteomics [65]. Several servers like mascot [66], sequest [67], SQID [68] are used for the analysis of data obtained from MS. This data can also be used to identify Post translational modifications(PTMs) in proteins/peptide that may help in the understanding of the role in biological pathways; SIMS server is also available to identify PTMs in MS data [69].

The HGP has also contributed significantly to our understanding of evolution. The successful completion of this project jump-started the whole genome sequencing of other eukaryotic organisms and bacterial species [70]. The resulting collection of whole genome sequencing data from a variety of living organisms ranging from microbes to human has led to the genealogical tree of life that strongly supports the notion that all species that exist nowadays arise from a common ancestor (14,71). Especially, genome analysis of Neanderthal is likely to provide more insightful results into the evolutionary aspects of human beings especially [72].

The accessibility of all the diseases genes in human, along with genes from the human pathogens that are the causative agents of infectious diseases, will have a direct influence on drug development efforts. The human genome contains nearly 30,000 genes and it is expected that most, if not all of them, would be targets of therapeutic interventions. Functional and structural analyses of these genes and their encoded proteins respectively is likely to increase the number of drugs being developed in the coming years. Pharmaceutical sector is actively engaged to exploit the yet unexplored potential of recent advancements in genomics [73]. Due to complexity of biological system, system based drug discovery is also an effective approach to design drugs [74].

The variation in the human population can be analyzed by the power of genomics which will contribute to the science of medicine. DNA sequences are already in use for diagnostic purposes to identify the association of unique sequence variants or Single Nucleotide Polymorphisms (SNPs) with a particular disease. Distinct from point mutations, SNPs are sequence variants that are frequently found in the human population. These genetic variants do not in itself cause disease; rather they contribute to disease susceptibility in an additive manner. More than 10 million SNPs in human population were identified till 2011. This data was used to study the impact of SNPs on pharmacogenomics [75]. Moreover, these SNPs are also linked with complicated responses such as personalized responses to drug therapy. Hence, it may be possible to elucidate the variants that makes humans more prone to develop diseases such as

diabetes and asthma. Moreover, SNPs can be identified that influence individual response to drugs, thus ultimately increasing the likelihood of developing personalized therapies to target the unique genetic make-up of particular patients. SNPs are present in elite controllers of HIV and restrict the binding of virus with co-receptor CCR5 to block viral entry. The survival rate in elite controllers is comparatively higher than progressors [76]. However, the associated social, ethical, legal and moral issues need to be recognized and addressed to protect privacy and to prevent discrimination.

5. Post Genomics era in Plant Biotechnology

One of the many factors that limit crop production is salinity. Plants respond to saline stress in a complex way and the response is mediated by many genes which are the components of different signaling pathways in which cross-talk has also been reported [77]. Hence, it is difficult to fully understand how plants respond to salinity. Advancements in the field of genomics has provided the much-needed knowledge for crop improvement. Genes responsive against salinity induced stress have been identified and characterized, signaling pathways have been mapped, thus ultimately providing the basis for enhancing the salinity stress response of existing plants [78]. The information is crucial in the development of stress tolerant crops through tools like gene pyramiding that has been applied in marker assisted breeding and genetic engineering [79]. The advent of Genome editing by CRISPR/Cas9, TALENs, etc. has enabled plant biologists to produce desired genetically engineered crops with improved productivity, yield, etc. Recent progress in genomics has led to increased understanding of plant responses against environmental stresses such as salinity stress and drought conditions [78]. This has in turn increased prospects for generating stress tolerant plant varieties such as wheat, rice etc.

The genome of potato had been sequenced firstly using homozygous DM1-3 518 R44 or DM and later on with a heterozygous diploid line RH89-039-16 or RH [80-82]. The availability of the whole genome sequence as well as associated annotation of almost 39000 potato genes has enabled the identification of candidate genes in those regions that are concerned with specific traits [83]. Genome sequence assisted in the identification of StCDF1 gene that is responsible for plant maturity as well as StSP6A gene that is required for tuber initiation in potato [84,85]. The study of genome also generated a collection of candidate resistance genes, thus significantly improving our ability for robust discovery along with the prospects of introgressive hybridization of R-genes in potato [86,87]. The integrated approach of biotechnology and genomics is a positive step to solve global food security concern. Oleic acid cultivars were genetically modified to enhance vegetable oil production. More than 40% increase in consumption of this oil is expected to be achieved by 2020 in the US population [88].

6. Artificial Chromosomes

To incorporate larger segment of DNA, Yeast artificial chromosome (YAC) was introduced. The system proved helpful in studying genes with the normal promoter [89–94]. The advancement in scientific knowledge and Human genome project has led to the synthesis of BAC (Bacterial Artificial Chromosome) that are used for functional analysis of proteins [95]. MAC (Mammalian Artificial Chromosome) was constructed a year after generation of YAC. In 1997, Human artificial chromosome was introduced [96], refined in 2010, and was later used in inserting HSV(Herpes simplex virus) into cancer cells making them susceptible to ganciclovir antiviral drug. The virus infected cells were cleaved afterwards [97]. Post genomic era has provided us numerous opportunities to deeply understand antiviral mechanisms, expression profiling, and pathway construction using NGS and single cell sequencing.

7. Conclusion

Genome sequencing and associated huge amount of data has transformed the World of Biotechnology. Nowadays, sequencing cost has reduced considerably enabling robust whole genome sequencing of living organisms. This recent progress has triggered the development of different Bioinformatics tools and software to analyze the huge biological data. This has aided in the better characterization of different viruses and facilitated vaccine development using sequencing data in reverse vaccinology. Moreover, these analytical tools have facilitated drug development and gene therapy using viruses. The Human Genome Project has greatly facilitated the understanding of the human genome; variations in the human genome associated with particular disease were able to be identified and a better understanding of the human evolution has been achieved by comparative genomes and phylogenomics. The ENCODE project, in itself dependent on human genome, aims to elucidate the functions/s of the non-coding regions in the human genome. Advancements in genomics has led to the identification and characterization of genes contributing to tolerance against salinity stress and drought conditions in plants thus providing an opportunity to generate genetically modified crop varieties with improved resistance against these abiotic stress factors. Biotechnology along with genomics can also be used to solve global food crisis. Finally, Yeast Artificial Chromosome and Bacteria Artificial Chromosome can be used to incorporate large DNA fragments. Hence, further advancements in genomics will no doubt have a significant impact in shaping the Biotechnology of tomorrow.

8. References

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Chapter 5

Plant Growth Promotion by Endophytic Actinobacteria Associated with Medicinal Plants

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Abstract

There is a lot of prospect for production of novel bioactive metabolites for application in medicine, pharmaceutical, agricultural and other industry from endophytic bacteria associated with medicinal plants. Actinobacteria are spore forming that can form a stable and persistent population in various ecosystems. Actinobacteria especially *Streptomyces* are prolific producers of several agriculturally important secondary metabolites that can be use as plant growth promoting and biocontrol agents. Endophytic actinobacteria associated with medicinal plants can directly promote the growth of plants through production of indole acetic acid, siderophore, solubilization of inorganic phosphate and fixing of free nitrogen. They can promote the plant under stress conditions by production of aminocyclopropane-1-carboxylic acid deaminase. They can also act as an agent for improving phytoremediation of toxic metals and organic pollutants. They may indirectly promote the plant growth by production of antifungal antibiotics and cell wall degrading enzymes. It is expected that endophytic actinobacteria associated with medicinal plants may produce bioactive metabolites that differ significantly from the soil dwelling actinobacteria. They may also participate in the host metabolic pathway and gain some genetic information and produce secondary metabolites similar to the host plants. Intensive research on characterization and identification of the untapped bioresource from endophytic bacteria especially actinobacteria is of outmost important for application in agriculture as the use of synthetic chemical pose serious risk to human health and environment. The use of plant growth promoting endophytic actinobacteria can emerge as novel sustainable and alternative tools.

Keywords: Endophytic actinobacteria; medicinal plants; *Streptomyces*; plant growth promoting activities

1. Introduction

Ethno-medicinal plants are the backbone of traditional medicine that has been used by mankind to treat a number of diseases since time immemorial. Numerous studies on the bioactivity of medicinal plants are still underway, since they constitute a rich source for production of novel secondary metabolites, for application in pharmaceutical, agricultural and other industries. In the past, research on medicinal plants focussed primarily on their ingredients; however, recently the focus has shifted to include the structure and function of several medicinal plant microbiomes. Endophytic bacteria associated with the medicinal plants may directly or indirectly involve in the production of bioactive phytochemicals [1]. Surprisingly, not only the plants themselves were able to produce compounds with phytotherapeutic properties, but their associated microbes, in particular endophytes, could as well [1,2].

Endophytes are microorganisms that reside within the interior tissues of plants without exhibiting negative effects on the host plant or the environment. However, some seemed to be latent pathogens and, conditionally, either induce or participate in host plant infection [3]. Almost all the plants have been found to be associated with one or more endophytes. They are able to associate with the host at a very early stage of plant development [4]. Endophytic bacteria have been isolated from various parts of the plants. However, majority of them are isolated from roots tissues followed by stem and leaf. The woody plants conferred far greater diversity in comparison to herbaceous plants. Plants that grow at tropical region harbour greater diversity than that grow at temperate region [5,6,7].

It is widely believed that the potential of secondary metabolites with biological activities from endophytic bacteria is just as great as that achieved from soil bacteria [8]. As a consequence of long term association of endophytes with the host plant, bacteria may participate in metabolic pathways and/ may gain some genetic information from the host plant, and produce biologically active compound(s) similar to the host plant [7,9,10,11]. Endophytes associated with medicinal plants have great potential to produce unique secondary metabolites, which can be exploited for application in pharmaceutical, agricultural and other industries.

2. Actinobacteria

Actinobacteria are aerobic spore forming gram-positive bacteria containing high guanine-cytosine (57-75%) in their genome, and belong to the order Actinomycetales that grow as branching filaments consisting of vegetative mycelia and aerial hyphae. They are ubiquitous and form a stable and persistant population in various ecosystems and play an important ecological role in soil nutrient cycling [12,13,14]. They are well known for the production of wide range of secondary metabolites, for use not just in pharmaceutical industries but agriculture as

well. The most extensively studied actinobacteria belong to genera *Streptomyces*. Actinobacteria are prolific producers of several agriculturally important secondary metabolites and several members have been considered as plant growth promoting (PGP) and biocontrol agents [15, 16,17].

Actinobactera can stimulate plant growth directly or indirectly. The main mechanisms by which they directly contribute to the plant growth are production of phytohormones such as indole-3-acetic acid (IAA), cytokinins and gibberellins; enhancing plant nutrition by solubilization of minerals such as phosphorus (P) and iron, production of siderophores and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase [17,18]. They indirectly benefit the plant by biocontrol of deleterious pathogens through the production of antibiotics and volatile compounds (VOCs), synthesis of fungal cell wall degrading extracellular enzymes, induction of systemic resistance and competition for nutrients and niches [17,18,19].

3. Actinobacteria from Medicinal Plants

Actinobacteria are able to associate with the host plant as endophyte at the early stage of plant development [4]. Outer plant tissues have greater diversity compared to other plant tissues [8,20,21,22]. Majority of actinobacteria are isolated from root tissues followed by stem and leaf [23-30]. The high rate of occurrence of actinobacteria in roots may be due to the fact that the actinomycetes are natural dwellers of soil and hence easily come in contact with the roots of plants, and may form symbiotic association with the host by entering the plant tissues. [5-7].

Among endophytic actinobacteria recovered from medicinal plants, Streptomyces accounts for the most abundant genus [5-7,26,28,30-34] followed by *Micromonospora, Actinopolyspora, Nocardia, Saccharopolyspora, Streptosporangium, Promicromonospora* and *Rhodococcus* [25,26,30,31]. Some rare genera, like *Dietzia, Microtetraspora, Actinocorallia, Verrucocsispora, Isoptericola* and *Kytococcus* [35,36] were also reported from medicinal plants.

Actinobacteria isolated from medicinal plants are producers of growth promoting metabolites, insect and pest repellents, antimicrobials against plant pathogens and protectors in stress conditions [7,10,11,16,28,37]. They also exhibited antimicrobial activity against multi drug resistant pathogens [38], antiviral [39], larvicidal [40], antimalarial [41,42] and other important activities such as antitumor [43], antidiabetic [44] and antioxidant [30].

4. Direct Plant Growth Promotion

Endophytic bacteria especially actinobacteria may directly contribute to growth of plants through PGP activities such as P solubilization, IAA, ACC deaminase and siderophore

production, and nitrogen (N) fixation [27,29,45-47] (**Table 1**). Out of 81 endophytic actinobacteria isolates from medicinal plant *Rhynchotoechum ellipticum*, 36 strains were positive for IAA production in the range of 7.4 to 52.3 µg/ml. Majority of IAA producer belong to genus *Streptomyces* and some to *Actinomycetes, Microbacterium, Micromonospora, Leifsonia, Brevibacterium, Pseudonocardia, Promicromonospora, Kocuria and Amycolatopsis* [30]. Similarly, *Gangwar* et al. [48] also found actinobacteria, mostly *Streptomyces* sp, capable of producing IAA in the range of 9.0–38.8 µg/ ml. Khamna et al. [49] reported that *Streptomyces* spp. isolated from medicinal plants produced IAA in the range of 11–144 µg/ml. Liu et al. [50] reported that 88 % endophytic actinobacteria isolated from medicinal plants *Ferula songorica* could fix free N while 19 % solubilize P. Dochhil et al. [51] demonstrated that IAA producing endophytic *Streptomyces* sp. CA10 and CA26 isolated from a folk ethno-medicinal plant *Centella asiatica* enhanced seed germination and seedling growth of French bean.

Endophytic bacteria having multiple PGP activities could successfully colonized the internal tissues and promote the growth of plants under greenhouse and field conditions. *Streptomyces* sp. En-1 endophytic to medicinal plant *Taxus chinensis* synthesize IAA via indole-3-acetamide (IAM) pathway. The strain could successfully colonize the intercellular tissue and promote the growth of *Arabidopsis* [52]. Endophytic bacteria strains *Sphingomonas, Pantoea, Bacillus* and *Enterobacter* isolated from the roots of elephant grass could solubilize inorganic P, fix N, and produce IAA and ammonia. Similarly, those strains were able to successfully colonized the roots of *Hybrid Pennisetum* and significantly promote the growth under salt stress conditions [53]. Endophytic bacterial strains *Paenibacillus* and *Bacillus* sp. (isolated from medicinal plant *Lonicera japonica*) possessing positive results for P solubilization, IAA, Siderophore, ACC deaminase productions enhance the growth and chlorophyll content of wheat plants under pot conditions [54]. Similarly, treatment of chilli and tomato with endophyte *Streptomyces* sp. having multiple PGP activities significantly enhance the growth under greenhouse conditions [37].

ACC deaminase producing endophytic bacteria can promote the growth of host plants by degrading the ACC, precursor of stress hormone ethylene, before its oxidation by plant ACC oxidase thus blocking stress ethylene production. As a result, bacteria can protect the host when plant is exposed to either biotic or abiotic stress conditions. ACC deaminase producing endophytic actinobacteria can effectively protect the host plants growth inhibition by flooding, high salt, drought, presence of pathogens, high levels of toxic metals and organic pollutants and low temperature [55,56].

Plant growth promotion by ACC deaminase producing endophytic bacteria was demonstrated by Sun et al. [56]. ACC deaminase producing wild type *Burkholderia phytofirmans* could promote the elongation of the roots of canola seedlings. Whereas, ACC deaminase (*acds*) gene deleted mutant strain failed to promote the growth of root growth. Tomato plant

treated with the ACC deaminase producing endophytic bacteria (*Pseudomonas fluorescens* and *Pseudomonas Migulae*) were more healthier than those plants treated with the mutant strains deficient in *acds* gene when grown under salt stress conditions (165 mM and 185 mM) [57]. The strains also delayed the flowers senescence in *Dianthus caryophyllus*, whereas senescence were quicker when treated with *acds* gene deleted strain [58]. Endophyte *Bacillus* sp from medicinal plant *Phyllanthus amaru* positive for IAA, ACC deaminase and siderophore production enhance germination, vigor index and growth of *Phyllanthus amarus* under salt stress conditions (160 mM) [59].

Endophytes have a great potential for use as an agent for improving phytoremediation and biomass production of non-food crops [60]. Endophytic strain *Pseudomonas* sp. A3R3 could successfully colonize the interior tissue of *Alyssum serpyllifolium* and *Brassica juncea* and increased the plant biomass and Ni accumulation in both plant when grown in Ni contaminated soils [61].

Table 1. PGP activities of endophytic actinobacteria from medicinal plants

Endophytic actinobacteria	Host	PGP activities	Reference
Streptomyces sp. En-1	*Taxus chinensis*	IAA production	51
Streptomyces albosporus, *Streptomyces cinereus*, *Micromonospora* sp. O6, *Saccharopolyspora* sp. O9	*Aloe vera, Mentha arvensis, Ocimum sanctum*	P solubilisation, IAA production	47
Streptomyces roseosporus	*Mentha arvensis, Ocimum sanctum*	P solubilisation, IAA production	47
Streptomyces aureus, Streptomyces griseorubroviolaceous, Streptomyces globisporus,	*Mentha arvensis, Ocimum sanctum*	IAA production	47
Streptomyces viridis	*Aloe vera, Mentha arvensis, Ocimum sanctum*	IAA production	47
Streptomyces olivaceus, Streptomyces sp. BPSAC101, *Streptomyces* sp. BPSAC121, *Streptomyces thermocarboxydus*	*Rhynchotoechum ellipticum*	P solubilization IAA and ammonia production	30
Brevibacterium sp. S10S2, *Janibacter* sp. R4S4, *Microbacterium* sp. S4S17	*Ferula sinkiangensis*	IAA and siderophore production, N fixation	29
Kocuria sp. R7S1	*Ferula sinkiangensis*	IAA production, N fixation	29

5. Indirect plant growth promotion

Endophytic bacteria, especially actinobacteria, can indirectly promote the growth of plant by production of antifungal antibiotics, cell wall degrading enzymes and VOCs. Actinobacteria are prolific producers of several agriculturally important secondary metabolites for use as biocontrol agents. Of about 23,000 bioactive secondary metabolites discovered in end-

ophytic microorganisms, approximately 10,000 metabolites are produced by endophytic actinobacteria and 7,600 are derive from the genus *Streptomyces* [62,63,64].

Endophytic actinobacteria isolated from medicinal plant Ferula *sinkiangensis* inhibit the growth of fungal pathogen *Alternaria alternate* [29]. Of 81 endophytic actinobacteria isolated from medicinal plant *Rhynchotoechum ellipticum*, 72 inhibit the growth of *Fusarium proliferatum, F. oxysporum f. sp. ciceri* and *F. oxysporum*. Majority of the strain showing antifungal activities belong to *Streptomyces* spp. *viz; Streptomyces olivaceus, Streptomyces* sp. *BPSAC101, Streptomyces* sp. BPSAC121 and *Streptomyces thermocarboxydus*. *Streptomyces olivaceus* and *Streptomyces* sp. BPSA 121 produce antifungal antibiotics ketoconazol, fluconazole and miconazole (**Table 2**). Seventeen strains showed positive results for presence of antibiotics biosynthetic gene cluster PKSI, PKSII and NRPS [30]. Presence of these biosynthetic gene clusters were also detected in endophytic actinomycetes isolated from 26 medicinal plants [31]. Antibiotic 6-prenylindole produced by endophyte *Streptomyces* sp. exhibit significant antifungal activity against plant pathogens, viz; *A. brassicicola* and *F. oxysporum*. Antifungal compound fistupyrone from *Streptomyces* sp. inhibit the infection of *A. brassicicola* in spring onion [65]. Li et al. [66] reported that antibiotic staurosporine extracted from endophytic *Streptomyces* strain CNS-42 showed a potent effect against *F. oxysporum f. sp. cucumerinum*. The *in-vivo* biocontrol assays showed a significant reduction in disease severity and increases in biomass and growth of cucumber plant. Endophytic Streptomyces sp. showed antifungal activity against *Alternaria* sp., *Colletotrichum truncatum, Geotrichum candidum, F. oxysporum* and *F. udum* [47,64]. Four peptide antifungal compounds Munumbicin A-D obtained from Streptomyces NRRL 3052, endophytic actinobacteria from medicinal plant snakevine, inhibit important agricultural fungal pathogens such as *P. ultimum, R. solani, Phytophthora cinnamomi* and *Sclerotinia sclerotiorum* (**Table 3**). The pepetide compounds contained common amino acids such as threonine, aspartic acid (or asparagine), glutamic acid (or glutamine), valine and proline, in varying ratios [40].

Production of fungal cell wall degrading enzymes by endophytic bacteria especially actinobacteria and their biocontrol activities against important plant fungal pathogens have been well documented in a number of literatures [27,30,50]. Endophytic bacteria isolated from ethnomedicinal plants exhibited antifungal activity against *F. oxysporum* through production of chitinase, pectinase and cellulase [45]. Similarly, cellulase and pectinase producing endophytic strains *Paenibacillus* and *Bacillus* sp. isolated from medicinal plant *Lonicera japonica* inhibit the growth of *F. oxysporum* [54]. Cell wall degrading enzymes and HCN producing endophytic actinobacteria such as *Streptomyces* sp. DBT204, *Streptomyces* sp. DBT 207, *Nocardiopsis* sp., and *Streptomyces thermocarboxydus* inhibit the growth of *R. solani, F. oxysporum, F. proliferatum, F. graminearum* and *Colletotrichum capsici* [37]. Chitinase producing endophytic *Streptomyces* sp. isolated from maize plant showed antifungal activity against *Fusari-*

um sp., *Pythium aphanidermatum, R. solani, Sclerotinia sclerotiorum*. The strain reduced the damping-off incidence caused by *P. aphanidermatum* in cucumber under greenhouse conditions [67]. Similarly, 3 endophytic actinomycetes isolated from cucumber roots identified as *Actinoplanes campanulatus, Micromonospora chalcea* and *Streptomyces spiralis* significantly promoted plant growth, yield and reduced seedling damping-off, and root and crown rots of mature cucumber caused by *P. aphanidermatum* under greenhouse conditions [68,69]. The three isolates causes plasmolysis, hyphal lysis and reduced the conidial germination of fungal pathogens by production of cell wall degrading enzymes such as chitinase, glucanase and cellulase. The strains could successfully colonize the internal tissues of roots, stems and leaves under field conditions [68,70,71].

Table 2: Antifungal metabolites production by endophytic actinobacteria from medicinal plants

Endophytic actinobacteria	Host	Antifungal metabolite	Target pathogen(s)	Reference
Streptomyces sp. NRRL 3052	*Kennedia nigriscans*	Munumbicins A, B, C and D	*Pythium ultimum, Rhizoctonia solani, Phytophthora cinnamomi, Sclerotinia sclerotiorum*	40
Streptomyces sp. MSU-2110	*Monstera sp.*	Coronamycin	*Pythium ultimum, Fusarium solani, Rhizoctonia solani*	71
Streptomyces sp. NRRL 30562	*Kennedia nigriscans*	Munumbicins E-4 and E-5	*Pythium ultimum, Rhizoctonia solani*	41
Streptomyces sp. Hedaya 48	*Aplysina fistularis*	Saadamycin/5,7-Dimethoxy-4-pmethoxylphenyl coumarin	*Fusarium oxysporum*	72
Streptomyces aurantiacus	*Impariens chinensis*	-	*Fusarium oxysporum, Curvularia lunata, Botrytis cinerea*	73
Streptomyces chryseus	*Potentilla discolour*	-	*Botrytis cinerea*	73
Streptomyces sp. SAUK6020	*Achyranthes aspera*	-	*Fusarium graminearum*	73
Streptomyces albogriseolus	*Cynanchum auriculum*	-	*Fusarium graminearum, Curvularia lunata, Botrytis cinerea*	73
Streptomyces ochraceiscleroticus	*Salvia militiorrhiza*	-	*Curvularia lunata Botrytis cinerea*	73
Micromonospora peucetia	*Ainsliaea henryi*		*Curvularia lunata, Botrytis cinerea*	73
Micromonospora sp. SAUK6030	*Stellera chamaejasme*		*Curvularia lunata, Botrytis cinerea*	73

Nonomuraea raseola	*Ainsliaea henryi*		*Fusarium oxysporum*, *Curvularia lunata*, *Botrytis cinerea*	73
Rhodococcus sp. SAUK6013	*Rhizoma arisaematis*	-	*Curvularia lunata*	73
Streptomyces sp. BPSAC34, *Leifsonia xyli*, *Microbacterium* sp. BPSAC21	*Eupatorium odoratum*, *Musa superb*, *Mirabilis jalapa*, *Curcuma longa*, *Clerodendrum colebrookianum*, *Alstonia scholaris*, *Centella asiatica*		*Rhizoctonia solani*, *Fusarium oxysporum*, *Fusarium graminearum*, *Fusarium prolifratum*	16
Brevibacterium sp. S1S8	*Ferula sinkiangensis*	-	*Alternaria alternate*, *Verticillium dahlia*	29
Microbacterium sp S4S17	*Ferula sinkiangensis*	-	*Alternaria alternate*	29
Kocuria sp. S3S2	*Ferula sinkiangensis*		*Alternaria alternate*	29
Streptomyces olivaceus, *Streptomyces* sp. BPSA 121	*Rhynchotoechum ellipticum*	Ketoconazol Fluconazole Miconazole	*Fusarium oxysporum*, *Fusarium proliferatum*	30
Streptomyces thermocarboxydus, *Streptomyces* sp. BPSAC101	*Rhynchotoechum ellipticum*	Fluconazole	*Fusarium oxysporum*, *Fusarium proliferatum*	30

Table 3: Antifungal activity of the Munumbicin A-D from Streptomyces NRRL 3052 against indicated pathogens (MIC µg/ml) [Adapted from Catillo et al. [40]]

Fungal pathogens	Munumbicin A	Munumbicin B	Munumbicin C	Munumbicin D
Pythium ultimum	2.0	0.2	4.0	0.4
Rhizoctonia solani	-	8.0	1.5	15.6
Phytophthora cinnamomi	-	6.2	1.5	15.6
Sclerotinia sclerotiorum	8.0	0.2	8.0	2.0

6. Conclusions and future perspectives

Intensive research on characterization and identification of the diverse population of endophytic actinobacteria associated with medicinal plants is of utmost importance, inorder to explore the enormous untapped bioresource for bioactive metabolites, for application in modern medicine, agricultural, pharmaceutical and other industries. It is expected that endophytes may produce novel secondary metabolites that differ significantly from soil-dwelling actinobacteria or other bacteria. As the use of synthetic pesticide and fertilizers pose serious

threat to human health and environment, the use of plant growth promoting endophytic actinobacteria can emerge as alternative tools for sustainable, organic and environmental friendly agricultural crop production.

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Advances in Biotechnology

Chapter 6

Viability of Probiotics in Dairy Products: A Review Focusing on Yogurt, Ice Cream, and Cheese

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Abstract

Probiotic is a dietary supplement of live microorganism that contributes to the health of the host. Commercially produced food biotechnology products may contain either a single probiotic strain or bacterial mixtures of various complexities to increase food nutritional and therapeutic properties. It is highly desirable that the viable number of probiotics in the final product to be at least 10^6–10^7 cfu/g to be accepted as the therapeutic minimum. Various ways were carried out to enhance the viability of probiotics. Therefore, the purpose of the present study is to review the importance of probiotics in dairy food and their viability in yogurt, ice cream and cheese during storage.

1. Introduction

Foods are functional when they provide additional properties other than nutritive values. Dairy products are established as healthy natural products and they form one of the four major food groups that make up a balanced diet [1]. Regular consumption of certain dairy products has beneficial effects in the prevention of disease [2] because they contain a number of active compounds with putative roles in both nutrition and health protection such as minerals, fatty acids, prebiotics, probiotics, carbohydrates and proteins/peptides.

Lactic Acid Bacteria (LAB) are friendly bacteria associated with the human gastrointestinal tract. Most of them are important as probiotic microorganisms. They are strictly fermentative dependent on carbohydrates for their energy supply and produce lactic acid from the carbohydrate catabolisms which is the major end-product of sugar fermentation. These bacteria are gram-positive, rod-shaped, non-spore-forming, catalase-negative organisms that are devoid of cytochromes and are of non-aerobic habit but are aero-tolerant, fastidious, acid-tolerant.

LAB and their metabolites play a key role in enhancing microbiological quality and shelf life of fermented dairy products [3,4]. LAB has an essential role in most fermented food for their ability to produce various antimicrobial compounds promoting probiotic properties [5].

The probiotic is living microbial feed supplements added to the diet [6]. It is now popularly referred to as being a mono-or mixed culture of live microorganisms e.g. as dried cells or as a fermented product. Common probiotics in use include *Bifidobacterium* spp. and members of LAB such as *Lactobacillus* species **(Table 1)**. These bacteria are added to fermented milk because they help to improve the balance of the intestinal microflora of the host upon ingestion [7,8]. In addition these probiotics contribute to the development of the immune system, improvement of normal intestinal morphology and maintaining a chronic and immunological balanced inflammatory response [9]. The growth of these probiotics showed inhibitory activities toward the growth of pathogenic bacteria via the creation of inhibitory compounds such as bacteriocins or reuterin, hydrogen peroxide, reduced pH as a result of accumulation of organic acids and competitive adhesion to the epithelium [10]. Probiotics also produce enzymes that help in the digestion of food in addition to B-complex vitamins production and neutralization of pathogenic microorganisms responsible for infections and diarrhea [11,12].

Viability and metabolic activity of the bacteria are important considerations in probiotic inclusion in foods. This is because the bacteria need to survive in the food during shelf life and gastrointestinal digestion i.e. acidic conditions of the stomach and degradation by hydrolytic enzymes and bile salts in the small intestine [13]. To ensure health benefits can be delivered by food containing probiotics, products sold with any health claims must meet the standard of a minimum level for probiotic bacteria ranging from 10^6 to 10^7 cfu/ml at the expiry date [14]. Therefore, the purpose of the present study is to review the importance of probiotics in dairy food such as yogurt, ice cream and cheese and their viability in these products during storage.

2. Probiotics

The word probiotic, derived from the Greek language, means for life is defined

as 'living microbial feed supplements added to the diet and offer beneficial effects on the host by enhancing their intestinal microflora balance' [6]. It is now popularly referred to as being a mono- or mixed culture of live microorganisms (e.g. as dried cells or as a fermented product) which usefully effects the host by enhancing the properties of the native microflora [15]. Common probiotics in use include *Bifidobacterium* spp. members of LAB and selected species of yeasts. To complement probiotics, "prebiotics" defined as selective non-digestible carbohydrate food sources, are becoming increasingly used in promoting the proliferation of bifidobacteria and lactobacilli [16].

3. Therapeutic Value of Probiotic in Dairy Food

3.1. Control of intestinal infections

Probiotic bacteria such as lactobacilli and bifidobacteria have antimicrobial activity [17]. Both *L. acidophilus* and *B. bifidum* for instance inhibit numerous of the generally known food borne pathogens [18-20]. The consumption of milk cultured with *L. acidophilus* or *B. bifidum* or both for preventative control of intestinal infections [19] can be occurred via:

- Inhibitory/antimicrobial substances production such as hydrogen peroxide, bacteriocins, organic acids, antibiotics and deconjugated bile acids.
- Competitive antagonist's action for example, through competition for adhesion sites and nutrients.
- Immune system stimulation.

The organic acids produced by the probiotics caused reduction in the pH and change the oxidation reduction potential in the intestine which leading to antimicrobial action. In addition, the limited oxygen content in the intestine can help the organic acids to inhibit especially pathogenic gram-negative bacteria type's e.g. coliform bacteria [21-23].

3.2. Reducing lactose intolerance

The lack of *β-D*-galactosidase in the human intestine results in the inability to digest lactose adequately follows by different degrees of abdominal pain and discomfort [24]. LAB used as starter cultures in milk during fermentation and probiotic bacteria such as *L. acidophilus* and *B. bifidum* produce *β-D* –galactosidase that digest lactose which helps consumers having better tolerance for fermented-milk products [24]. This utilization is referred to intra-intestinal digestion by *β-D*-galactosidase. Increased digestion of lactose may not only occur by hydrolysis of the lactose before consumption, but also in the digestive tract after ingesting of milk containing *L. acidophilus* [24]. Thus the continued utilization of lactose inside the gastrointestinal tract is governed by the survival of the lactobacilli.

3.3. Reduction in serum cholesterol levels

The consumption of fermented milk could significantly reduce serum cholesterol [25]. This is good news for hypercholesterolemic persons since substantial decrease in plasma cholesterol level plays a role in reduction heart attacks risk [26]. Appreciable amounts of cholesterol metabolism occur in the intestines before passage to the liver. This could provide some explanation on the association between the presence of certain *L. acidophilus* strains and some *bifidobacteria* species with the ability to reduce cholesterol levels inside the intestine. Cholesterol co-precipitates with de-conjugated bile salts as the pH drops as a result of lactic acid production by LAB [27]. The role of *bifidobacteria* cultures in reducing serum cholesterol is poorly known. Feeding of *bifidobacteria* to rats reduced serum cholesterol which may involve HMG-CoA reductase [28]. Sudha et al. [29] suggested a factor is formed in the milk during fermentation that inhibits cholesterol synthesis in the body. Alternatively, *L. acidophilus* may de-conjugate bile acids into free acids which are excreted faster from the intestinal tract than are conjugated bile acids. Subsequently, the production of fresh bile acids from cholesterol can decrease the total cholesterol level in the body [27]. A third hypothesis is that at lower pH values the production of lactic acid by LAB resulted in co-precipitation of cholesterol with de-conjugated bile salts cause reduction of cholesterol [29].

3.4. Anti-carcinogenic activity

probiotics are known to have antitumour action related to the inhibition of carcinogens and/or inhibition of bacteria that convert pro-carcinogens to carcinogens [19,30], improvement of the host's immune system [22,31] and/or reduction of the intestinal pH to decrease microbial activity. Studies in rats showed that probiotic bacteria in yogurt and fermented milk inhibited tumor formation and proliferation [19,30].

3.5. Prevention of colon cancer

Probiotics have shown capability to reduce risk of colon cancer owing to their ability to bind with heterocylic amines; carcinogenic substances that formed in cooked meat [30]. Most human studies have reported that probiotic may apply anti-carcinogenic effects by reducing the activity of ß-glucuronidase, an enzyme which produces carcinogens in the digestive system [32]. Although human intervention studies demonstrate the reduced presence of biomarkers associated with colon cancer risk. The evidence that probiotics decrease colon cancer occurrence in humans is lacking [33]. Thus the subject of probiotic uptake and cancer prevention is still open to further investigation.

3.6. Anti-diarrhea effects

Diarrhea can have many causes and its effects on flushing out the bacteria living in the intestine leaves the body vulnerable to opportunistic harmful bacteria. It is important to

replenish the body with probiotics during and after the incidence of diarrhea. The advantages of probiotics in the inhibition and treatment of a range of diarrhea illnesses, such as acute diarrhea caused by rotavirus infections, antibiotic-associated diarrhea, and travelers' diarrhea have been extensively studied [34]. LAB may possibly reduce diarrhea in some ways including competition with pathogens for nutrients and space in the intestines [34]. For instance *L. casei* and *B. bifidum* effectively prevent or treat infantile diarrhea [34] by several ways:

1) Compete with pathogens for nutrients and space in the intestines.

2) Some metabolism by-products such as acidophilin and bulgarican produced by *L. casei, L. acidophilus* and *L. bulgaricus* have a direct effect against inhibition of pathogens growth.

3) Enhance immune system which has effect against diarrhea, particularly through alleviation of intestinal inflammatory responses and intestinal immunoglobulin A (IgA) responses which cause create gut-stabilizing effect [31,34].

3.7. Improving immune function and preventing infections

Lactic acid bacteria are assumed to have some valuable effects to enhance immune function. These include the improvement of immune function by increasing the number of IgA producing plasma cell, increasing or educating phagocytosis other than increasing the proportion of T lymphocytes and natural killer cell [34]. They may protect against pathogen and to prevent or treat infections such as postoperative infections [35], respiratory infections [36], and the growth of *Helicobacter pylori,* a bacterial pathogen responsible for type B gastritis and peptic ulcers.

3.8. Anti-inflammatory effects

Probiotics have been shown to modulate inflammatory and hypersensitivity reactions. They can affect the intestinal flora and may have beneficial effects in inflammatory bowel disease (IBD), which includes ulcerative colitis, Crohn's disease and pouchitis [34]. Clinical studies suggest that they can prevent reoccurrences of IBD in adults [34], enhance remediation of milk allergies and decrease the risk of atopic eczema in children [37].

4. Application of Probiotics in Dairy Foods

Growing consumer knowledge of roles of diet in health has aroused amongst others the demand for foods containing probiotic. A number of dairy food products including frozen fermented dairy desserts [38], yogurt [39], cheeses [40], freeze-dried yogurt [41], ice cream [42] and spray dried milk powder [40] have been utilized as delivery vehicles for probiotic to consumer. Hence the selection and balancing of LAB is important to ensure food and dairy products maintain their desirable flavor, texture and nutritional value characteristics, because

these parameters may be affected by the initial composition of the milk flora and starter culture [43].

To elicit health effects, probiotic organisms must be viable (~ 10^9 cfu/ day) at the time of consumption [44]. Therefore, it is important to minimize the decline in the numbers of viable bacteria during storage period. Dairy foods present ideal delivery system of food for probiotics to the human gut because it offers suitable environment and nutrients to promote growth or support viability of these cultures. The fermented dairy products are the most popular food delivery systems for probiotic. However the low pH, the presence of H_2O_2 and inhibitory substances produced by the bacteria and the aerobic conditions of production and packaging may result in the decreases in the survival of probiotics in the final product. In fact the required level of viable cells of probiotic bacteria in many commercial dairy products cannot be guaranteed and therefore, failed the prerequisite for successfully delivery of probiotics [45].

5. Yogurt

The most common functional dairy products are those containing probiotic bacteria, quite frequently enriched with prebiotics, such as yogurt [46]. Yogurt is fermented milk obtained by lactic acid bacteria fermentation of milk and is a popular product throughout the world. It is recognized as a healthy food due to the beneficial action of its protein and its rich contents of potassium, calcium, protein and B vitamins.

Yogurt is formed during the slow fermentation of milk lactose by the thermophilic lactic acid bacteria *S. thermophilus* and *L. delbrueckii ssp. Bulgaricus*. However, these bacteria are not indigenous to humans and cannot colonize the intestine to promote human health. Thus probiotics, mainly *Lactobacillus acidophilus* and *Bifidobacterium* spp. are added to improve the fermentation process for production probiotic yogurt [47] and offer many advantages for the consumer. *S. thermophilus* and *L. delbrueckii ssp. Bulgaricus* are required to convert milk to yogurt whereas *L. acidophilus* and *Bifidobacterium* are added to increase the functional and health-promoting properties. Some researchers proposed that yogurt containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* could be regarded as members of the probiotic because both bacteria provide health benefits to the host [48]. These bacteria are able to release β- galactosidase enzymes that improve the digestion of nutrients in the intestine and modulate immune responses for human health [49].

The food biotechnology industry has in recent years developed a huge number of commercial products containing a single probiotic strain or bacterial associations of various complexities [18,50]. The development of yogurt with new flavors and products with health benefits has the potential to increase sales and to consumers satisfaction. Yogurts in the marketplace are available to satisfied different consumer groups. For example, fat free dairy products for consumers with cardiovascular problems and lactose free dairy products for

lactose intolerant people. In addition, folic acid enriched yogurt taken during initial stages of pregnancy help to prevent neural tube defects such as anencephaly, spina bifida, heart defects, facial clefts, limb deficiencies and urinary tract abnormalities [51].

6. Viability of Probiotic in Yogurt

Commercially produced food biotechnology products may contain either a single probiotic strain or bacterial mixtures of various complexities. Thus, the addition of probiotic increases yogurt's nutritional and therapeutic properties [52]. It is important that probiotic yogurt must contain living probiotic strains in adequate concentration at the time of consumption [14]. However, the key problems associated with incorporating probiotic bacteria into milk during fermentation are slow growth in milk and low survival rate during storage [53]. One of the strategies applied to improve the growth of probiotic bacteria is the addition of prebiotic substances with proper selection of starter cultures [53,54]. In order to provide functional properties and additional nutrients for bacteria growth in probiotic yogurts many other supplements with active components have been studied such as plant extracts, phenolic compounds and antioxidative substances [55-59]. Recently, cocoa powder and stabilizers are used as natural food additives to increase the survival of probiotic bacteria during passage through gastric tract [60]. In addition, lipid fraction of cocoa butter found to protect *B. longum* from environment stress [61]. Chocolate can also enhance the survival of *L. helveticus* and *B. longum* (91% and 80% respectively) compared to milk (20% and 30%) in low pH environment [62].

Several studies have demonstrated the effect of phenolic compounds on the growth and metabolism of probiotic in yogurt [60-62]. The bacterial species and strain in addition to chemical structure and concentration of the polyphenols play a significant role in sensitivity of probiotic to the phenolic compounds [63]. *L. plantarum* and *L. casei* Shirota strain found to be able to metabolize phenolic compounds [64,65]. Kailasapathy et al., [66] reported that the amount 5 or 10 g/100 g of added fruit mixes (mango, mixed berry, passion fruit and strawberry) in yogurt did not affect *B. animalis* ssp. *lactis* LAFTIs B94 growth except on *L. acidophilus* LAFTI L10 yogurt with 10 g/100 g passion fruit or mixed berry. However, the reduction in *L. acidophilus* counts was higher than the plain yogurt ($p<0.01$) which could be related to the chemical composition of these fruits. On the other hand, [67] found that the addition of passion fruit peel powder (0.7 g/100g) had no significant effect on viability of *L. acidophilus* LAFTI L10 in yogurt during 28 days of storage. The differences between the amounts of added passion fruit in previous studies could explain the discrepancy in the probiotic viability results obtained. Previous study observed that immobilized *L. casei* cells on fruit pieces (apple or quince) could be promising application in dairy food processing [68]. Immobilized *L. casei* cells on fruit pieces found to supports further the chances of *L. casei* survival for a long period of storage up to 129 days and can be adapted to the acidic condition which usually acts as inhibitor towards bacteria growth [68].

Chromatographic studies were used to evaluate the effect of *S. thermosphilus* and *L. bulgaricus* in yogurt on six phenolic compounds Catechin gallate (CG), epigallocatechin (EGC), catechin (C), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG) and epicatechin gallate (ECG) in green and black teas [69]. The chromatographic profiles of green and black tea phenolic compounds after the treatment with *S. thermosphilus* and *L. bulgaricus* yogurt bacteria showed no significant alteration ($p<0.05$) of these phenolic compounds compared to before treatment. This indicated that yogurt bacteria did not affect significantly ($p< 0.05$) the composition of green and black teas phenolic compounds [69]. This was in agreement with Najgebauer-Lejko, [70] who found the concentrations of green tea infusion (5%, 10% or 15%) did not influence the viability of *S. thermophilus* and *B. animalis* ssp. *lactis* BB-12 in yogurt during 21 days of storage. However, the presence of green tea maintained the viability of bifidobacteria in yogurt at the average above 7 log cfu/g for extra 2 weeks compared to plain yogurt. On the other hand, Michael et al. [71] reported that the count of *L. bulgaricus* decreased below the recommended concentration of 6 log cfu/ml in yogurt after 2 weeks of storage. However, the presence of plant extract (0.5% and 1%; Cegemett® Fresh) increased the viability of this bacteria to more than 2 folds (> 6 log cfu/ml) until 4 weeks of storage which could related to prebiotics or sodium acetate existing in plant extract. The addition of plant extract (0.5%) did not adversely affect the viability of *S. thermophilus* during 50 days of storage [71]. Another study found that the addition of plant extract (garlic or cinnamon) in bio-yogurt did not affect the viability of *Lactobacillus* spp and *S. thermophilus* during 21 days of storage [72]. However, *B. bifidum* increased significantly ($p<0.05$) in the presence of these plant extracts as compared to the absence over 21 days of storage [73]. This meant that bacteria may behave differently from each other in the presence of phenolic compounds [63].

do Espírito Santo et al. [74] reported an increased ($p<0.05$) in the counts of *L. delbrueckii* subsp. *bulgaricus* (from 5.0 to 9.2 Log cfu/ml) in skim milk yogurt co-fermented by *L. acidophilus* L10 and the addition of fruit fibers such as apple or banana (1%) had no inhibitory effect on the viability of *L. delbrueckii* subsp. *bulgaricus*. Yet, in some cases as in yogurt co-fermented by the *B. animalis* subsp. *lactis* HN019, the presence of fibers from apple or banana have stimulated *L. delbrueckii* subsp. *bulgaricus* growth compared to the absence. This could be resulted of symbiotic relationship between apple or banana fibers and *B. lactis* HN019 that lead to enhance the viability of *L. delbrueckii* subsp. *bulgaricus*. In general, the presence of either apple or banana fibers showed an increase in the numbers of probiotics (*L. acidophilus* L10 *and B. animalis* subsp. *lactis* BL04, HN019 and B94) by no less than 1 Log cfu/ml compared to the absence. This could be related to their high contents of pectins and fructooligosaccharides that have prebiotic effect to enhance the bacteria growth [75,76].

Recently, Buriti et al., [77] studied the fermented whey-based goat milk and goat cheese beverages prepared using probiotic culture (*B. animalis* subsp. *lactis* BB-12, *L. rhamnosus* Lr-32 and *S. thermophilus* TA-40) with added guava or soursop pulps and with or without addition

of partially hydrolysed galactomannan (PHGM) from Caesalpinia pulcherrima seeds. It was observed that both *B. animalis* and *L. rhamnosus* maintained good viability in the presence of either guava or soursop pulps. Although, including dietary fiber ingredients into food during processing has been widely used to increase the viability of probiotic during storage of products [74] however, *B. animalis* and *L. rhamnosus* showed inability to metabolize the PHGM, since no significant difference ($p>0.05$) between with and without PHGM. Similarly, Buriti et al. [78] found that PHGM was not fermented under *in vitro* conditions by the same probiotic strains. Oleuropein is a bioactive natural product from olives with variety of health beneficial properties. Zoidou et al. [79] detected that inclusion of oleuropein into yogurt during fermentation did not either metabolize by LAB or inhibit their growth and its remained stable in the final products.

7. Ice cream

Ice cream is a frozen dairy product produced from a combination of served ingredients other than milk. The composition of ice cream varies depending upon the ingredients used in its preparation. In many countries, the percentage composition of a good ice cream is 11–12% milk fat, 10–12% milk non-fat solids (MSNF), 12% sugar, 5% corn syrup solids, 0.3% stabilisers-emulsifiers [80].

Ice cream is a delicious and nutritious frozen dairy dessert with high calorie food value [81] and 82 g provides approximately 200 calories, 3.99g protein, 0.31g calcium, 0.10g phosphorus, 0.14mg iron, 548 IU vitamin A, 0.038mg thiamine and 0.23mg riboflavin [82]. Ice cream has nutritional properties but owns no therapeutic value [83]. Recently, the increasing demand from consumers for healthier and functional food has led to produce ice cream containing special ingredients with recognized nutritional and physiological properties such as dietary fibers [84], probiotics [85,86], lactic acid bacteria [87], prebiotics [58,88] alternative sweeteners [89], low glycemic index sweeteners [90] and natural antioxidants [55].

The main ingredient of ice cream is cow milk and this unfortunately may make dairy ice cream off limits to many consumers who suffer from lactose intolerance. Thus, replacing cow's milk with vegetables milk in general would help address two nutritional issues related to cow's milk: lactose intolerance and cholesterol content. Several researchers have used vegetable milk such as soy and coconut milk to produce probiotic ice cream with nutritional and theraputical properties [91-94]. Other studies found that the addition of plant ingredients such as watermelon seeds, ginger extract and black sesame could increase the overall acceptability of ice cream as well as enrich it with antioxidant activity [93,95,96].

Consumption ice cream containing probiotic strains could reduce bacteria levels in the mouth responsible for tooth decay [97]. Singh et al. [98] reported that consumption of probiotic ice-cream containing *B. lactis* BB12 and *L. acidophilus* La5 was associated with significant

reduction in the levels of *Streptococcus mutans* in salivary of school children with no significant effect on lactobacilli levels. The pH and coliform counts of human faces of volunteers fed with synbiotic ice cream were significantly reduced ($p<0.01$) after two weeks of ingestion [99]. The pH reduction may be attributed to the production of short chain fatty acids by the colonic microbiota and probiotic bacteria [100,101]. In addition, consumption of ice cream containing probiotics such as *L. acidophilus* increased the faecal lactobacillus counts during 15 days of ingestion [99]. Ice cream prepared with probiotic culture such as *L. acidophilus* LA-5, *B. lactis* BB-12 and *Propionibacterium jensenii* 702 had a significant influence on the gastrointestinal tolerance (*in vitro*) after exposure to both highly acidic conditions (pH 2.0) and 0.3% bile [61]. This indicated that probiotic ice cream could improve the balance of the intestinal microflora of the host upon consumption [7,8] followed by immune system development [9].

8. Viability of Probiotic in Ice Cream

The growth and viability of probiotic bacteria are influenced by the temperature of the cultures medium. The effectiveness of probiotics ice cream consumption on consumer's health is associated with bacteria viability. Therefore, it is importance not only to reduce cell death during the freezing process but also to maintaining stability of bacteria during storage. Since ice cream is a whipped product, incorporation of large amounts of air into the mix resulting in oxygen toxicity, one of the most important factors of bacteria cell death. The viable counts of *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in probiotic ice cream found to be significantly lower after freezing compared to prior to freezing [102]. The decline in bacterial counts in ice cream after freezing may occur due to the freeze injury of cells leading to the death of these cells. However, the mechanical stresses of the mixing and freezing process may have caused a further reduction in bacterial cells counts.

During ice cream freezing process, the probiotics can be lethally injured by damaging cell walls or rupturing their membranes because of the ice formed in the environment or inside the cell [103]. The rate of dehydratation of bacterial cells depends on the absorbency of the cell membrane and the surface area in relation to its volume. Thus, increase freezing rates may cause small ice crystals size with less damaging effects towards bacterial cells [103]. Rapid freezing of the ice cream mix obtained after inoculating with the probiotics contributes to maintain their stability in the recommended therapeutic doses. Some studies reported that the viability of the probiotic bacteria in ice cream after freezing is an important parameter to ensure compliance to the food industry standards and to meet consumer expectation [42,104].

Da Silva et al. [105] study the viability of *B. animalis* in goat's milk ice cream during storage. It was found that *B. animalis* decreased about 1 log and registered a rate of survival (84.3%) approximately 7 cfu/g during the first 24 hours of frozen storage. The viable cells counts of these bacteria were decreased about 1.26 log cycles with 84.7% survival rate during 120 days of frozen storage. This means that *B. animalis* had ability to maintain satisfactory

viability in goat's milk ice cream during frozen storage (≥ 6.5 log cfu/g). The viability of novel probiotic *Propionibacterium jensenii* 702 included with *L. acidophilus* (La- 5), *B. animalis* subsp. *lactis* BB-12 in ice cream made from goat's milk was examined by Ranadheeraa et al. [102]. The viable counts of probiotic were found to be significantly lower by 56.14% for *L. acidophilus* and 66.46% for *B. lactis* after freezing whereas *P. jensenii* showed higher survival rate with 88.72%. This suggested that *P. jensenii* 702 may have mechanisms allowing survival during freezing which are not possessed by *L. acidophilus* and *B. lactis* [102]. These mechanisms may include ability of *P. jensenii* to dehydrate rapidly and thus decrease the formation of intracellular ice crystals that can damage cytoplasmic membranes and lead to cell death [106]. Anyhow, the final product of probiotic ice cream made from goat's milk found to be able to maintain satisfactory viability (10^7 to 10^8 cfu/g) over 52 weeks of the storage [102]. A mixture of human-derived probiotic strains of *L. acidophilus*, *L. agilis* and *L. rhamnosus* was used in ice cream manufacture [107]. The study stated that the viable cells counts of these bacteria remained constant in ice cream during 6 months of storage without any major loss of bacterial cells between in presence and in absence of sweeteners (sucrose or aspartame).

Previous study reported that *L. johnsonii* La1 and *L. rhamnosus* GG showed high survival rate in retail-manufactured ice cream with no decrease in the population inoculated initially (7 log cfu/g and 8 log cfu/g respectively) during storage up to 8 months for *L. johnsonii* and 1 year for *L. rhamnosus* [86,108]. Moreover, the sugar level (15% and 22% w/v), fat content (5% and 10% w/v) and even different storage temperatures (-16 °C and -28 °C) did not affect significantly on the viability of both probiotic bacteria [86,108]. The effects of inulin and different sugar levels on survival of probiotic bacteria in ice-cream were investigated by Akın et al. [85]. Ice cream produced by adding 10% w/w of fermented milk with commercial freeze-dried mixed probiotic culture consisting of *S. thermophilus*, *L. bulgaricus*, *L. acidophilus* LA-14 and *B. lactis* BL-01 to ice cream mix with different concentrations of sugar (15%, 18% and 21%; w/w) and inulin (1% and 2%). The results showed that ice cream content 18% sugar had the highest viable cells counts of bacteria. Yogurt bacteria in ice cream showed viability above 10^7 and 10^6 cfu/g for *S. thermophilus* and *L. bulgaricus* respectively during 90 days of storage. Moreover, the addition of inulin did not affect significantly on numbers of *S. thermophilus* or *L. bulgaricus*. However, the viability of *L. acidophilus* and *B. lactis* in ice cream found to increase from 10^5 cfu/g to 10^6 cfu/g after addition of 2% of inulin [85]. Likewise, Akalin and Erişir, [88] indicated that the survival of *L. acidophilus* La-5 and *B. animalis* BB-12 can be improved significantly ($p<0.05$) by addition of oligofructose in low-fat ice cream stored at -18°C for 90 days. *B. animalis* BB-12 maintained a minimum level of 10^6 cfu/g in only ice cream with oligofructose during storage period. Recently, Leandro et al. [87] reported that using of inulin to replace fat partially or totally in ice cream does not affect the viability of *L. delbrueckii* UFV H2b20 after processing and during storage. However, *L. delbrueckii* UFV H2b20 found to be differing from *L. acidophilus* La-5 and *B. animalis* BB-

12 which exhibited stability after freezing process and after 40 days of storage at -16 °C [88]. This suggested being associated with low overrun presented by the ice cream formulations. Similar observation has been demonstrated for *L. rhamnosus* [109]. Another study stated that incorporating fructo-oligosaccharides into probiotic ice cream significantly increased ($p<0.01$) survival of *L. acidophilus* and *Saccharomyces boulardii* during two weeks of freezing storage [83]. Non fermented probiotic ice cream made from vegetable milk (soy or coconut milk) improved the growth and viability of *B. lactis* and *L. acidophilus* during 30 days of storage at -20°C [94]. Furthermore, the study indicated that the survival of both probiotics was higher in soy milk ice cream than coconut milk ice cream which probably due to soy milk proteins that provide physical protection against freezing damage through encapsulating probiotics with stable network looks like a gel structure [110].

Several studies on survival of probiotic in ice cream during freeze storage have focused on the protective effects of encapsulation. Survival of free and microencapsulated *L. casei* (Lc-01) and *B. animalis* (BB-12) in symbiotic ice cream containing resistant starch as a prebiotic substance was studied [111]. The viable cells counts of free *L. casei* (Lc-01) and *B. animalis* (BB-12) in ice cream showed a decreased by 3.4 and 2.9 log respectively after 6 months of storage. However, encapsulated *L. casei* and *B. animalis* showed reduction by only 1.4 and 0.7 log throughout the storage period. Ice cream prepared by using encapsulated *L. casei* and *B. animalis* maintained viability of probiotic between 10^8 and 10^9 cfu/g overall shelf life. This indicated that encapsulation can significantly maintain high viability of probiotic bacteria in ice cream over storage. The observation is in line with Shah and Ravula, [112] who noted an improvement in counts of microencapsulated *L. acidophilus* MJLA1 and *Bifidobacterium* spp. BDBB2 compared to free cells in frozen fermented dairy dessert during 12 weeks of storage. Sahitya et al. [113] revealed that encapsulated *L. helveticus* 194 and *B. bifidum* 231 showed significantly ($p<0.05$) higher log counts (7.96 and 8.06 log10 cfu/g respectively) than non-encapsulated bacteria (6.06 and 6.33 log10 cfu/g respectively) at the end of 90 days of storage. In addition, co-encapsulated *L. helveticus* 194 and *B. bifidum* 231 along with prebiotics (3% Fructooligosaccharides) increased probiotic viability during storage at -20°C (Sahitya et al., 2013). Lately, Karthikeyan et al. [114]. evaluated the survivability of *L. acidophilus* (LA-5) and *L. casei* (NCDC-298) in ice cream using microencapsulation technique. Unencapsulated free *L. acidophilus* (LA-5) and *L. casei* (NCDC-298) showed about 3 log reduction over 180 days of storage at - 23°C with final cells counts of 6 log cfu/g and 7 log cfu/g respectively. However, microencapsulated improved the viability of *L. acidophilus* (LA-5) with only one log reduction during the entire shelf life and final bacteria counts of 8 log cfu/g whereas microencapsulated *L. casei* (NCDC-298) remained constant over storage with about 9 log cfu/g. Similar behavior has been displayed by *B. Lactis* (BB-12) with 30% increase in their viability in ice cream after microencapsulated with calcium alginate and whey protein for 6 months of storage [115].

9. Cheese

Cheese is a kind of fermented milk-based food product. It can also be regarded as a consolidated curd of milk solids in which milk fat is entrapped by coagulated casein [116]. The Food and Agriculture Organization of the United Nations (FAO) defines cheese as "the fresh or matured product obtained by the drainage (of liquid) after the coagulation of milk, cream, skimmed or partly skimmed milk, buttermilk or a combination thereof" [117]. Cheese contains, in a concentrated form, many of cow milk's nutrients and provided many essential nutrients such as protein and calcium; it also contains phosphorus, fat zinc, vitamin A, riboflavin and vitamin B12. Several bifidobacteria strains have been successfully incorporated into cheeses [118,119]. The addition of probiotic bacteria does not generally affect the gross chemical composition of cheese (i.e. salt, protein, fat and moisture) and pH [12;122]. Similarly, the primary proteolysis in cheese not influenced by added of probiotic cultures which in many cheeses occurred as a result of activity of the coagulant agent (except for high cook cheeses) and to a minor range by plasmin and subsequently residual coagulant and enzymes from the starter microflora [123]. However, addition of probiotic in cheese reported to effect on the changes of secondary proteolysis and the increases in free amino acid content as well as free fatty acid profile of cheese which directly contribute to cheese characteristics [120,124,125]. Most cheeses containing probiotic lactobacilli and bifidobacteria which have high lactic acid and acetic acid content due to lactose fermentation [120-122,125]. Bifidobacteria produce acetic and lactic acid in a ratio of 2:3 whereas lactobacilli produce lesser acetic compared to bifidobacteria [126]. Probiotic cheese provided an opportunity for lactose intolerant individuals due to a complete lactose hydrolysis that observed in several cheeses such as Crescenza, Canestrato Pugliese and Cheddar-like cheeses [127-129].

Probiotic cheese is believed to reduce the risk of heart disease and certain cancers [130,131]. Conjugated linoleic acid (CLA) is found in cheese, and recent scientific research supports potential roles for CLA isomers in reducing the risk of certain cancers and heart disease, enhancing immune function and regulating body weight/ body fat distribution [132]. Cheese with *L. rhamnosus* HN001 and *L. acidophilus* NCFM found to be beneficial in improving the immune response of healthy elderly subjects [133]. Probiotic fresh cheese allows *B. bifidum, L. acidophilus* and *L. paracasei* to exert significant immunomodulating effects in the gut [134]. The pure cultures of *B. bifidum* and *L. paracasei* were identified in small intestine of mice fed with probiotic fresh cheese whereas *L. acidophilus* was mainly identified in the large intestine [134].

Probiotic cheese reduces the risk of dental caries (decay) which usually results from the breakdown of tooth enamel by acids produced during the fermentation of sugars and starches by the plaque bacteria [135]. The short-term consumption of probiotic cheese containing *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* LC 705 reduced caries-associated

salivary microbial counts such as *Streptococcus mutans* by 20% and salivary yeast by 27% in young adults [136]. The protective effect of cheese against dental caries may also be explained by an antibacterial effect of components produced during metabolic activities of probiotic bacteria in cheese (e.g., fatty acids, organic acid, peptides etc.).

10. Viability of Probiotic in Cheese

Probiotic bacteria can be included into cheese during manufacture in two ways either as a starter (depended on the ability to produce adequate lactic acid in milk) or as adjunct to the starter culture which is more favourable option to incorporate probiotic with the starter bacteria during cheese making. A few approaches have been applied to improve the survival of probiotic in cheeses one of them is the use of different combination of starter and probiotic [131]. The development of probiotic cheeses can be very strain dependent as many of the probiotic strains showed poor performance in the cheese environment. Strain selection plays a key role in successful development of probiotic cheese. In addition, processing conditions, cooking procedure, the aerobic environment, temperatures of ripening and storage are affecting viability of probiotic bacteria a well as the concentration of these bacteria in the final product provides a therapeutic dose to consumers [131]. *Lactobacillus acidophilus* (La-5) is a probiotic bacterium that important to be survived in cheeses during production and storage of probiotic cheeses. In order to exert the beneficial effects of probiotic foods at the minimum probiotic therapeutic daily dose intake 100 g of a food product containing 6 or up 7 log cfu/g [137]. The viability of probiotic culture of *L. acidophilus* fund to be above 6.00 log cfu/g during storage in minas fresh cheese, festivo cheese, white brined cheese, argentinian fresco cheese, semi-hard argentinean fresco cheese, petit suisse cheese and Tallaga cheese [121,138-143].

Tharmaraj and Shah, [144] found the best combination of probiotic bacteria can be used in cheese-based dips when combined *L. acidophilus*, *B. animalis* and *L. paracasei* subsp. *paracaseiin* together (inoculation at 9 log/g). The *L. acidophilus* and *B. animalis* showed a high level of population required for health benefit through 10 weeks of storage period. However, the presence of *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* in cheese-based dips with the above mentioned combination had no significant effect of the bacteria in the combination and can be inoculated at level of 7 log to keep the viability above 6 logs during 10 week of storage. The viability of *L. casei*, *L. rhamnosus* GG or probiotic mix YO-MIX™ 205, including *L. bulgaricus*, *L. acidophilus*, *Bifidobacterium* spp. and *S. thermophilus* added to cottage cheese during storage was observed by Abadía-García et al. [145]. All the added probiotic bacteria persisted viable in cottage cheese throughout 28 days of storage. Cottage cheese including *L. casei* or YO-MIX ™205 showed higher viable cell counts of 8 $\log_{10}$ cfu/g over the last 2 weeks of storage at 8 °C. Conversely, *L. rhamnosus* GG remained constant at levels of 6 $\log_{10}$ cfu/g over the whole storage period [145]. Six batches of Cheddar cheeses inoculated with different probiotic bacteria used as an adjunct including *B. longum* 1941, *B. animalis* subsp. *lactis*

B94, *L. casei* 279, *L. casei* L26, *L. acidophilus* 4962 or *L. cidophilus* L10 [118]. The viability of probiotic in all cheese batches were remained at the level of 8-9 $\log_{10}$ cfu/g at the end of the production process. The amounts of starter lactococci in cheese batches inoculated with *B. animalis* B94, *L. casei* L26 or *L. acidophilus* were significantly reduced ($p < 0.05$) by the ripening temperature at 8 °C compared to those at 4 °C after 24 weeks. However, the probiotic cells in cheeses with different strains of probiotic were not significantly ($p > 0.05$) different during the ripening period (24 weeks) and ripening temperature (4 °C and 8 ° C). [146] found that the combination of *L. paracasei* A13 with probiotic (*B. bifidum* A1, *L. acidophilus* A3) and starter (*Lactococcus lactis* A6 and *S. thermophilus* A4) in Argentinian fresh cheese improved viability of *L. paracasei* A13 by approximately half log order during the production process at 43 °C and another half log order during the first two week of storage at 5 °C. In addition, increase storage temperature to 12°C (temperatures usually found in retail display cabinets in supermarkets) had positive effect on the growth of *L. paracasei* A13 by almost 2 log orders from day 30 until day 60 [146].

The impact of two different techniques (pre-incubation step or directly to the vat) for the inoculation of probiotics mixture (*L. acidophilus*, *L. paracasei* and *B. lactis*) on the viability of these probiotics during semi-hard cheese ripening for 60 days was investigated by [119]. They found no significant differences in the counts of each probiotic strain at the end of the ripening regardless their addition as lyophilised or after pre-incubation. In addition, *L. paracasei* strain registered the highest cell counts ~10^9 cfu/g followed by *L. acidophilus* and *B. lactis* with cell concentration of 10^8 cfu/g/ and 10^7 cfu/g respectively [119]. This study was in line with previous study conducted by Bergamini et al. [121] who found no significant differences between using the two techniques in inoculation of probiotic bacteria in semi-hard Argentinean cheese (freeze-dried powder or after pre-incubation). Lyophilized or freeze-dried powder technique is a more effective process because it is easier, cheeses are not over acidified and the probiotic population at the end of ripening is relatively similar to that in pre-incubation in substrate composed of milk [119,122]. Recent study found a new invention process consisting in an edible sodium alginate coating as carrier of probiotic (*L. rhamnosus*) and prebiotic (fructooligosaccharides) which was effective in manufacture functional Fiordilatte cheese [147]. Research results indicated that the a consumption of 100 g of coated Fiordilatte cheese provide a daily dose of probiotics equal to 10^9 cfu/100g which recommended for health purpose. However, the functional acceptability limits for the coated Fiordilatte cheese with probiotics and prebiotics were 8 days at 4 °C, 6 days at 9 °C and 5 days at 14 °C [147].

Besides the acceptable probiotic viable counts, the behavior of probiotics in presence of prebiotics in cheese have been widely studied [147-149]. The addition of both inulin and oligofructose combined in petit-suisse cheese showed satisfactory probiotic viable counts of *L. acidophilus* and *B. animalis* subsp. *lactis* during 30 days of storage [148]. This performance has not observed in other studies where inulin had no significant effect on growth and survival

of *L. paracasei* in a synbiotic fresh cream cheese [149]. Likewise, the presence of inulin or a mixture of inulin and fructooligosaccharides (50:50) in the synbiotic cheeses was not affected the viability of *L. casei* 01 and *B. lactis* B94 during 60 days of ripening period [150]. Therefore, the improvement of probiotic cheeses in presence of prebiotics such as inulin, oligofructose and fructooligosaccharides could be very strain and cheese type dependent. In addition, the populations of *L. acidophilus* in Caprine Coalho cheese naturally enriched with conjugated linoleic acid (CLA) were no statically significant ($p>0.01$) compared to Caprine Coalho cheese prepared without CLA-enhanced milk during 60 days of storage [149]. However, the stability of CLA content (isomer C18:2 cis-9, trans-11) in Caprine Coalho cheese was observed during the ripening period. This could provide healthier fatty acid profile, offering an increased CLA, oleic and linoleic acid levels along with a lower content of total saturated fat [149].

11. Conclusion and Recommendations

Dairy food is a promising food matrix for probiotics. Generally, probiotic yogurt developed for the market considered to be competitive as compared with probiotic cheese or ice cream. In addition, a number of studies regarding to including plant materials to probiotic yogurt have been successfully established to increase the viability of probiotic during production and storage. However, such an approach has not developed sufficiently in probiotic cheese or ice cream which could have a significant impact on probiotic survival. The interaction between phenolic compounds from plants extracts and probiotic bacteria has not been fully understood yet. The bacterial species and strain in addition to chemical structure and concentration of the polyphenols play a significant role in sensitivity of probiotic to the phenolic compounds. Furthermore, strain selection and possible process modifications should be carefully assessed to promote probiotic cells in dairy food during manufacture and storage to ensure health benefits can be delivered to consumers on daily consumption. More additional studies might be needed to evaluate *in vivo* therapeutic properties of probiotic yogurt, ice cream and cheese.

Table 1: Examples of probiotic bacteria used in probiotics dairy products.

Lactobacillus species	Bifidobacterium species	Other
L. acidophilus	*B. bifidum*	*Streptococcus thermophilus*
L. casei	*B. longum*	*Propionibacterium jensenii*
L. helveticus	*B. lactis*	*Propionibacterium freudenreichii subsp. shermanii*
L. plantarum	*B. adolescentis*	*Lactococcus lactis ssp. lactis*
L. rhamnosus	*B. infantis*	*Enterococcus faecium*
L. agilis	*B. breve*	*Lactococcus lactis ssp. cremoris*
L. johnsonii	*B. animalis*	*Leuconostoc mesenteroides ssp. dextranicum*
L. paracasei		*Pediococcus acidilactici*
L. gasseri		

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