Environmental Chemistry for a Sustainable World

Divya Arora
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Pharmaceuticals from Microbes

Impact on Drug Discovery

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Preface

I play with microbes. There are, of course, many rules to this play but when you have acquired knowledge and experience it is very pleasant to break the rules and to be able to find something nobody has thought of (Alexander Fleming)

Artist view of gut microbes, copyright Isabelle Hartweg, INRA 2018

In Europe, an estimated 25,000 people a year die from antibiotic-resistant bacteria.[1] In the USA, at least 2 million illnesses and 23,000 deaths a year can be attributed to antibiotic resistance. The overuse of antibiotics has led to the evolution of new resistant microbial strains and, in turn, to the lack of pharmaceutical drugs for healing acute infections. The severity of drug resistance has created havoc in scientific and medical fraternities. Natural products have an immense potential to curb this threat because natural substances have already served human civilization since immemorial times as therapeutic agents. Natural drugs that are a hallmark of modern pharmaceuticals include quinine, theophylline, penicillin G, morphine, paclitaxel, digoxin, vincristine, doxorubicin and cyclosporine. Secondary metabolites

from microbes are drug reservoirs that still need to be explored. Indeed, the high chemical diversity of natural metabolites makes them suitable candidates for drug discovery. Most current antibiotics were discovered fortuitously. Now, integrative biology, a new discipline combining expertise from remote fields to study natural systems, provides a more rational way to discover new natural drugs.

This book provides advanced knowledge on drugs derived from various microorganisms. The book focuses on pharmaceuticals from marine microbes, antiprotozoal and anthelmintic drugs, bioactive molecules from *Nocardia* species or other microbes, siderophores against antimicrobial resistance, metabolites from endophytic fungi and gut microbiota, and probiotics.

The first chapter by Pandey discusses the pharmacological significance of marine microbes and provides the concise scientific knowledge regarding pharmaceutical products. The natural products obtained from marine microbes have been elaborated for their bioactive potential. In Chap. 2, Singh et al. review the progress made in the discovery and development of antiprotozoal and anthelmintic drug candidates of microbial origin. Dhakal et al. represent the information regarding the diversity of bioactive molecules derived from *Nocardia* species along with their biological significance. Simultaneously the biosynthetic mechanism and metabolic engineering to produce bioactive metabolites from *Nocardia* spp. are described and discussed in Chap. 3. In Chap. 4, Mohanta et al. review the various bioactive compounds isolated from microbes having therapeutic importance, followed by an overview of recent advancements and developments in drug delivery strategies.

Ribeiro and Simões discuss the main classes of siderophores and their mechanism of action to combat the resistance among pathogens. In Chap. 5, they also describe the recent efforts to develop drugs that can interrupt the assimilation of iron by bacteria, interfering at different levels with the bacterial iron metabolism, a process that is vital to cellular homeostasis. In Chap. 6, Sharma et al. describe the potential of endophytic fungi, which act as a store house of naturally occurring bioactive secondary metabolites and can be explored to obtain bioactive metabolites for generating novel bioactive leads. In Chap. 7, Bhushan et al. represent the roles of gut microbiota, probiotics, and their specialized molecules in human health and disease. The role of primary and secondary metabolites of the human microbiota and their impacts on hosts' physiology are emphasized in this chapter. In addition, they also describe the mode of actions of newly identified effector molecules, i.e., polysaccharides, outer membrane proteins, pili, muropeptides, and CpG-rich DNA, both for human microbiota and probiotics. Elshaghabee et al. focus on understanding the role of gut microbiota in both pathogenesis of nonalcoholic fatty liver disease and also in their management through dietary intervention with different probiotic strains, reducing the risks associated with nonalcoholic fatty liver disease in Chap. 8.

We extend our sincere gratitude to all the authors who have put considerable efforts into their contributions and for their timely responses and consistent cooperation during the manuscript writing and revision process. We also extend our thanks to the Springer Nature team from acceptance of proposal to the production of the book.

We hope this book will be useful to all researchers, students, professors, and scientists working in the domain of bioactive secondary metabolites. Finally, we acknowledge the almighty God and our family members, who encourage us to take such venture and motivate us in the completion of this book.

1. https://www.theguardian.com/society/2018/jan/23/number-of-new-antibiotics-has-fallen-sharply-since-2000

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Chapter 1
Pharmacological Potential of Marine Microbes

Abhishek Pandey

Contents

Abstract The extensive growth of world population is a global issue, which has increased consumption of the existing resources as well as drugs. Therefore, there is a strong need for the search of new resources to develop safe and effective pharmaceuticals to fulfill increasing demand of the world population in order to combat various health ailments. Marine environments have diverse resources for the discovery of new drugs. Since a long time, various biological active compounds have been discovered from marine sources to prevent a wide range of diseases and disorders. Marine microbes represent a huge reservoir for exploration of novel bioactive compounds to provide future drugs as arsenal against cancer, microbial and protozoal infection, severe inflammation, and other major diseases.

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Since the last two decades, marine microbiologists have identified different microbes including bacteria, fungi, actinomycetes, and microalgae-cyanobacteria (free-living and symbiotic) as a source of bioactive compounds. This chapter selectively highlights status and major critical research on various compounds of pharmaceutical importance derived from marine microorganisms. Antibacterial, antifungal, antiviral, anticancer, antimalarial activities were reported for 55 marine compounds. Similarly, 43 marine compounds were reported for anti-inflammatory, antituberculosis, anticholinesterase, antidiabetic activities. Additionally, 15 marine compounds bind to a variety of receptors and miscellaneous molecular targets.

1.1 Introduction

The ocean encompasses more than 70% area of the earth. It is one of the major habitats for the variety of marine organisms due to the diversified natural environment of different sea zones around the world. Marine environment is enriched with natural products as a prominent source of bioactive substances for the treatment of several human ailments. Marine organism undergoes vast physical and chemical conditions in the marine environment; thus, a high diversity is found in nature of molecules, which they produce. However, unlike the long historical medical uses of terrestrial plants, marine microorganisms have a limited history in the pharmacological application. Since the last two decades, researchers have discovered a variety of novel metabolites from marine microbes such as polyketides, alkaloids, peptides, proteins, lipids, shikimates, glycosides, and isoprenoids, which exhibit numerous pharmacological activities such as anticancer, antitumor, anti-inflammatory, antidiabetic, cytotoxic, and antibiotic properties Rateb and Ebel (2011). In contrast to marine macroorganism, marine microorganisms have attracted more attention as potential key compound producers, having the advantage of a renewable and sustainable source of bioactive compounds as they can be cultured at large scale and can even be envisaged as amazing microbial factories for the production of natural products (Blunt et al. 2015).

Since ancient time marine resources have been exploited as a source of medicine. Figure 1.1 illustrates the manifold of bioactive compounds produced in the marine environment. From a historical perspective dye, Tyrian purple was the first marine product extracted from marine mollusks by the Phoenicians about 1600 BC. For a long time, the marine natural product field had attention on metabolites derived from macroorganisms; famous examples are marine biopolymers like agar and carrageenan, vitamins A and D extracted from fish liver oil, or polyunsaturated fatty acids like eicosapentaenoic acid and docosahexaenoic acid. Literature survey reveals that about 30,000 compounds are known to obtain from marine resources, and as an outcome of extensive research since the last decade,

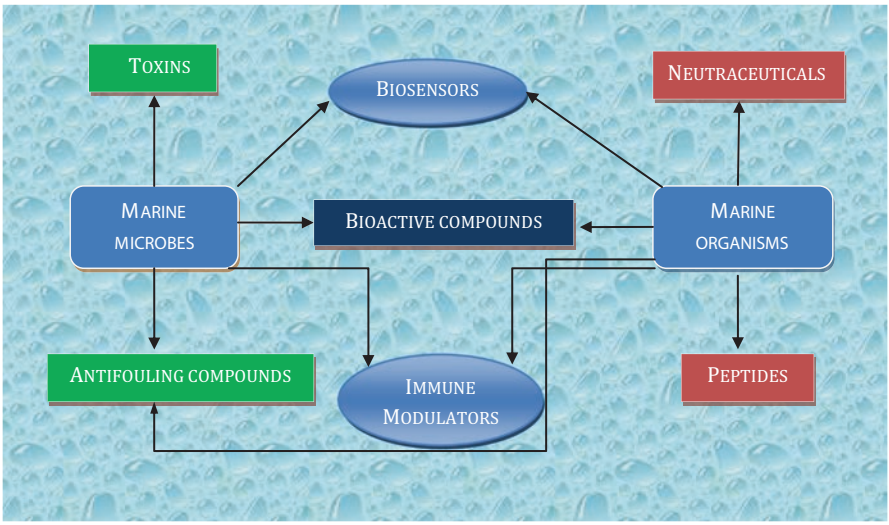

Fig. 1.1 Bioactive compounds produced in the marine habitat

more than 1000 compounds have been isolated mainly from marine invertebrates. Simultaneously, marine microorganisms have attracted more and more attention as a prominent source of bioactive compounds. In 1949, the first secondary metabolite isolated from a marine-derived fungal strain, famous cephalosporin C, is derived from the culture of a *Cephalosporium* sp. isolated from the Sardinian coast. Despite the discovery of such significant drug from marine microbes, the rate of discovery of bioactive compounds from marine microbial resources was extremely slow. Since the 1980s, researchers have focused on the pharmacological potential of bioactive compounds derived from marine microbes. Marine microbes represent an alternative reservoir for the production of bioactive secondary metabolites that could be used for the treatment of several human ailments. These compounds are characterized by structural features, affinity to binding from receptor and mechanism of action. In conclusion, the marine environment is the least explored area of the planet especially as far as the medicinal importance of microorganism is concerned.

Marine microbiology is one of the most important and fast-growing research areas of modern science. Recent development in molecular biology and remote sensing including deep-sea exploration unfolds the concepts of abundance and diversity of microbial life, interaction of marine microbes with other organisms, symbiosis, pathogenicity, and isolation of compounds of pharmaceutical significance. However, marine microbes cause disease or infection apart from this; they have extremely beneficial properties such as the development of new pharmaceutical products in the emerging field of marine pharmacology. This chapter sets the scene

for the discussion of the pharmacological significance of marine microbes to provide concise scientific knowledge about marine microbe-derived pharmaceutical products for the treatment of various human ailments.

1.2 Marine Microbes: The Latent Sources of Bioactive Compounds from Marine Macroorganisms

Most aquatic organisms act as host for various microorganisms within their membranes where they remain in the extra- and intracellular space. In some instances, these symbiotic microorganisms may constitute up to 40% of the biomass, for example, of sponges such as the Mediterranean *Aplysina aerophoba* (Vacelet 1975). On the other hand, many invertebrates are filter feeders and consume microorganisms from the inhaled seawater by phagocytosis. The relationships of marine invertebrates and marine microorganisms that may serve as food or that live either permanently or temporarily inside of marine macroorganisms are highly complex (Wilkinson 1992; Hentschel et al. 2000). Marine-derived fungi and bacteria are prominent source of biologically active secondary metabolites with antifungal, antibacterial, antiviral, cytotoxic, and immunosuppressive activity. Microorganisms not only serve as food for filter feeders or (in the case of cyanobacteria and chemoautotrophic bacteria) enrich the diet of their hosts by carbon and nitrogen fixation but may perhaps also be involved in the biosynthesis of natural products that are recovered, for example, from sponges. Sponges of the genus *Halichondria* such as *H. okadai* or *H. melanodocia* provide a well-known example of the importance of microalgae for the typical natural products recovered from these invertebrates. Both *Halichondria* species contain the protein phosphatase inhibitor okadaic acid. Numerous scientific evidence for a microbial origin of natural products isolated from marine macroorganisms exists for numerous invertebrates. For example, symplostatin 1, a close structural analog of dolastatin isolated from the marine mollusk *Dolabella auricularia* and currently in phase II clinical trials, was found to be a metabolite of the blue-green alga (cyanobacterium) *Symploca hydnoides*. Staurosporine recently isolated from the Micronesian tunicate *Eudistoma toealensis* and its predatory flatworm *Pseudoceros* sp. (Schupp et al. 1999) was so far known only from actinomycetes such as *Saccharothrix aerocolonigenes* subsp. *staurosporea* (formerly known as *Streptomyces staurosporeus*). Like dolastatin 10, staurosporine derivatives have received considerable interest due to their pronounced cytotoxic activity. The sponge *Theonella swinhoei* collected in the Philippines contains the cyclic peptide theopalauamide and the macrolide swinholide. Differential centrifugation of several cellular fractions from the tissue of Theonella swinhoei revealed the presence of theopalauamide in filamentous bacteria whereas swinholide A contain mostly unicellular bacteria as evidenced by spectroscopic and microscopic analysis of the various fractions. Both compounds showed pronounced antifungal and cytotoxic activity (Bewley et al. 1996; Bewley

and Faulkner 1998). Based on the above discussion or even identity of highly unusual natural products from marine invertebrates and microorganisms, it is tempting to assume that, rather than reflecting a mere chemical coincidence, compounds such as dolastatin 10 and staurosporine are introduced into the respective invertebrates through the food chain or originate from symbiotic (sensu lato) bacteria or microalgae.

1.3 Antibacterial Activity

After the discovery of penicillin, exhaustive studies have been conducted chiefly on soil-derived bacteria and fungi, which confirmed that microorganisms are a valuable source of structurally novel bioactive compounds. The escalating need for new antimicrobial agents to regulate emerging diseases or resistant strains of microorganisms sparked a number of researchers to explore the sea for new bioactive compounds. Throughout the years, extensive screening programs were developed worldwide, and great efforts have been devoted to the isolation of new metabolites from marine microorganisms. As part of an ongoing global research to discover novel antimicrobials to treat various infections caused by resistant pathogenic bacteria, numerous studies contributed novel antibacterial marine natural products isolated from marine fungi and bacteria. Marine fungi and bacteria represent a key producer of antibacterial compounds in the diverse marine environment. In the search for the potential antibacterial compound, Xiong and co-workers (2009) reported the bioactive potential of marine fungus *Cladosporium* sp. F14 with plenty of reported biologically effective compounds including methanephrine, cis-1-chloro-9-octadecene, 16-nitrobicyclo [10.4.0] hexadecane-1-ol-13-one 13-bromotetradecanoic acid 2-phenazinol, 6-amino-morphinan-2,4-diol-6-one, N-formyl- and pyrrolo [1,2-a]-pyrazine-1,4-dione, and hexahydro-3-(phenylmethyl) with antibacterial activity against 6 bacterial species including *Bacillus* sp., *Vibrio* sp., and *Micrococcus* sp. A preliminary study carried out on *Cladosporium* sp. resulted in the separation of the compound, cladospolide E, a nine-membered lactone, which showed the potential of antibacterial activity against several pathogenic bacteria including *E. coli*, *Bacillus thuringiensis*, *B. subtilis*, *Mycobacterium smegmatis*, and *S. aureus* (Gao et al. 2010).

Moreover, compound 6-oxo-de-O-methyllasiodiplodin is isolated from a copper-colored alga endophytic fungus (ZZF36) from the South China Sea, identified as a polyketide, which possesses notable antibacterial effect against *B. subtilis*, *S. aureus*, and *Salmonella* enteritidis with an IC_{50} value between 6.25 and 12.5 μg/mL (Yang et al. 2006). The researchers first time separated a marine-based fungus *Nigrospora* sp. from Similan Islands in Thailand. This fungal species develop as plant endophytes particularly. Chemical investigation of *Nigrospora* sp. resulted in the isolation of four novel marine metabolites called nigrospoxydons and nigrosporapyrone, with nine known compounds in potato dextrose agar (PDA) medium.

Crude fungal extracts of *Nigrospora* sp. revealed sturdy antibacterial activity against standard *S. aureus*- and methicillin-resistant *S. aureus* with MIC (minimum inhibitory concentration) value ranging from 64 to 128 mg/ml (Trisuwan et al. 2008). A novel antibacterial compound dioxopiperazine, dehydroxybisdethiobis-methylthiogliotoxin, and the earlier reported bisdethiobis-methylthio-gliotoxin and gliotoxin were isolated from the broth of a marine-originated fungus of the species *Pseudallescheria.* These isolated compounds showed potent antibacterial activity against the methicillin multidrug-resistant *Staphylococcus aureus* (Li et al. 2006). A bacteriostatic novel compound spirodioxynaphthalene (ascochytatin) was discovered by Kanoh et al. (2008). These bacteriostatic molecules were isolated from cultures of a marine fungus *Ascochyta* sp. NGB4 (separated from a floating scrap of rotting rope that had been obtained at a fishing port in Nagasaki area, Japan). Ascochytatin exhibits bacteriostatic effect by inhibiting the bacterial growth regulatory system and consequently inhibits the *Bacillus subtilis* growth. Cultures of the marine bacterial isolate *Brevibacillus laterosporus* PNG276 obtained from Papua New Guinea produce a unique lipopeptide named tauramamide, collectively with its methyl and ethyl esters. These isolated compounds exhibited potent MIC (minimum inhibitory concentration) values of 0.11 mM and moderately particular activity against the important Gram-positive human pathogen *Enterococcus* sp. (Desjardine et al. 2007). Marinispora (strain NPS008920), a member of the new bacterial genus, was isolated from a sediment sample collected in Cocos Lagoon, Guam. Biochemical examination of this strain yielded a series of novel 2-alkylidene-5-alkyl-4-oxazolidinones and lipoxazolidinones A, B, and C. These compounds revealed a wide range of antimicrobial activities, and the outcomes obtained were similar to antibiotic linezolid (Zyvox) (Macherla et al. 2007).

Approximately 100 bacteria were extracted from the abdominal region of fish found at Balochistan Coast, Pakistan. Further, investigation on these isolated bacteria yielded an isolate of *Pseudomonas stutzeri* (CMG 1030) that demonstrated pronounced inhibitory activity on various pathogenic bacteria such as MRSA strains, whereas chemical analysis of the ethyl acetate extract yielded a new antibacterial metabolite called zafrin (4b-methyl-5,6,7,8–tetrahydro-1 (4b-H) – phenanthrenone) which exhibited significant activity against numerous clinical and environmental microorganisms. The minimum inhibitory concentration of zafrin (235.85–589.62 mM) compared favorably with other novel antimicrobials such as 2,4-diacetylphloroglucinol (2.38–4.76 mM) (Isnansetyo et al. 2003). Surprisingly, the inhibition rate of zafrin against *Bacillus subtilis* was faster than commercial reference standards such as ampicillin, vancomycin, or tetracycline. Zafrin exerts bactericidal effect; its pattern of lysis resembles that of compounds such as nisin (14.91 mM) and Triton X-100, which disrupt the cell membrane. Therefore, it was concluded that the mode of action of zafrin is via the disruption of the cytoplasmic membranes since the molecule is amphiphilic in nature (Uzair et al. 2008).

1.4 Antifungal and Antiviral Activity

Symbiotic characteristic of the marine microbes with macroorganism is a distinguished phenomenon for the production of bioactive compounds. Various experimental studies have proved that compound xestolactone B, isolated from marine fungus *Penicillium* cf. *montanense* associated with the sponge *Xestospongia exigua*, exhibited notable antifungal effect against filamentous fungal pathogens of *C. albicans* (Edrada et al. 2002). Likewise, an anthracycline-related pentacyclic compound, seragikinone A, isolated from an unknown marine fungus extracted from the rhodophyte *Ceratodictyon spongiosum* revealed weak antifungal effect against *C. albicans* (Shigemori et al. 1999).

A fungal strain of marine origin *Zopfiella marina* has been reported to yield zofimarin an antifungal diterpene glycoside, whereas another leading antifungal drug griseofulvin has been reported to derive from marine fungus *Penicillium waksmanii* (Pietra 1997). Marine endophytes *Chaetomium* sp. of algal symbiosis have been reported to produce three new fungal polyketide metabolites, chaetocyclinones, collectively with three discovered compounds. The compound chaetocyclinone A displayed dose-dependent antifungal activity tested against selected phytopathogenic fungi *Phytophthora infestans* (Losgen et al. 2007). Similarly, a marine sponge Myxilla incrustans derived fungus Microsphaeropsis sp. which yielded an eremophilane derivative, microsphaeropsin, showed significant antimicrobial activity against Ustilago violacea and Mycotypha microspora (Holler et al. 1999). A group of researchers finds out that crude culture solvent extract of fungal species *Fusarium* sp. displayed potent antimicrobial effect. Moreover, the results of preliminary screening from *Fusarium* sp. revealed presence of new antifungal compound fusarielin E with confirmed structure elucidation (Gai et al. 2007). Chemical investigation of fungal broth of a marine-derived fungal species PSU-F44 of genus *Penicillium* from *Annella* sea fan yielded two novel metabolites, penicipyrone and penicilactone, together with three known macrolides (+)-brefeldin A, (+)-brefeldin C, and 7-oxobrefeldin A. Antimicrobial assay of penicilactone, brefeldin A, and 7-oxobrefeldin A showed that compound brefeldin A exhibited remarkable antifungal activity against *M. gypseum* SH-MU-4 with minimum inhibitory concentration value of 228.57 mM, whereas the remaining compounds were inactive (minimum inhibitory concentration >700 mM) (Rukachaisirikul et al. 2009). A macrolide phomolide B is isolated from a fungus *Phomopsis* sp. which exerted *E. coli* inhibition effect (Du et al. 2008). The lipopeptide molecule hassallidin A isolated from *cyanobacterium Hassallia* sp. significantly inhibited *C. albicans* and *A. fumigates* (minimum inhibitory concentration = 4.8 μM) when pharmacologically tested. Similarly, the fatty acid majusculoic acid isolated from marine cyanobacterial mat assemblage inhibited *C. albicans* (minimum inhibitory concentration = 8 μM) but found less potent than reference drug fluconazole (Macmillan and Molinski 2005; Neuhof et al. 2005). Kunze and coworkers (Kunze et al. 2008) investigated biological properties of cyclopeptide pedein A and B isolated from *Chondromyces pediculatus* (myxobacteria). The cyclopeptide pedein A exhibited significant antifungal activity (minimum inhibitory concentration = 0.6–1.6 μg/mL) against *R. glutinis*, *S. cerevisiae*, and *C. albicans* with undetermined molecular mechanism of action.

The extensive ongoing research on marine fungi has afforded impressive in-depth on the exploration of antiviral compounds of marine source. Marine-derived fungal species such as *Fusarium heterosporum* and a *Phoma* sp. yielded novel active compounds equisetin and phomasetin, respectively; both these metabolites displayed remarkable HIV-1 integrase inhibition activity in bioassay-based experiments (Singh et al. 1998). Similarly, sansalvamide A, a topoisomerase blocker, was isolated from the marine fungus *Fusarium* sp. and significantly inhibited pathogenic poxvirus *Molluscum contagiosum* (cause severe lesions in HIV patients) by inhibiting DNA relaxation, DNA binding, and covalent complex formation which consequently exhibited significant therapeutic potential (Hwang et al. 1999).

Stachyflin terpenoid compound isolated from the fungus *Stachybotrys* sp. RF-7260 exhibited significant in vitro antiviral activity against influenza A virus (H1N1). The inhibitory concentration of stachyflin (IC_{50} = 0.003 μg/mL) was comparable with standard anti-H1N1 drug amantadine. The structural analysis revealed that *M. Stachyflin* as a pentacyclic terpenoid possesses a novel cis-fused decalin; this unique structural feature is responsible for inhibiting the fusion between the viral envelope and the host cell membrane (Minagawa et al. 2002).

Wu et al. (2009) delineate two new compounds, asperxanthone and asperbiphenyl, isolated from a fungal strain, *Aspergillus* sp. (MF-93). These isolated compounds exert moderate inhibitory activity against a typical tobacco mosaic virus of the *Tobamovirus* group. In addition Muftah et al. (2011) reported a 12-membered new macrolide balticolid isolated from the culture broth of fungal strain *Ascomycetes* sp. 222, which showed a moderate anti-HSV-1 activity with an IC_{50} value of 0.45 μM. Table 1.1 summarizes the various examples of antifungal and antiviral compounds isolated from marine microbes.

Table 1.1 Antifungal and antiviral compounds isolated from marine microbes

Compound	Microorganism	Activity	References
Trichodermaketone A	*Trichoderma konongii*	Antifungal	Mi-Hee et al. (2008)
Fumigaclavin	*Penicillium viridicatum*	Antifungal	Mi-Hee et al. (2008)
Seragikinone A	*Rhodophyte ceratodictyonspongiosum*	Antifungal	Punyasloke et al. (2006)
Xestodecalactone A	*Penicillium* cf. *montanense*	Antifungal	Punyasloke et al. (2006)
Chaetocyclinone A	*Chaetomium* sp.	Antifungal	Losgen et al. (2007)
Balticolid	Ascomycetes	Antiviral	Muftah et al. (2011)
Asperxanthone	*Aspergillus* sp.	Antiviral	Wu et al. (2009)
Equisetin	*Fusarium heterosporum*	Anti-HIV	Singh et al. (1998)

1.5 Anti-inflammatory Activity

Inflammation is a defensive reaction to injury and arises from the resultant cell damage. Physical and chemical agents which provoke the inflammatory response include mechanical trauma, radiation, direct chemical damage, secondary chemical or biological damage, invading organisms, and antibody-antigen reactions. Inflammatory reactions are generally divided into two types, acute and chronic. In acute inflammation following the initial insult to the tissue, there may be transient vasoconstriction, but this is followed by invariant response of local vasodilatation with increase blood flow. The mediators of the dilation include histamine, kinins, and prostaglandins. Damage to mast cell in the tissue releases histamine, serotonin, and inflammatory mediators (Denko 1992). Figure 1.2 describes various local and systemic inflammatory mediators such as histamine, serotonin, prostaglandins, cytokines, leukotrienes, bradykinin, etc. These inflammatory mediators are responsible for the inflammatory conditions, and targeted inhibition of these inflammatory mediators is the primary aim in research of new anti-inflammatory compounds.

New marine microbe-derived bioactive compounds have been emerged as potential anti-inflammatory agents, since they could be helpful in combating chronic inflammatory degenerative conditions. Chemical investigation of a marine fungus, *Penicillium steckii* 108YD142, revealed the presence of new tanzawaic acid Q together with four known analogs tanzawaic acids A, C, D, and K. These compounds act by inhibiting nitric oxide synthesis, and the new tanzawaic acid inhibited

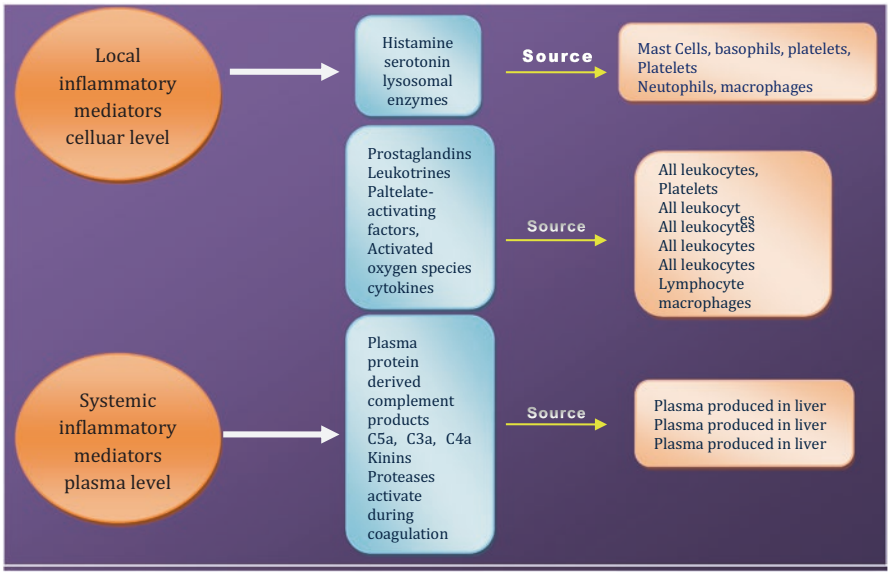

Fig. 1.2 Various local and systemic inflammatory mediators responsible for inflammatory conditions

the lipopolysaccharide-induced nitric oxide synthase, cyclooxygenase-2 proteins, and mRNA characters in RAW 264.7 macrophages.

Additionally, tanzawaic acid also diminished the mRNA levels of inflammatory cytokines. In conclusion, the results of this study demonstrated that the new tanzawaic acid and its derivative restrain lipopolysaccharide-induced inflammation. This is the first literature on the anti-inflammatory activity of tanzawaic acid (Shin et al. 2015). In recent discovery, an anthraquinone derivative, questinol, was successfully isolated from the broth extract of the fungus *Eurotium amstelodami* (isolated from an unknown marine organism) obtained from the Sungsan seashore in Jeju Island, Korea. Questinol exhibited potential anti-inflammatory effect by targeting various inflammatory mediators such as inhibition of production of pro-inflammatory cytokines, interleukin-1β, tumor necrosis factor-α, and interleukins-6 as well as suppressing the expression level of inducible nitric oxide synthase in a dose-dependent manner. through the western blot analysis (Yang et al. 2014). All these findings coined questinol as a promising anti-inflammatory agent. A new naphthalene derivative vaccinal is isolated from marine fungus *Pestalotiopsis vaccinii* endogenous with the mangrove plant *Kandelia candel* (L.) Druce (Rhizophoraceae) showed potent COX-2 inhibitory activity. Similarly, a new benzoic acid, 2-(2-hydroxypropanamido) benzoic acid separated from the fermentation treatment of fungus *Penicillium chrysogenum*, displayed remarkable anti-inflammatory and analgesic activities (Wang et al. 2014a, b). Taori et al. (2007) reported lyngbyastatin, a peptide isolated from marine cyanobacteria *Lyngbya* spp. from South Florida. This isolated lyngbyastatin selectively and potently inhibited porcine pancreatic elastase (IC_{50} = 3–10 nM) and confirms its therapeutic potential in pathophysiological conditions where elastase hyperactivity is involved.

Marine actinomycetes also have been exploited as a source of biologically active secondary metabolites with anti-inflammatory properties. Oh et al. (2008) purified a novel polyketide salinipyrone A from the marine actinomycete *Salinispora pacifica*, which moderately inhibited interleukin-5 (IC_{50} = 10 μg/mL) in a mouse splenocyte model of allergic inflammation, with low cell cytotoxicity. Similarly, cyclomarins are three cyclic heptapeptides (A, B, and C), isolated from the marine bacterium actinomycete, belonging to *Streptomyces* sp., of the Californian coast. Cyclomarin A, constituted of three common and four unusual amino acids, showed potent anti-inflammatory in both in vivo and in vitro assays, managing to inhibit edema and pain similarly to the drug hydrocortisone (Renner et al. 1999). A moderate anti-inflammatory effect has been reported also in cyclomarin C, whose total synthesis was recently experimented and reported (Wen et al. 2005). For this reason both cyclomarins A and C and their derivatives can develop as potential naturally occurring anti-inflammatory therapies. Peptide molecules salinamides (A, B, C, D, and E) from marine actinomycetes, belonging to *Streptomyces sp*., are isolated from the surface of the jellyfish *Cassiopea xamachana*, found in Florida waters (Trischman et al. 1994). Salinamides A and B are the two major bicyclic metabolites, with potent topical anti-inflammatory activity and moderate antibiotic activity against Gram-positive bacteria, and could be used in the treatment of tissue inflammation and some infections. Salinamides C, D, and E are the minor

metabolites; structures of these compounds were established through spectral and chemical techniques, whereas salinamide D has a similar structure but contains a valine residue in place of the isoleucine present in salinamide A; salinamides C and E are represented by monocyclic peptides, which exert a moderate anti-inflammatory activity (Moore et al. 1999). Marine actinomycetes found in the Caribbean brown alga *Lobophora variegate* yielded two bioactive lobophorins A and B with antibiotic, anticancer, and anti-inflammatory properties; lobophorins showed potent anti-inflammatory effect higher than indomethacin (Jacobson and Jacobs 1992).

1.6 Anticancer Activity

Several research studies on biological activities of marine microbe-derived compounds confirm their noteworthy contribution in the treatment of cytotoxicity on different cancer cell lines. One of the significant domains of marine microbe's research is dedicated to the development of new anticancer molecules. In this search, marine fungi have afforded a number of potential pharmacological compounds and thus render a precious resource of new anticancer drugs. Among all these secondary metabolites isolated from marine fungi, none of them currently is available commercially except plinabulin, a synthetic cyclic dipeptide analog of halimide, which is under phase II of clinical trials for the treatment of non-small cell lung cancer (Imhoff 2016). A new anticancer compound aspochalasin V (aspochalasin, 20-β-methylthio-aspochalsin Q) was isolated from the culture broth of *Aspergillus* sp., obtained from the gastrointestinal tract of a marine isopod *Ligia oceanica* (Dinghai in Zhoushan, Zhejiang Province of China). This is the first report about methylthio-substituted aspochalasin derivative. Apochalasin V exhibited moderate cytotoxic activity against the prostate cancer cell line and HCT116 cell line with IC_{50} values of 30.4 and 39.2 μM. Selectively (Liu et al. 2014) *Neosartorya pseudofischeri*, a type of fungal species, which was isolated from the inner tissue of starfish *Acanthaster planci*, has been reported to yield a novel gliotoxin in glucose-peptone-yeast (GluPY) extract medium. Gliotoxin exhibited pronounced inhibitory activities against two multidrug-resistant bacteria, *S. aureus* and *Escherichia coli* (E. coli), as well as significant cytotoxic effect against human embryonic kidney (HEK) 293 cell line and human colon cancer cell lines, HCT-116 and RKO (a poorly differentiated colon carcinoma cell line). Liang et al. (2014) studied the impact of cultivation parameters of marine fungus on secondary metabolites. Marine microorganisms are latent reservoirs of new drug candidates. A novel depsipeptide named thiocoraline was isolated from the mycelial extract of the bacterium *Micromonospora marina* symbiotic with a marine soft coral in the Indian Ocean. It acts by inhibition of DNA polymerase-a (Newman and Cragg 2004). The extract of the marine fungus *Curvularia* sp. (strain no. 768) demonstrated cytotoxic effect against human tumor cell lines. Additionally, chemical examination of *Curvularia* sp. yielded the unique macrolide apralactone A, a 14-membered phenyl acetic

acid macrolactone, along with 6 curvularin macrolides. The isolated macrolides were tested against 36 human tumor cell lines, including 14 different solid tumor types. The investigated macrolides revealed concentration-dependent activity. The novel macrolide apralactone A exhibited moderate concentration-dependent cytotoxic effect with a mean effective concentration (EC_{50} value of 9.87 mM). The most effective metabolite (+)-(10*E*,15*R*)-10,11-dehydrocurvularin revealed concentration-dependent cytotoxicity with a mean EC_{50} value of 1.25 mM, combined with notable in vitro tumor cell selectivity toward 9 of the 36 experimented tumor cell lines. Chemical investigation of marine fungus *Aspergillus* carbonarius yielded two distinct secondary metabolites, carbonarones A and B. Both compounds carbonarones A and B were tested against human leukemia cell lines K562 by the application of sulforhodamine B (SRB) assay. The cytotoxic effects were also evaluated against P388 (murine leukemia), A549 (human lung carcinoma), BEL-7402 (human hepatoma), and HL-60 (human promyelocytic leukemia) cell lines. Both compounds showed moderate antiproliferative activity against K562 cell lines with EC_{50} values of 244.54 and 121.39 mM, respectively, while they were inactive against the other cell lines tested (EC_{50} >436.68 mM) (Zhang et al. 2007).

Fungus *Aspergillus ustus* isolated from the marine sponge *Suberites domuncula* reported to yield seven new drimane sesquiterpenoids together with three known compounds. The crude extract of *A. ustus* exhibited cytotoxic activity against the murine lymphoma cell line L5178Y at a concentration of 10 mg ml^{-1}. Similarly, experimental study of fungus *Arthrinium arundinis* ZSDS1-F3 (obtained from sponge in Xisha Islands, China) yielded two new 4-hydroxy-2-pyridone alkaloids, arthpyrones (1–2). Compounds 1 and 2 had significant in vitro cytotoxicities against the K562, A549, Huh-7, H1975, MCF-7, U937, BGC823, HL60, and Hela cell lines, with IC_{50} values ranging from 0.24 to 45 μM (Liu et al. 2009).

1.7 Antituberculosis and Anticholinesterase Activity

Tuberculosis is infectious disease caused mainly by strains of bacteria known as *Mycobacteria*. *Mycobacterium tuberculosis* is the second most cause of death in the world. Marine resources have been poorly investigated for the search of antituberculosis drugs recently; they are noticed as potential source of antituberculosis bioactive moieties. Recently, racemic dinaphthalenone derivatives (±)-asperlone A and (±)-asperlone B were isolated from the cultures of *Aspergillus* sp. from the leaves of *S. apetala* (collected in Hainan Island, China). Both these compounds showed a potent antituberculosis effect by inhibition of *Mycobacterium tuberculosis* protein tyrosine phosphatase B with IC_{50} values of 4.24 ± 0.41, 4.32 ± 0.60 μM, respectively, which offers them as lead compounds for the development of new antituberculosis drugs (Xiao et al. 2015). Zhang et al. (2008b) have reported isolation of two new dimeric naphtha-γ-pyrones 8′-O-demethylniger-one and 8′-O-demethylisonigerone

from *Aspergillus carbonarius*, a marine-derived fungus. These isolated compounds showed weak antimycobacterial activity against *Mycobacterium tuberculosis* with minimum inhibitory concentration value (43 and 21.5 μM, respectively). Cyclomarin A, isolated from the marine bacterium actinomycetes, belonging to *Streptomyces* sp. constituted of three common and four unusual amino acids, showed potent anti-inflammatory as well as significant antimycobacterial activity. Cyclomarin A proved to be bactericidal against *Mycobacterium tuberculosis* by targeting its caseinolytic protease, resulting in a potent antitubercular compound (Renner et al. 1999; Schmitt et al. 2011).

Penicilliumine is a new compound isolated from the fermentation *Penicillium commune* 366,606 (a marine-derived fungus isolated from the seawater collected at Qingdao, China). Penicilliumine significantly abolishes the acetylcholinesterase activity by 18.7% (±0.26%) and 32.4% (±2.08%) at the concentration of 50 μM, respectively, when compared with 43.6% (±2.12%) inhibition rate of the standard drug tacrine (He et al. 2014).

1.8 Antimalarial Activity

Very little work has been done toward the search of marine microbe-derived bioactive with antimalarial activity. Some of the major research findings of marine antimalarials have been described in this section. Two reports were contributed during 2007 by the Panama International Cooperative Biodiversity Groups project: McPhail et al. (2007) investigated a novel lipopeptide dragomabin from the cyanobacterium *Lyngbya majuscula* with moderate antimalarial effect (IC_{50} = 6.0 μM). Additionally, dragomabin also produces differential toxicity to malarial parasite versus mammalian host cells. Linington et al. (2007) discovered Panamanian *Oscillatoria* sp. that yielded new cyclic hexapeptides venturamides A and B. These hexapeptides demonstrated significant in vitro antiplasmodial activity against the W2 chloroquine-resistant strain of the parasite (IC_{50} = 5.6–8.2 μM), with mild cytotoxicity to mammalian cells, the first example of the identification of cyanobacterial peptides with selective antimalarial activity. Na et al. (2008) discovered a new marine *Streptomyces* sp. H668 polyether from Hawaii that exhibited in vitro antimalarial activity (IC_{50} = 0.1–0.2 μg/mL) against both *Plasmodium falciparum* chloroquine-susceptible (D6) and chloroquine-resistant (W2) clones, with minimal cytotoxicity toward mammalian cells. A group of researchers (Clark et al. 2008) proved that a novel acylproline derivative, tumonoic acid I isolated from the Papua New Guinean marine cyanobacterium *Blennothrix cantharidosmum*, demonstrated moderate antimalarial activity against *Plasmodium falciparum* (IC_{50} = 2 μM). Pontius et al. (2008a) isolated a heterocyclic-substituted xanthone, chaetoxanthone B, from cultures of a marine-derived fungus *Chaetomium* sp. that showed selective activity toward *Plasmodium falciparum* K1 strain (IC_{50} = 0.5 μg/mL).

1.9 Antidiabetic Activity

Diabetes mellitus is a common and often life-threatening disorder with increasing number of patients globally. Because of the severity of this metabolic disorder and the availability of inadequate antidiabetic drugs, the constant search for new molecules, particularly from the marine environment, has drawn the attention of the biomedical researchers. In contrast, marine microbes are poorly studied for antidiabetic activity, but they may have a pivotal role in the research for new antidiabetic drugs for a future point of view. Usually, diabetes mellitus is of two types, type 1 and type 2 (Fig. 1.3), and gestational diabetes strike females during pregnancy. In type 1 diabetes, the beta cells are impaired due to an autoimmune response, and the insulin production is terminated. Therefore, type 1 diabetes is also known as insulin-dependent diabetes because it causes patients dependency to take insulin injections for the entire life period. In type 2 diabetes, the body does not produce adequate insulin for proper functioning, or the pancreatic β-cells do not respond to insulin (insulin resistance) (Lauritano and Ianora 2016). As diabetes is a metabolic disorder, screening of antidiabetic agents involve evaluation of the functioning of specific enzymes required in sugar metabolism, in both rat models and patients, e.g., α-amylase, β-glucosidase, N-acetyl-glucosaminidase, aldose reductase, hexokinase, glucose 6-phosphatase, dipeptidyl peptidase IV, glucose transporter 4, and glycogen synthase kinase-3 (Bidon-Chanal et al. 2013).

Protein tyrosine phosphatase 1B (PTP1B) is another well-known target for antidiabetes trial. This enzyme family includes about 100 proteins that catalyze

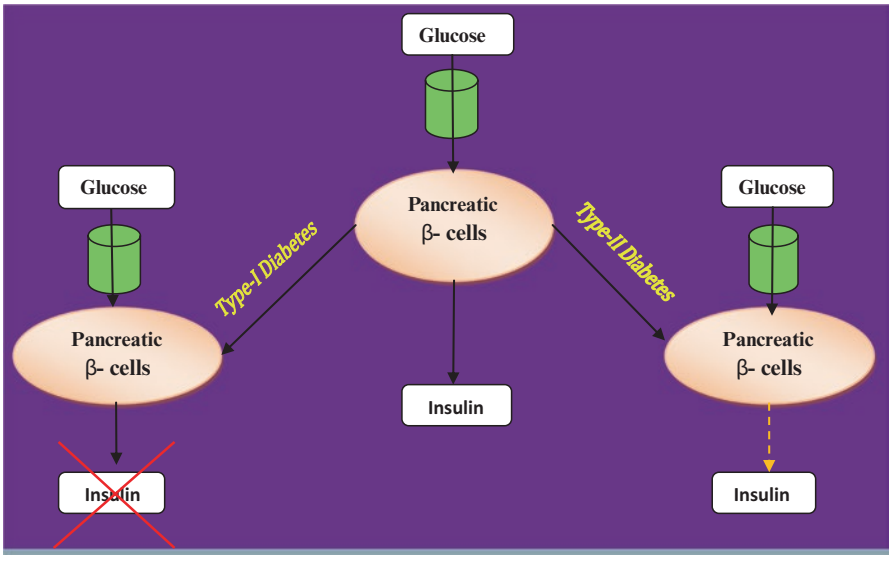

Fig. 1.3 In type 1 diabetes, β cells are destroyed, and insulin is not produced, whereas in type 2 diabetes, the body does not produce enough insulin, or cells do not react to insulin

dephosphorylation of phosphotyrosine residues in protein substrates, such as the insulin receptor PTP1B which antagonizes insulin signaling by reducing the activation state of the insulin receptor kinase, thereby inhibiting post-receptor signaling in insulin-responsive tissue. For this reason, this enzyme is associated with the development of type 2 diabetes (Barde et al. 2015). Therefore, in the search of targeted inhibition of enzyme PTP1B, researchers have carried out bioassay-guided study of the marine-derived fungus *Cosmospora* sp. SF-5060, resulting to the isolation of compound aquastatin A. The compound exhibited potent inhibitory activity against protein tyrosine phosphatase 1B (PTP1B); studies confirmed that protein tyrosine phosphatase 1B, an intracellular non-receptor type protein tyrosine phosphatase, negatively controls insulin and leptin receptor which interfered signaling pathways subsequently responsible for the development of type 2 diabetes. Thus, inhibition of this pathway may serve an excellent, novel remedy for type 2 diabetes and obesity. Aquastatin A was found to inhibit protein tyrosine phosphatase 1B activity in a competitive and selective manner as described by kinetic analyses and trial over a small panel of other PTPs, respectively. In addition, hydrolyzing the analysis of the compound confirms the inhibitory activity of molecule due to the presence of dihydroxy pentadecyl benzoic acid moiety (Seo et al. 2009).

A couple of new sulfur-containing benzofuran derivatives, eurothiocins A and B, were isolated from the fungus *Eurotium rubrum* SH-823 collected from a *Sarcophyton* sp. soft coral in the South China Sea. These compounds shared a methyl thiolester moiety, which was considerably rare in natural secondary metabolites. Both eurothiocins A and B showed potent inhibitory effects against α-glucosidase activity than acarbose, which was the clinical α-glucosidase inhibition. Recently researchers (Pandey et al. 2013) have investigated compounds with inhibitory effects on β-glucosidase in bacteria. This enzyme performs a key role in the degradation of polysaccharides and the processing of glycoproteins and glycolipids, representing a focused target for the treatment of diabetes and obesity. They have stated that bacteria symbiotic with the marine sponge, *Aka coralliphaga*, yielded a huge number of glucosidase inhibitors. Marine actinomycetes (e.g., *Streptomyces* sp.) have also investigated as an origin of numerous enzyme inhibitors and other bioactive compounds. *Streptomyces corchorusii* subsp. rhodomarinus revealed significant α-amylase inhibition, while another *Streptomyces* strain (*Streptomyces* sp.) collected at a depth of approximately 100 m from Otsuchi Bay in Iwate Prefecture was found to produce two novel compounds, pyrostatins A and B, with specific inhibitory activity against N-acetyl-glucosaminidase (Imada 2005).

1.10 Novel Metabolites Produced by Marine Actinomycetes

Actinomycetes are the most economic and biotechnological important prokaryotes. They are the key producer for the generation of the majority of discovered bioactive secondary metabolites. Actinomycetes represent themselves as a prominent source of antibiotics, antitumor agents, and immunosuppressive agents including enzymes.

Table 1.2 Novel metabolites produced by actinomycetes

Compound	Source	Activity	References
Abyssomicins	*Verrucosispora* sp.	Antibacterial	Riedlinger et al. (2004)
Aureoverticillactam	*Streptomyces aureoverticillatus*	Anticancer	Mitchell et al. (2004)
Bonactin	*Streptomyces* sp.	Antibacterial, antifungal	Schumacher et al. (2003)
Caprolactones	*Streptomyces* sp.	Anticancer	Stritzke et al. (2004)
Chandrananimycins	*Actinomadura* sp.	Antialgal, antibacterial, anticancer, antifungal	Maskey et al. (2003)
Chinikomycins	*Streptomyces* sp.	Anticancer	Li et al. (2005)
Chloro-dihydroquinones	Novel actinomycete	Antibacterial, anticancer	Soria-Mercado et al. (2005)
Diazepinomicin (ECO-4601)	*Micromonospora* sp.	Antibacterial, anticancer, anti-inflammatory	Charan et al. (2004)
3,6-disubstituted indoles	*Streptomyces* sp.	Anticancer	Sanchez Lopez et al. (2003)
Frigocyclinone	*Streptomyces griseus*	Antibacterial	Bruntner et al. (2014)
Glaciapyrroles	*Streptomyces* sp.	Antibacterial	Macherla et al. (2005)
Gutingimycin	*Streptomyces* sp.	Antibacterial	Maskey et al. (2004)

Because of the enormous properties of actinomycetes in this concern, significant endeavors have been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past five decades. Numerous novel metabolites with great pharmacological significance have been isolated from marine actinomycetes in the past few decades. Some outstanding examples of novel compounds isolated from marine actinomycetes are shown in Table 1.2.

1.11 Miscellaneous Pharmacological Activities of Marine Microbes

Table 1.3 lists marine compounds with miscellaneous pharmacological activities, molecular chemistry, IC_{50} value, and molecular mechanism of action. Table 1.3 shows only two marine microbe-derived natural products deoxyrubralactone and saproxanthin and myxol with a pharmacological activity and molecular mechanism of action. In contrast, although a pharmacological activity was described and an IC_{50} value for inhibition of an enzyme or receptor determined, detailed molecular mechanisms of action studies were unavailable for the following 13 marine compounds

Table 1.3 Marine microbes/compounds with miscellaneous pharmacological activities

Compound/microorganism	Chemistry	Pharmacological activity	IC_{50} value	Mechanism of action	References
1-Deoxyrubralactone/fungus	Shikimate	X and Y DNA polymeraseinhibition	12–60 μM	Specific inhibition of DNA polymerase β and κ	Naganuma et al. (2008)
Saproxanthin and myxol/bacterium	Polyketide	L-glutamate toxicity inhibition	3.1–8.1 μM	Lipid peroxidation inhibition	Shindo et al. (2007)
Botrytis sp. α-pyrone derivative/ fungus	Polyketide	Tyrosinase inhibition	4.5 μM	Undetermined	Zhang et al. (2007)
Cephalosporolides H and I/fungus	Polyketide	Xanthine oxidase and steroid inhibition	b0.29 mM	Undetermined	Li et al. (2007)
Chaetominedione/fungus	Alkaloid	Tyrosine kinase inhibition	b200 μg/mL	Undetermined	Abdel-Lateff (2008)
Circumdatin I/fungus	Alkaloid[s]	Ultraviolet A-protecting	98 μM	Undetermined	Zhang et al. (2008a)
Diapolycopenedioic acid xylosyl ester/bacterium	Terpenoid	Lipid peroxidation inhibition	4.6 μM	Undetermined	Shindo et al. (2007)
Irregularasulfate/bacterium	Terpenoid	Calcineurin inhibition	59 μM	Undetermined	Carr et al. (2007)
Kempopeptins A and B/bacterium	Peptide	Elastase and chymotrypsin inhibition	0.32–8.4 μM	Undetermined	Taori et al. (2008)
Lyngbyastatin 4/bacterium	Peptide	Elastase and chymotrypsin inhibition	0.03–0.3 μM	Undetermined	Taori et al. (2007)
Malevamide E/bacterium	Peptide	Extracellular Ca^{2+} channel inhibition	9 μM	Undetermined	Adams et al. (2008)
Monodictysin C/fungus	Shikimate	CYP1A inhibition	3.0 μM	Undetermined	Krick et al. (2007)
Monodictyochromes A and B/fungus	Shikimate	CYP1A inhibition	5.3–7.5 μM	Undetermined	Pontius et al. (2008b)
Penicillium waksmanii A, B, and C	Alkaloid	Histamine H3 receptor agonists	0.12–0.2 μM	Undetermined	Kushida et al. (2007)
Saliniketals A and B/bacterium	Polyketide	Ornithine decarboxylase induction	1.9–7.8 μg/mL	Undetermined	Williams et al. (2007)

IC Inhibitory concentration

included in Table 1.3: botrytis sp. α-pyrone derivative, cephalosporolides H and I, chaetominedione, diapolycopenedioic acid xylosyl ester, irregularasulfate kempopeptins A and B, lyngbyastatin 4, malevamide E, monodictysin C, monodictyochromes A and B, and *Penicillium waksmanii* A, B, and C.

1.12 Supply Issue of Bioactive Compounds from Marine Resources

Limited scientific knowledge of marine microbial flora and specific environmental conditions required for the culture media development and isolation of marine microbes have been a difficult task to pharmaceutical industries to ensure the availability of marine-derived active pharmaceutical ingredients at commercial levels. Collection of samples from the deep-sea environment has been a challenging task and renders great blockades in the research of drug development from the marine atmosphere; hence only the marine resources available at mangroves, superficial coral reefs, and seashore with ease of collection have been exploited for marine drug discovery. In conclusion, problem of continuous supply is a serious obstacle in the development of marine-based natural products that are currently under clinical trials or in preclinical evaluation**.** The quantity of many highly bioactive compounds in marine invertebrates is often minute, sometimes accounting for less than 6–10% of the wet weight. For example, in order to obtain approximately 1 g of the promising anticancer agent trabectedin (also known as ecteinascidin 743 or ET-743), close to 1 metric tonne (wet weight) of the tunicate *E. turbinate* has to be harvested and extracted (Mendola 2000). Similarly, for the halichondrins (e.g. halichondrin B), which are powerful cytostatic polyketides of sponge origin, the ratio of biomass to yield of product is even less favorable. In order to obtain as little as 300 mg of a mixture of two halichondrin analogs, 1 metric tonne of the sponge *Lissodendoryx* sp. had to be collected and extracted (Hart et al. 2000).

This already causes considerable difficulties and delays in clinical studies where Gram quantities of compounds are generally needed but will prove to be an overwhelming obstacle once one of these compounds is licensed as a drug. For example, provided that the halichondrins make it to the market as new anticancer drugs, the annual need for these compounds is estimated to be in the range of 1–5 kg per year, which corresponds to roughly 3000–16,000 metric tonnes of sponge biomass per year (Hart et al. 2000). It is obvious that such large amounts of biomass of either sponges, tunicates, or other pharmacologically promising marine invertebrates can never be harvested from nature without risking extinction of the respective species. Therefore, marine microbes represent an alternative source for the production and development of bioactive compounds with a regular supply. Furthermore, identification, cultivation, and isolation of marine microbes either from the surrounding seawater or from the tissue of host organism through the application of molecular

methods and development of special culture media required for the growth of marine microbes can play a pivotal role to resolve the major problem of supply of many compounds of pharmacological significance.

1.13 Conclusion

It is vivid and clear from the past and ongoing research that marine microbial flora has an excellent plethora of bioactivities. However, there is still a long way ahead. Researchers have discovered a diversified range of bioactive compounds from marine microbial resources such as antibacterial, antifungal, antiviral, cytotoxic, antimalarial, etc. Nevertheless, extensive scientific investigations are still required to exploit the marine microbe-derived pharmaceuticals to combat against acquired immune deficiency syndrome (AIDS), diabetes, immunosuppression, neurological disorder, cardiac disease, inflammation, aging processes, and numerous life-threatening diseases. Therefore, interdisciplinary collaborative research is required among scientist fraternity of different discipline including pharmaceutical scientists, marine microbiologists, and biotechnologists to discover lead molecules as well as strengthen the marine microbe-based biomedical research. Due to limited scientific knowledge about marine environment, marine microbes have not been given the significant attention as they deserve, and a very limited insight into the capabilities and pharmacological potential of marine microorganisms is available in the literature to date. There is still scope for a higher magnitude of research and investigation to explore the potential of marine microorganisms as producers of novel drugs. Numerous studies have proved that natural products isolated from higher marine organisms like marine invertebrates are generally of microbial origin, fungi, bacteria, or cyanobacteria. Nevertheless, these microorganisms, which are, for example, in a symbiotic relationship with higher organisms, normally cannot be cultivated alone in a pure culture. Their growth depends directly on the activity of their hosts. It is worth giving serious consideration to the exploitation of marine microbial life (symbiotic and free living) and the associated secondary metabolites, aided by genomic analyses, applying metabolic approach, careful method development of culture media and employing combined biomedical and biotechnological efforts, which would lead to the discovery of some novel, lead compounds of a varied degree of bioactivity. In conclusion, the ongoing and future research on isolation of novel bioactive molecules from marine microbial flora will minimize the dependency on terrestrial's resources of drugs, and successful preclinical trials will certainly impart an alternative strategy for the search of novel pharmaceuticals to impart treatment of various diseases and disorders affecting such a large sector of the human population.

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Chapter 2
Antiparasitics from Microorganisms

Nasib Singh, Pooja Devi Gautam, Puja Kumari Chauhan, Tanvir Kaur, Karan Singh, Joginder Singh, and Sumit Singh Dagar

Contents

Abstract Parasitic protozoa and helminth worms are major public health problems in many countries of the world, particularly in the tropical regions. Most of these infections are considered neglected tropical diseases by the World Health

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Organization and are responsible for significant mortality and morbidity in socio-economically under-developed populations. Malaria, leishmaniasis, lymphatic filariasis, schistosomiasis, toxoplasmosis, amoebic dysentery, trypanosomiasis, Chagas' disease and soil-transmitted helminthiasis are devastating diseases.

Here we review antiparasitic drugs and potential drug candidates derived from microorganisms. Avermectins, ivermectin, paromomycin and amphotericin B are microbial metabolites or semi-synthetic derivatives which remain the mainstay of antiparasitic chemotherapy. Among several species of bacteria and fungi, *Streptomyces* spp. are the most prolific producer of antiparasitic agents. Many microbially derived bioactive compounds have progressed in various phases of drug discovery and development phases and are expected to strengthen our current arsenal against parasitic diseases. The untapped microbial resources, i.e. marine, endophytic, endolichenic and extremophilic microbes, could afford novel leads for drug development against parasitic infections. In coming years, microbial bioprospecting, metagenomics, drug repurposing, genome mining, combinatorial chemistry supported by public–private partnerships and multidisciplinary collaborations are poised to accelerate the antiparasitic drug development process. Emphasis is also warranted on developing and utilizing high-throughput screening assays and novel target-based drug discovery approaches.

2.1 Introduction

Protozoan parasites and helminth worms are responsible for significant mortality and morbidity in billions of people annually, majority of which belongs to developing and under-developed countries of the world. Many of these endemic diseases are categorized as neglected tropical diseases by international and national health organizations (Singh et al. 2016; Hotez 2017; WHO 2018). India, Bangladesh, Nepal, Brazil, Iraq, Egypt, Iran and many least-developed African countries are most severely affected by parasitic diseases due to poor socioeconomic status of the populations in tropical and subtropical environments (Molyneux et al. 2017). In addition, USA, Canada, Australia, Japan, China, South-East Asian countries, Latin American countries and several central European countries are also reported for the incidences of parasitic infections. Malaria, visceral leishmaniasis (kala-azar), human African trypanosomiasis or sleeping sickness, Chagas disease and toxoplasmosis are ranked higher in terms of mortality and morbidity data among protozoan infections (Table 2.1). These are caused by *Plasmodium* spp., *Leishmania* spp., *Trypanosoma brucei*, *T. cruzi* and *Toxoplasma gondii*, respectively (Andrews et al. 2014; Khare et al. 2016; WHO 2018). Malaria and visceral leishmaniasis are among the deadliest protozoal diseases in the world. Helminth parasites (parasitic worms) are multicellular metazoan organisms categorized in three groups, viz. cestode (tapeworms), trematode (flukes) and nematode (roundworms). It is estimated that nearly 60% of the world population carries helminth parasites, most of which are

Table 2.1 Main human protozoan parasites, helminth parasitic worms and the resultant diseases or clinical conditions

Parasites	Parasite species	Diseases/clinical conditions
Protozoa (unicellular)	*Plasmodium* spp.	Malaria
	Leishmania spp.	Visceral leishmaniasis (kala-azar)
		Post-kala-azar dermal leishmaniasis
		Cutaneous leishmaniasis
		Mucocutaneous leishmaniasis
	Trypanosoma brucei complex	Human African trypanosomiasis (sleeping sickness)
	Trypanosoma cruzi	Chagas disease
	Entamoeba histolytica	Amoebic dysentery
	Trichomonas vaginalis	Trichomoniasis
	Toxoplasma gondii	Toxoplasmosis
Helminths (multicellular)	*Ascaris lumbricoides* (roundworm)	Ascariasis
	Taenia spp. (tapeworm)	Cysticercosis
	Ancylostoma duodenale (old world hookworm)	Hookworm disease
	Enterobius vermicularis (pinworm)	Enterobiasis
	Hymenolepis nana (dwarf tapeworm)	Hymenolepiasis
	Necator americanus (new world hookworm)	Hookworm disease
	Strongyloides stercoralis (threadworm)	Strongyloidiasis
	Trichuris trichiura (whipworm)	Trichuriasis
	Schistosoma haematobium, S. japonicum, S. mansoni (blood fluke)	Schistosomiasis (bilharzia)
	Wuchereria bancrofti, Brugia malayi	Lymphatic filariasis (elephantiasis)
	Loa loa (eye worm)	Loa loa filariasis
	Onchocerca volvulus	Onchocerciasis (river blindness)

chronic and asymptomatic in nature (Hotez et al. 2008; McSorley and Maizels 2012; Molyneux et al. 2017; WHO 2018; DNDi 2018). Lymphatic filariasis, schistosomiasis, fascioliasis, onchocerciasis and soil-transmitted helminthiases are the most prevalent and serious helminth infections (Molyneux et al. 2017; GAHI 2017). In children of developing countries, parasitic worms especially nematodes cause chronic infections resulting in anaemia, diarrhoea, impaired cognition and stunted growth (Sorobetea et al. 2018).

The relationship among host, vectors, alternate hosts and tissues affected is depicted in Fig. 2.1. The history, biology, transmission, epidemiology, pathogenesis, immunological and chemotherapeutics aspects of protozoa and parasitic worms have been extensively reviewed previously by Demain and Sanchez (2009), Blunt et al. (2013), Nagle et al. (2014), Andrews et al. (2014), Sundar and Chakravarty (2015), Molyneux et al. (2017) and Preston and Gasser (2018). In this review, we will present the progress made in discovery and development of microbial-derived antiprotozoal and anthelmintic drug candidates during the last 10 years.

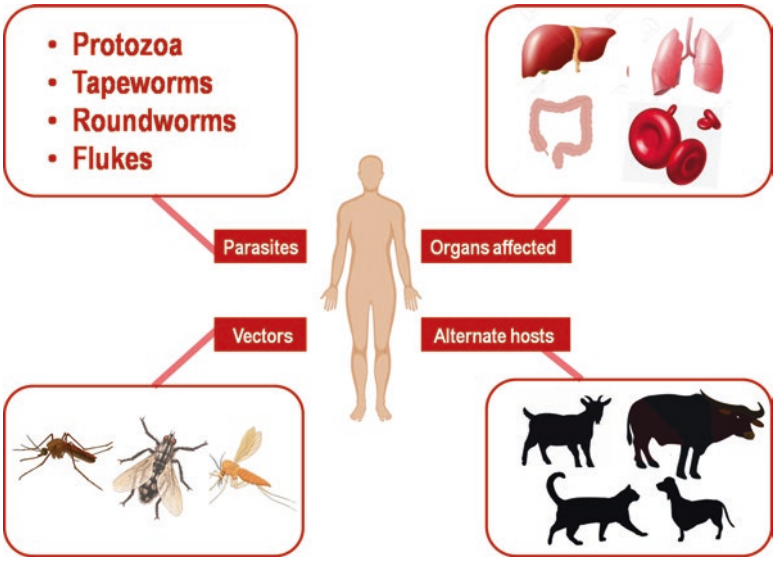


Fig. 2.1 Human parasitic infections caused by parasitic protozoa and parasitic worms (helminths). As depicted, several arthropod vectors (mosquitoes, sand fly, tsetse fly and several others) are involved in the transmission of these diseases. Some parasites require alternate hosts for the completion of their life cycles. These parasites exert detrimental effects on liver, lungs, intestine, blood cells, lymphatic system, eyes, and muscles among several tissues

2.2 Global and National Status of Parasitic Infections

Of the 20 neglected tropical diseases identified by World Health Organization, 3 diseases, viz. Chagas disease, human African trypanosomiasis (sleeping sickness) and leishmaniasis are caused by unicellular protozoan parasites, whereas 8 diseases, viz. dracunculiasis (guinea-worm disease), echinococcosis, food-borne trematodiases, lymphatic filariasis, onchocerciasis (river blindness), schistosomiasis, soil-transmitted helminthiases and taeniasis/cysticercosis are due to metazoan helminth parasites (WHO 2018). Soil-transmitted helminths (STHs) are mainly represented by *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), *Necator americanus* and *Ancylostoma duodenale* (hookworms; WHO 2018; Silver et al. 2018). In particular, hookworm disease is the major public health problem in millions of people in India, South-East Asia, sub-Saharan Africa, Latin America and Caribbean countries (Stutzer et al. 2018; Silver et al. 2018). As per World Health Organization estimates, nearly 241 million children belonging to age group 1–14 years in India are at risk of parasitic worms in infections. India accounts for 222 million, 102 million and 67 million cases of ascariasis, hookworm disease and trichuriasis, respectively in 2016 (GBD 2016).

Parasitic diseases are a major public health problem in several states of India. Among these, vector-borne diseases such as malaria, visceral leishmaniasis (kala-

azar) and lymphatic filariasis are most devastating, especially in rural areas due to poor socioeconomic status of the people. Visceral leishmaniasis is caused by obligate unicellular protozoan parasite *Leishmania donovani* and transmitted by *Phlebotomus argentipes* (sand fly). This disease is endemic in Bihar, Jharkhand, West Bengal and Uttar Pradesh states with 90% of all cases are reported from Bihar alone (Dube et al. 2009; Sundar and Chakravarty 2010, 2015). A total of 13,530 cases of visceral leishmaniasis were reported from India in 2016 which is about 45% of total global occurrence (GBD 2016; Hotez and Damania 2018). There were 1.09 million cases of malaria reported in India in year 2016 (NVBDCP 2018). Lymphatic filariasis is endemic in 256 districts of 21 states/union territories of India (NVBDCP 2018).

2.3 Chemotherapeutics Against Parasitic Infections

Unfortunately, no vaccines are available for human use against any of protozoal and helminth diseases. Moreover, parasitic diseases received underinvestment and little priority in drug development programmes funded by corporate groups and pharmaceutical industries. This is evident from the fact that from 2000 to 2011, only 4% new therapeutic products were indicated for neglected tropical diseases out of total 850 approved products (DNDi 2018). The main drugs currently used against protozoal parasites are sodium stibogluconate, paromomycin, miltefosine, amphotericin B, metronidazole, artemisinin, mefloquine, nifurtimox, benznidazole, melarsoprol and eflornithine (Nagle et al. 2014; Sundar and Chakravarty 2015; Field et al. 2017). In case of helminth infections, avermectins, ivermectin, benzimidazoles, albendazole, mebendazole, levamisole, pyrantel pamoate, diethylcarbamazine and praziquantel are the mainstay of chemotherapeutic interventions (Hotez et al. 2008; Campbell 2012; GAHI 2017; DNDi 2018). The mechanism of action of these chemotherapeutics is highly parasite-specific in nature (Fig. 2.2). Unfortunately, many of these drugs suffer from severe toxicity, inferior efficacy and emergence of drug resistance in the target parasites. Therefore, the discovery and development of new drugs with higher efficacies remain a constant endeavour to ensure health for all.

2.3.1 Antiparasitic Drugs Developed from Microbial Metabolites

Microorganisms have been and will remain the most promising, rich, credible and infinite source of healthcare products for the mankind. Satoshi Omura, Nobel laureate, has rightly called microbial metabolites as “splendid gifts” from microorganisms. Nearly 50% of all antibiotics are obtained from different species of filamentous actinobacterium *Streptomyces* (Manivasagan et al. 2014). Historical uses of yeasts,

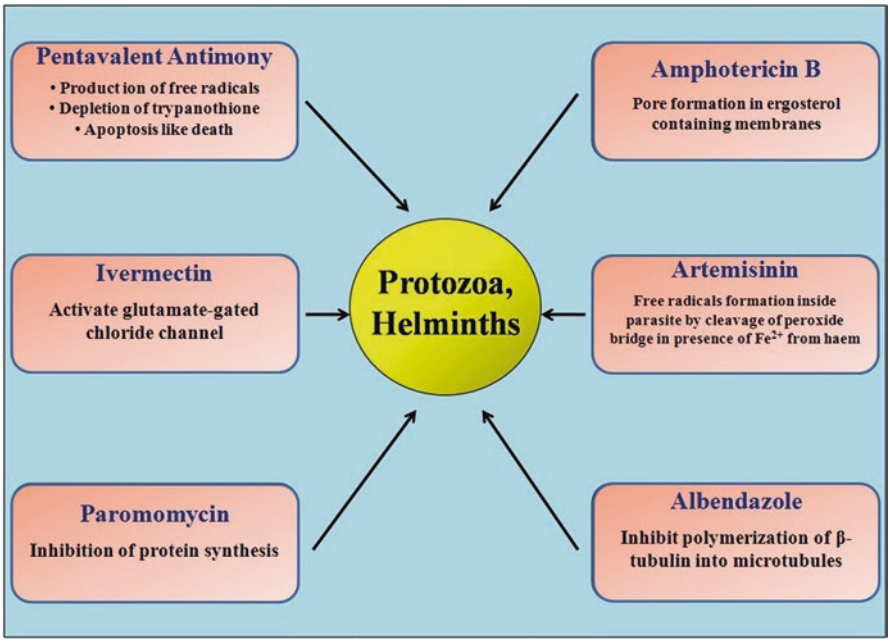


Fig. 2.2 The mechanism of action of some clinically important antiparasitic drugs derived from microbial sources, medicinal plants and by chemical synthesis

moulds, mushrooms, algae, bacteria and diatoms for medicinal use are available in the ancient literature. Microorganisms represent a highly diverse, unique and promising natural resource for antiparasitic drug discovery and development due to their unparallel capabilities to synthesize an array of novel bioactive metabolites (primary and secondary), amenability towards genetic manipulation, feasibility of large-scale cultivation at industrial scale and simpler downstream processing. Discovery of fungal metabolite penicillin in 1929 from mould *Penicillium notatum (P. chrysogenum)* by Sir Alexander Fleming is considered as the most significant application of microorganisms towards human health. Since then, thousands of bioactive metabolites have been isolated and developed as clinically approved drugs by public institutions and pharmaceutical industries. More than 40,000 metabolites have been isolated from bacteria and filamentous fungi (Rateb et al. 2013). Microbial metabolites showing antimicrobial (antibacterial, antifungal, antimalarial, antileishmanial, antiviral), anthelmintic, anti-inflammatory and other bioactivities are extensively reported in literature and reviewed in detail elsewhere (Demain and Sanchez 2009; Newman and Cragg 2016). Intensive search of novel microbial metabolites is made essential due to several drawbacks of currently available drugs *viz*. high cost, severe toxicity and emergence of drug resistance.

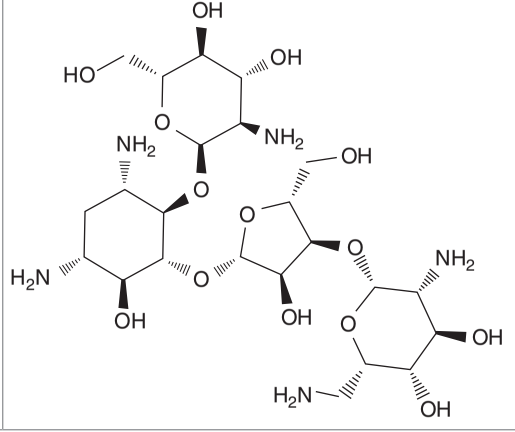
Microorganisms were also investigated as source of anthelmintics and antiprotozoan drugs. Avermectins were isolated from soil filamentous bacterium *Streptomyces avermectinius* in 1950–1980s (Burg et al. 1979; Omura 2008; Campbell 2012).

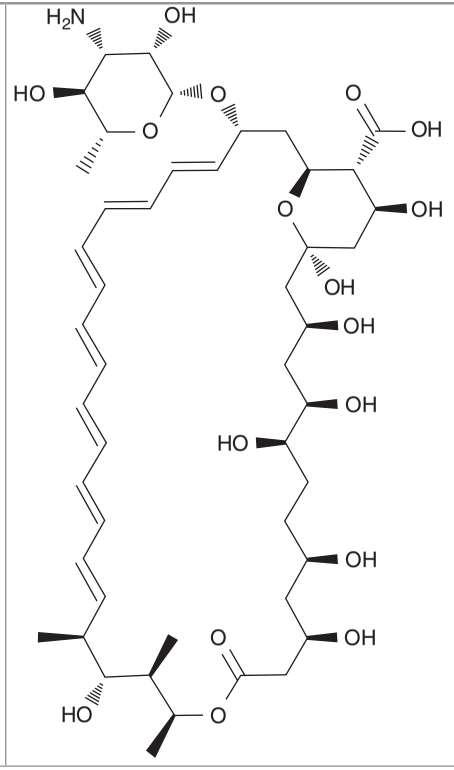
The avermectins are 16-membered macrocyclic lactone derivatives having potent anthelmintic (especially for lymphatic filariasis and river blindness) and insecticidal properties. Ivermectin, abamectin, selamectin and doramectin are some important synthetic derivatives of avermectins (Demain and Sanchez 2009; Campbell 2012). These remain the mainstay of treatment options available against the parasitic helminths infections of human and animals (Table 2.2). In 2015, William C. Campbell and Satoshi Ōmura were awarded Nobel Prize in Physiology or Medicine for the discovery of avermectin. Paromomycin, amphotericin B (both antileishmanial), clindamycin and spiramycin are other clinically approved and widely accepted drugs derived from the microorganisms. In the microbial world, filamentous soil actinobacteria, soil fungi, algae and mushrooms are known as the most efficient producers of secondary metabolites having immense pharmaceutical importance. In the recent times, the focus has also shifted to untapped microbial resources such as marine actinobacteria, microalgae, marine fungi, diatoms, endophytic bacteria and fungi, lichen fungi, symbiotic microbes, invertebrate-dwelling microbes, etc. for discovering novel chemical skeletons suitable for drug development against parasitic diseases (Newman and Cragg 2016; Tchokouaha Yamthe et al. 2017). More than 20,000 bioactive compounds have been isolated and evaluated from marine organisms in the last five decades (Agrawal et al. 2017).

2.3.2 *Discovery of New Drug Candidates from Microbial Resources*

Microorganisms are blessed with unprecedented metabolic capabilities which are evident from highly diverse array of compounds synthesized by them. Many of these compounds are resultant of secondary metabolism and exhibit potent activities against different life stages of protozoa and helminth parasites. Inhibition of *Leishmania* parasites was exhibited by preussomerin, palmarumycin, epiepoformin, kojic acid, purpureone, hypocrellin A, terrenolide S, fucoidan, gallinamide A, dihydroauroglaucin (Table 2.3). Fucoidan, a polysaccharide from brown algae, was highly effective against *L. donovani* infection in mice (Kar et al. 2011). Potent antiplasmodial activity (87% parasite inhibition in mice) was observed with violacein, a pigment derived from *Chromobacterium violaceum* (Lopes et al. 2009). Similarly, jogyamycin, cytochalasin, trichothecene, metacycloprodigiosin and salinosporamide A exhibited high to moderate inhibitory activity against different *Plasmodium* species. Trypanosomacidal activity was reported in several studies as mentioned in Table 2.3. Studies have found excellent potential in jogyamycin, sagamilactam, cytochalasin H, aureothin, destomycin and several other compounds of microbial origin against *Trypanosoma* parasites. However, most of these reports represent only in vitro antiparasitic activities of microbe-derived compounds. This necessitates the need to put impetus on evaluation of active entities in biologically relevant experimental models to establish efficacy levels and further development of lead molecules in drug discovery and development pipeline.

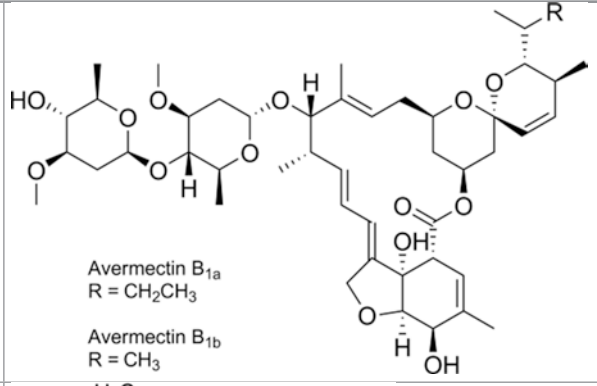
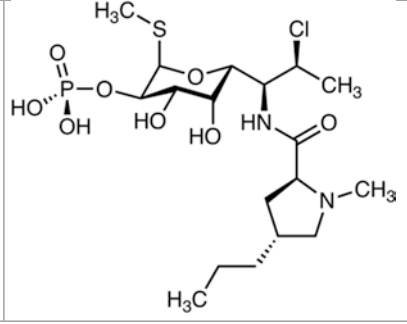
Table 2.2 Microbial metabolites used for the treatment of protozoal and helminth diseases of humans and animals

Chemical name	Source organism	Effective against	Chemical class	Structure	References
Paromomycin	*Streptomyces krestomuceticus*	*Leishmania* spp.	Aminoglycoside–aminocyclitol antibiotic		Sundar et al. (2007) and Ben Salah et al. (2013)

Amphotericin B	*Streptomyces nodosus*	*Leishmania* spp.	Polyene antibiotic		Sundar and Chakravarty (2010)

(continued)

Table 2.2 (continued)

Chemical name	Source organism	Effective against	Chemical class	Structure	References
Avermectins & Ivermectin (semi-synthetic)	*Streptomyces avermectinius*	Helminth parasites	Macrocyclic lactones		Burg et al. (1979), Omura (2008), and Campbell (2012)
Clindamycin	*Streptomyces lincolnensis*	Malaria parasite	Semisynthetic lincosamides		Andrews et al. (2014)

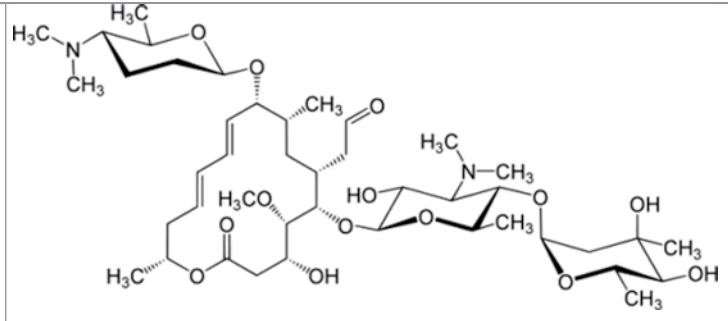
Spiramycin	*Streptomyces ambofaciens*	*Toxoplasma*	Macrolide antibiotic		Robert-Gangneux and Darde (2012)

Table 2.3 Microbial origin antiparasitic compounds under laboratory evaluation and/or in preclinical drug development phases

Metabolites/products	Microbial source	Nature of organism	Activity against	Antiparasitic activity	Reference
Preussomerin, Palmarumycin	*Edenia* sp.	Endophytic fungus	*Leishmania donovani*	IC_{50} 0.12–8.40 μM	Martínez-Luis et al. (2008)
Violacein	*Chromobacterium violaceum*	Bacterium	*Plasmodium chabaudi*	87% inhibition at 7.5 mg/kg in mice	Lopes et al. (2009)
Jogyamycin	*Streptomyces* sp.	Bacterium	*Plasmodium falciparum, Trypanosoma brucei brucei*	IC_{50} 1.5–12.3 nM	Iwatsuki et al. (2012)
Cytochalasin, Trichothecene	*Diaporthe* sp., *Verticillium* sp., *Xylaria* sp.	Endophytic fungi	*P. falciparum*	IC_{50}<20 nM	Calcul et al. (2013)
Epiepoformin; 2,5-dihydroxybenzaldehyde	*Geosmithia langdonii*	Filamentous fungus	*L. donovani*	IC_{50} 3–10 μM	Malak et al. (2014)
Nafuredin	*Aspergillus niger* FT-0554	Filamentous fungus	*Haemonchus contortus*	90% egg reduction at 2 mg/kg	Omura and Shiomi (2007)
Cry protein Cry5B	*Bacillus thuringiensis*	Bacterium	*Ancylostoma ceylanicum*	90% elimination at 100 mg/kg in hamster model	Conlan et al. (2012)
Cry protein Cry5B	*B. thuringiensis*	Bacterium	*Ascaris suum*	ED_{50} 0.094 μg/mL	Urban et al. (2013)
Kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone)	*Aspergillus* spp.	Filamentous fungus	*L. amazonensis*	IC_{50} 27.9 μg/mL	Rodrigues et al. (2014)
Hypocrellin A	*Hypocrella bambusae*	Parasitic fungus	*L. donovani*	IC_{50} 0.27 μg/ml	Ma et al. (2004)
Purpureone (ergochrome)	*Purpureocillium lilacinum*	Endophytic fungus	*L. donovani*	IC_{50} 0.17 μg/ml	Lenta et al. (2016)

10-acetyl trichoderonic acid A and 6′-acetoxy-piliformic acid	*Nectria pseudotrichia*	Endophytic fungus	*L. braziliensis*	IC_{50} 21–28 μM	Cota et al. (2018)
(22*E*,24*R*)-stigmasta-5,7,22-trien-3-*β*-ol and Terrenolide S	*Aspergillus terreus*	Endophytic fungus	*L. donovani*	IC_{50} 11–27 μM	Elkhayat et al. (2016)
Dragonamide E	*Lyngbya majuscula*	Cyanobacterium	*L. donovani*	IC_{50} 5 μM	Balunas et al. (2009)
Crude and methanolic extract	*Lyngbya aestuarii and Aphanothece bullosa*	Cyanobacterium	*L. donovani*	IC_{50} 24–26 mg/ml	Kumar et al. (2013)
Dihydroauroglaucin and Auroglaucin	*Eurotium repens*	Fungus	*P. falciparum*	IC_{50} 1–3 μg/ml	Gao et al. (2012)
Dihydroauroglaucin and Auroglaucin	*Eurotium repens*	Fungus	*L. donovani*	IC_{50} 6–8 μg/ml	Gao et al. (2012)
Atomaric acid (meroditerpene)	*Stypopodium zonale*	Brown alga	*L. amazonensis*	IC_{50} 20 μM	Soares et al. (2016)
Sagamilactam	*Actinomadura* sp.	Bacterium	*Trypanosoma*	IC_{50} 0.25 μM	Kimura et al. (2016)
Prodigiosin	*Serratia marcescens*	Bacterium	*Radopholus similis, Meloidogyne javanica*	LC_{50} 79–83 μg/ml	Rahul et al. (2014)
Metacycloprodigiosin	*Streptomyces spectabilis*	Bacterium	*P. falciparum* K1	IC_{50} 0.005 μg/ml	Isaka et al. (2002)
18-des-hydroxy Cytochalasin H	*Diaporthe phaseolorum*	Endophytic fungus	*Trypanosoma cruzi, Schistosoma mansoni, L. amazonenses*	50–80% inhibition, IC_{50} 9.2 μg/ml	Brissow et al. (2017)

(continued)

Table 2.3 (continued)

Metabolites/products	Microbial source	Nature of organism	Activity against	Antiparasitic activity	Reference
Butyrolactone I	*Aspergillus terreus* F7	Endophytic fungus	*Schistosoma mansoni*	100% inhibition aqt 235.6 μM	da Silva et al. (2017)
Gallinamide A	*Schizothrix* sp.	Cyanobacterium	*L. donovani*	IC_{50} 8.4 μM	Linington et al. (2009)
Viridamide A	*Oscillatoria nigro-viridis*	Cyanobacterium	*P. falciparum, L. mexicana, T. cruzi*	IC_{50} 5.8 μM	Simmons et al. (2008)
				IC_{50} 1.1 μM	
				IC_{50} 1.5 μM	
Valinomycin	*Streptomyces* sp.	Marine bacterium	*T. brucei, L. major*	IC_{50} 0.0032 μM	Pimentel-Elardo et al. (2010)
				IC_{50} 1.1 μM	
Diketopiperazines	*A. fumigatus, Nectria inventa*	Marine fungi	*T. brucei*	IC_{50} 0.002–40 μM	Watts et al. (2010)
Fucoidan (polysaccharide)	*Laminaria japonica, Undaria pinnatifida*	Brown algae	*L. donovani*	100% inhibition at 200 mg/kg/day dose in mouse model	Kar et al. (2011)
Aureothin, cellocidin, destomycin A, echinomycin, hedamycin, irumamycin, LL-Z 1272β, venturicidin A, O-methylnanaomycin A and virustomycin A	Na	Soil microorganisms	*T. brucei brucei, T. b. rhodesiense*	IC_{50} 0.45–300 ng	Otoguro et al. (2008)
Cyclooctadepsipeptide PF1022A and emodepside	*Rosellinia* sp. PF1022	Filamentous fungus	Nematodes	na	Krücken et al. (2012)
Echinomycin A, Tirandamycin A	*Streptomyces* sp.	Actinobacterium	*Entamoeba histolytica*	EIC_{50} 44–46. μM	Espinosa et al. (2012)
Salinosporamide A	*Salinispora tropica*	Marine actinobacterium	*P. falciparum*	IC_{50} 40 nM	Prudhomme et al. (2008)

IC inhibitory concentration, *EC* effective dose

Microbial metabolites can also be of significant importance with regard to anthelmintic activities against cestodes, trematodes and nematodes. However, the studies which evaluated their potential are limited due to complex life cycle, multiple life stages and multicellular nature of these parasites. A compound nafuredin isolated from fungus *Aspergillus niger* was found effective against *Haemonchus contortus* resulting in 90% egg reduction at 2 mg/kg dose (Omura and Shiomi 2007). Another compound, Cry5B from *Bacillus thuringiensis* showed inhibition of hookworm (Conlan et al. 2012) and *Ascaris suum* (Urban et al. 2013). Similarly, cyclooctadepsipeptide and emodepside produced by fungus *Rosellinia* sp. were inhibitory to roundworms (Krücken et al. 2012). Other microbial metabolites investigated for their antiparasitic efficacy in vitro and in vivo models are mentioned in Table 2.3.

2.3.3 New Drug Targets and Screening Assays/Technologies

Drug screening assays are well established for protozoan parasites due to simpler life cycle, lesser life stages and easy growth manipulations. Traditional assays based on cell viability and new reporter gene-based approaches are most widely used for screening of microbial metabolites, plant products and synthetic compounds against *Leishmania, Plasmodium, Trypanosoma, Toxoplasma, Entamoeba* and *Giardia* species. However, in order to expedite the screening process, high-throughput methods are being developed and evaluated by several research groups (Table 2.4). The focus of antiparasitic drug discovery efforts has also been shifted to discover and asses novel drug targets in these parasites.

2.4 Conclusion

The existing chemotherapeutics against protozoal and helminth infections have toxic side effects, inferior efficacy and not affordable to the poor populations of the developing countries. Drug development against parasitic diseases has received lesser attention from the corporate sector and pharmaceutical organizations. Moreover, the number of lead molecules which are in drug development and clinical trial phases are limited. As most of the parasitic diseases involve multiple hosts, reservoirs and invertebrate vectors, the drug candidates should be able to target the multiple life stages of the parasites. Drug development process is also slow due to stringent regulatory requirements and financial constraints. Microorganisms are highly amenable to genetic manipulation, epigenetic modifications, switching of biosynthetic genes and “one strain many compounds” approach in order to obtain a diverse chemical diversity of primary and secondary metabolites which can accelerate the drug discovery process. In addition, cocultivation of microbial species in culture media and single cell culture strategies could be the key approaches towards

Table 2.4 Drug screening assays, high-throughput technologies and drug targets for antiparasitic drug discovery and development

Antiparasitic compound screening assays and technologies
MTT, XTT and other viability based in vitro assays
Reporter gene-based assays: Green fluorescent protein (GFP), enhanced GFP, red fluorescent protein, yellow fluorescent protein, luciferase, β-lactamase, β-galactosidase
Fluorescence-based high-throughput screening assays
High-throughput flow cytometry based assays
Cell culture- based in vitro and semi-in vivo assays
Automated live-cell phase contrast microscopic assay
Fragment-based phenotypic lead discovery
Caenorhabditis elegans and zebra fish-based assays
INVAPP/Paragon system
Worm assay system
The Worminator assay system
Drug targets in parasites
Replication and translation processes
Tryparedoxin
Gene expression machinery
Apicoplast
Cytoskeleton proteins
Cell membrane proteins
Mitochondrial membrane
Proteasomes
Kinome-based targets
Cell cycle proteins
Secretory proteins and secretory pathways
Cytochrome bc1
Enzymes of intermediary metabolism
Phosphofructokinase
Phosphoglycerate mutase
Phosphoglycerate kinase
Calcium-dependent protein kinases
Cysteine proteases
Dihydroorotate dehydrogenases
Glucose phosphate isomerase
Thioredoxin reductase

Adapted from Dube et al. (2009), Singh and Dube (2015), Muller and Hemphill (2016), and Partridge et al. (2018)
MTT 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, *XTT* 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt, *INVAPP* INVertebrate Automated Phenotyping Platform

achieving the future goals of effective antiparasitic drugs. Cost-effective, reliable, biologically relevant and automated high-throughput screening systems are necessary for rapid advancement in the search and development of novel antiprotozoal and anthelmintic drug candidates. The endolichenic fungi, marine microbes, human gut microbiota, extremophilic microbes, archaea, endophytic microbes and anaerobic microbes are underexplored groups which can prove a diverse, promising and sustainable resource for future drug discovery and development against parasitic diseases. With increased emphasis on microbial metabolites discovery amalgamated with recombinant DNA technology, improved screening technologies, metabolic engineering, combinatorial chemistry, genome mining and drug repurposing, better and cheaper drugs against parasitic diseases are now in closer sight than earlier.

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Chapter 3
Bioactive Compounds from *Nocardia*: Biosynthesis and Production

Dipesh Dhakal, Anil Shrestha, Nguyen Huy Thuan, Vijay Rayamajhi, Ravindra Mishra, Rubin Thapa Magar, and Jae Kyung Sohng

Contents

Abstract The infection caused by drug-resistant pathogens, such as cancer and cardiovascular diseases, are major causes of mortality in the world. Diverse bioactive molecules isolated from microbial or plant sources are used for the ailment of such infections or disease conditions. Since a few years, a group of microorganisms named "rare actinomycetes" are more frequently unveiled as excellent sources of bioactive molecules. *Nocardia* spp. are important members of "rare actinobacteria" and characterized as the prominent microbial source for the isolation of diverse bioactive molecules with pharmaceutical values. *Nocardia* spp. are catalase positive, aerobic, and nonmotile Gram-positive filamentous bacteria. They contain high guanine plus cytosine (G+C) content in their genome. *Nocardia* have been

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studied for a long time, primarily for strain characterization and taxonomic classification of new isolates. Most species are reported as unusual causes of diverse clinical diseases in both humans and animals. Hence, most species are clinical isolates, whereas only few species have been isolated from common natural habitats, such as soil and water.

Recently, novel strains belonging to rare actinobacteria have been explored for isolating and characterizing diverse bioactive metabolites. Hence, there is emerging interest in bioactive molecules from such rare actinobacteria, as *Nocardia* spp. Here, we present bioactive molecules derived from *Nocardia* species and biosynthetic mechanism of few such biomolecules, such as nocardicins, nargenicin, nocardiothiocin, and nocobactin. For commercial use, there is a requirement of large-scale production by microbial fermentation, chemical synthesis, or semisynthetic processes. Hence, advances in genetic engineering of *Nocardia* spp. for enhancing the production titer or structurally diversifying pharmaceutically important biomolecules are also presented. Moreover, with current technological advances, it is feasible to explore the genomic, proteomic, transcriptomic, and metabolic information of most of *Nocardia* spp.

3.1 Introduction

The infection caused by drug-resistant pathogens, cancer, and cardiovascular diseases are top causes of mortality in the world. Cardiovascular disease deaths are the major cause of death, whereas 80% of the deaths occur in low- and middle-income countries (Shi et al. 2016). In terms of global mortality rate, cancer comes next to cardiovascular diseases. In 2012 alone, approximately 14.1 million new cancer cases and 8.2 million cancer deaths occurred worldwide (Ferlay et al. 2015). In addition, infections due to pathogens as bacteria, fungi, parasites, virus, etc. are noted causes of mortality in both developed and undeveloped countries. Particularly in the case of the inhabitants of low-income countries, there is high prevalence of HIV/AIDS, tuberculosis, pneumonia, malaria, and other pathogenic infections (Dye 2014). Natural products obtained from plants or microorganism, native or structurally modified, and natural or synthetic are utilized for treatment of such infections or disease conditions (Dhakal and Sohng 2017; Newman and Cragg 2016).

Among all the microorganisms, actinobacteria are major sources of biomolecules with pharmaceutical importance, such as antibacterials, antiviral agents, anticancer agents, anti-parasite, immunosuppressant, etc. (Genilloud 2017: Demain and Vaishnav 2011; Lam 2007). Actinobacteria are Gram-positive filamentous bacteria possessing a high G+C content in their genome (Dhakal et al. 2017). The combination of tip extension and branching of hyphae is most common physiological phenomenon for their growth. The name "actinobacteria" is derived from Greek, whereas *aktis* or *aktin* stands for ray and *mukes* stands for fungi, because they produce mycelium while many of them reproduce by sporulation, similar to filamentous fungi.

However, like all bacterium the actinobacteria possess prokaryotic nucleoid and peptidoglycan cell wall, which differentiate them significantly from fungi (Barka et al. 2016). Among all actinobacteria, *Nocardia* spp. possess numerous distinct morphological features including presence of fragmenting hyphal forms (Shivlata and Satyanarayana 2015). They are catalase positive, aerobic, and nonmotile, with characteristic presence of short-chain spores, cyclic menaquinones in bacterial cell membranes, and distinct distribution of mycolic acids in their cell walls (Luo et al. 2014; Goodfellow 1992; Goodfellow et al. 2012). They produce primary mycelia usually fragmenting into rod- to coccoid-shaped fragmented hyphal forms. Upon growth on standard agar media, *Nocardia* spp. form diverse morphologies as smooth to rough, clear margined to irregular colonies and exhibit diverse range of colors (Luo et al. 2014; Shivlata and Satyanarayana 2015).

Nocardia species (Fig. 3.1) have been studied from long past, but most of the studies are focused on characterization and taxonomic classification of new isolates. They are frequently associated as the causative agent for wide spectrum of clinical diseases in humans and animals, infecting cattle, horses, dogs, and swine. They are less frequently characterized as environmental isolate from soil and water (Brown-Elliott et al. 2006; McNeil and Brown 1994). The human infection is considered opportunistic, usually occurring through contact through skin or subcutaneous tissues or direct inhalation through contaminated air. The most reported cases include the lungs (pulmonary nocardiosis) or the whole body (systemic nocardiosis). These nocardial infections usually occur in immunocompromised patients; however, there are few reports of infection to immunocompetent individuals (Dhakal and Sohng 2015a; Dhakal et al. 2016b). Due to their predominance in diverse locations, various *Nocardia* spp. have frequently been isolated from clinical samples (Jannat-Khah et al. 2010; Sánchez-Herrera et al. 2012) and a variety of natural habitats, including soil (Kurup and Sandhu 1965; Sohng et al. 2008), sludge (Yamamura et al. 2005), mangrove sediments (Lee et al. 2014), and seawater (El-Gendy et al. 2008).

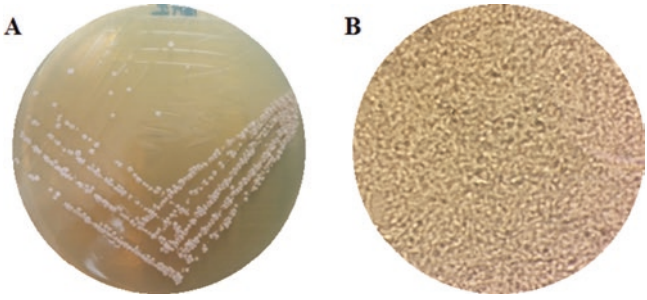

Fig. 3.1 *Nocardia* species: (**a**) growth in plate; (**b**) observation under microscope

3.2 Classification and Taxonomy

Nocardia spp. are taxonomically classified as phylum, *Actinobacteria;* class, *Actinobacteria;* order, *Actinomycetales*; suborder, *Corynebacteriaceae*; family, *Nocardiaceae;* and genus, *Nocardia.* Besides genus *Nocardia,* the family *Nocardiaceae* includes other morphologically or biochemically related genera as *Gordonia*, *Micropolyspora*, *Millisia*, *Rhodococcus*, *Skermania*, *Smaragdicoccus,* and *Williamsia* (http://www.bacterio.net/-classifphyla.html#Nocardia). Among the entire genus, *Actinoplanes, Actinomadura, Micromonospora, Saccharopolyspora, Streptosporangium, Streptoverticillium,* and *Nocardia* are categorized as "rare actinomycetes." These "rare actinomycetes" are more importantly characterized as sources of bioactive molecules (Sharma et al. 2016).

The limitations in classical taxonomy methods have led to a lot of confusion and controversies in the taxonomy of *Nocardia*. The first reported isolation of *Nocardia* strain was from a case of bovine farcy in Guadeloupe in 1888 by Edmond Nocard. The strain was later named as *Nocardia farcinica* (Luo et al., 2014). However, Gordon and Mihm found that these strains exhibit common morphological observations and physiological characterizations. Thus, these strains were not easily distinguished from strains of *Nocardia asteroides*, another well-established species. It was proposed to merge the two species, and *N. asteroides* were chosen as the type species of the genus *Nocardia* (Gordon and Mihm 1957, 1962). Later, Tsukamura showed that the strains *N. asteroides* could be divided into 3 distinct subgroups as *N. farcinica*, *N. asteroides* A, and *N. asteroides* B, based on 57 discrete differentiating characteristics (Tsukamura 1977). It clearly indicated that the previously defined species *N. asteroides* consists of highly heterologous group, whereas independent status can be allocated to *N. farcinica*.

Around the 1990s, molecular identification methods were applied in taxonomy and strain characterizations. This revolutionarily changed the situation of ambiguities of classifications based on colony morphologies or biochemical properties. Hereafter, taxonomic classifications were based on the new methods like restriction endonuclease analysis. Similarly, the taxonomic status of many *Nocardia* spp. was determined by the sequence analysis of different housekeeping genes and 16S rRNA genes (Conville et al. 2000; Roth et al. 2003). The multilocus sequence analysis (Maiden et al. 1998) was applied in the identification of *Nocardia* species, which exhibited a more powerful differentiation between different *Nocardia* species (McTaggart et al. 2010; Tamura et al. 2012). Although multilocus sequence analysis is more cost intensive, the taxonomic studies and species identification of *Nocardia* are more reliable (McTaggart et al. 2010). Recently by utilizing the whole genome analysis, multilocus sequence analysis, and DNA-DNA hybridization, the phylogenetic analysis of 72 validated *Nocardia* spp. was performed. Few of the strains were reclassified based on the analysis (Tamura et al. 2018). Till date, more than 200 species have been identified and reported based on various isolation strategies, biochemical studies, and taxonomical classification methods (National Center for Biotechnology Information/Taxonomy).

3.3 Bioactive Compounds from *Nocardia* spp. and Their Biological Significance

Nocardia species belong to class of "rare actinomycetes," which are an important microbial reservoir for the isolation of bioactive compounds with clinical importance (Dhakal et al. 2015; Dhakal and Sohng 2015a). Particularly pathogenic species as *Nocardia* have attracted increasing interest for several reasons. They are assumed more active biologically and possessing discrete metabolic pathways divergent than nonpathogenic producer strains. The pathogenic behavior is believed to be associated with competition mechanisms with their hosts, which in turn lead to the generation of diverse molecules for their survival and maintenance (Mikami 2007). Hence, numerous antibacterial, antivirus, antifungal, immunosuppressive, and cytotoxic agents were isolated from different species of *Nocardia,* and their bioactivities were evaluated and reported (Dhakal and Sohng 2015a). They include compounds with diverse chemical structures as beta-lactams, polyketides, terpenoids, siderophores, etc. The major bioactive chemical entities isolated from diverse *Nocardia* spp. during the past decades, along with their most promising biological importance, are presented in Table 3.1.

3.3.1 Nocardicins

Nocardicins (Fig. 3.2) were isolated from the fermentation broth of *Nocardia uniformis* subsp. *tsuyamanensis*. They exhibited moderate activity against numerous Gram-negative bacteria (Akoi et al. 1976). The structural elucidation confirmed presence of monocyclic beta-lactam ring along with presence of p-hydroxyphenylglycine and oxime units, which are uncommon chemical structures. Among all the molecules, structurally similar major compounds are named as nocardicin A and B (Hashimoto et al. 1976). Unlike most of the bicyclic beta-lactams, nocardicin A shows resistance to beta-lactamase, which may be due to presence of more stable monocyclic beta-lactam ring in its structure (Kojo et al. 1988). It may be the reason for remarkably higher activity of nocardicin A in vivo than in vitro experimental models (Kamiya 1977).

The biological importance of the compound generated interest on study of its biosynthetic mechanism. The biosynthetic origins of nocardicin A were established to be L-serine and two units of non-proteinogenic amino acid, p-hydroxyphenylglycine by precursor incorporation studies (Townsend and Salituro 1984; Hosoda et al. 1977). The homoseryl side chains were reported to be derived from L-methionine (Townsend and Brown 1981, 1983). Subsequently, it was observed that amine oxidation led to formation of the oxime and the inversion of configuration at the seryl beta-carbon results to the stereochemical course of beta-lactam ring formation (Townsend et al. 1983; Townsend and Brown 1982).

Table 3.1 Major bioactive molecules isolated from *Nocardia* spp

Compound	Compound type	Source	Main biological activity	References
Nocardicin A-B	Beta-lactam	*N. uniformis* ATCC 21806	Antibacterial	Akoi et al. (1976) and Hashimoto et al. (1976)
Nargenicin A1	Macrolide	*N. argentinensis* 31306, *Nocardia* sp. CS682	Antibacterial	Celmer et al. (1980) and Sohng et al. (2008)
Tubelactomycin A	Macrolide	*N. vinacea* MK703-102F1	Antimycobacterial	Igarashi et al. (2000)
Brasilinolide A	Macrolide	*N. brasiliensis* IFM 0406	Immunosuppressive	Shigemori et al. (1996) and Tanaka et al. (1997b)
Brasilinolide B	Macrolide	*N. brasiliensis* IFM 0466	Antifungal	Mikami et al. (2000)
Brasilicardin A-D	Terpenoid	*N. brasiliensis* IFM 0406	Immunosuppressive, cytotoxic	Shigemori et al. (1998) and Komatsu et al. (2004)
Nocardicycline A-B	Anthracycline	*N. pseudobrasiliensis* IFM 0624	Antibacterial	Tanaka et al. (1997a)
Brasiliquinone A-D	Benz[α] anthraquinone	*N. brasiliensis* IFM 0667	Cytotoxic	Tsuda et al. (1996, 1997) and Nemoto et al. (1997)
Nocarasin A-C	Benzenoid	*N. brasiliensis* IFM 0667	Cytotoxic, Antibacterial	Tsuda et al. (1996, 1997)
Nocathiacin I–III	Thiazolyl peptide	*Nocardia* sp. ATCC 202099	Antibacterial	Leet et al. (2003) and Li et al. (2003)
Transvalencin A	Thiazolidine	*N. transvalensis* IFM 10065	Antifungal	Hoshino et al. (2004)
Nocardithiocin	Thiopeptide	*N. pseudobrasiliensis* IFM 0761,	Antimycobacterial	Sakai et al. (2015)
		N. pseudobrasiliensis IFM 0757		Mukai et al.

(continued)

Table 3.1 (continued)

Compound	Compound type	Source	Main biological activity	References
Brasilidine A	Indole alkaloid	*N. brasiliensis* IFM 0089	Cytotoxic	Kobayashi et al. (1997)
Nabscessins A-B	Aminocyclitol	*Nocardia abscessus* IFM 10029	Antifungal	Hara et al. (2017)
Diketopiperazines	Diketopiperazines	*Nocardia ignorata* DP94	Cytotoxic	Noël et al. (2017)
SO-75R1	Anthracycline	*N. brasiliensis* IFM 0075	Antibacterial	Maeda et al. (1992)
HS-6	Macrolide	*N. otitidiscaviarum* IFM 0273	Cytotoxic	Mikami et al. (1990)
Nocobactin NA	Siderophore	*N. farcinica* ATCC 3318	Cytotoxic	Sakagami et al. (2005)
BE-32030 A-E	Siderophore	*Nocardia* sp. A32030	Antitumor	Tsukamoto et al. (1997)
Formobactin	Siderophore	*Nocardia* sp. ND20	Free radical scavenging	Murakami et al. (1996)
Amamistatin A-B	Siderophore	*N. asteroides* IFM 0959	Antitumor	Suenaga et al. (1999)
Asterobactin	Siderophore	*N. asteroides* IFM 0959	Antitumor	Nemoto et al. (2002)
Brasilibactin A	Siderophore	*N. brasiliensis* IFM 0995	Cytotoxic	Tsuda et al. (2005)
Nocardimicin A-F	Siderophore	*Nocardia* sp. TP-A0674	Muscarine M3 receptor binding	Ikeda et al. (2005)
Nocardimicin G-I	Siderophore	*N. nova* JCM 6044	Muscarine M3 receptor binding	Ikeda et al. (2005)
Transvalencin Z	Siderophore-like	*N. transvalensis* IFM 10065	Antibacterial	Mukai et al. (2006)
Aamycin	–	*Nocardia* sp. ALAA2000	Antibacterial	El-Gendy et al. (2008)
Glucolipsin B	–	*N. vaccinia* WC65712	Glucokinase activator	Qian-Cutrone et al. (1999)
Intervenolin	–	*Nocardia* sp. ML96-86F2	Anti-*Helicobacter pylori* activity	Kawada et al. (2013)

Gunsior et al. (2004) determined the biosynthetic gene cluster and characterized the functional role of several enzymes involved in biosynthesis of nocardicins (Gunsior et al. 2004). There were several genes in biosynthetic gene cluster of nocardicins as shown in Table 3.2. Different enzymes had specific role in specific biosynthetic steps. Primarily, a nonribosomal peptide synthetase is responsible for assembly of two units of L-pHPG, and one unit of serine to yield a D-, L-, D-tripeptide, or possibly nocardicin G by coercive action of five domains of NocA and NocB (Davidsen and Townsend 2012). This biosynthetic step was characterized

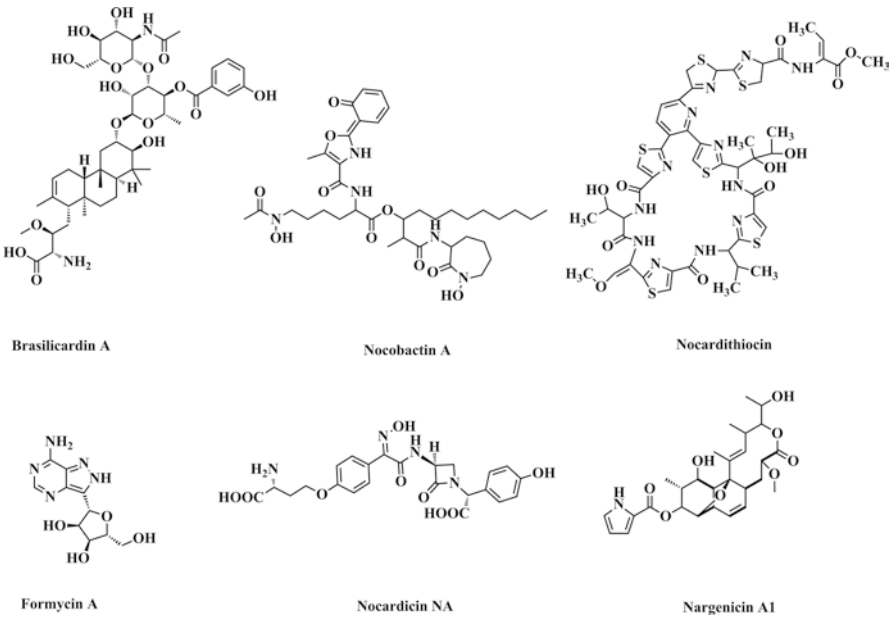

Fig. 3.2 Bioactive natural compounds produced by different *Nocardia* spp. Brasilicardin A, an immunosuppressant isolated from *Nocardia brasiliensis*; nocobactin A, a siderophore molecule isolated from *Nocardia farcinica*; nocardiothiocin, a thiopeptide antibiotics from *Nocardia pseudobrasiliensis*; formycin A, an unusual metabolite from *Nocardia interforma*; nocardicin A, a monocyclic beta-lactam from *Nocardia uniformis* subsp. *tsuyamenensis*; nargenicin A1, a macrolide antibiotics isolated from *Nocardia* sp. CS682

by in vivo gene inactivation studies, whereas further enzymatic in vitro reaction studies showed that Nat catalyzes addition of the homoseryl side chain. This step is crucial for transfer of 3-amino-3-carboxypropyl group from S-adenosyl-L- methionine to the diverse substrates such as nocardicin E, F, and G, whereas nocardicin G is the most preferred substrate (Reeve et al. 1998). Further, in vivo gene inactivation studies revealed that NocL was first example of a prokaryotic cytochrome P450 catalyzing N-oxygenation, particularly catalyzing the formation of oxime of nocardicin A (Kelly and Townsend 2002). The in vitro enzymatic assays using spinach ferredoxin and spinach ferredoxin-reductase established that nocardin C was most suitable substrate for NocL; meanwhile, the conversion of nocardicin G to E or F was not observed (Kelly and Townsend 2005). Hence, the biosynthetic steps for biological production of nocardicin A1 were assigned in order as shown in Fig. 3.3.

Table 3.2 Biosynthetic gene cluster of nocardicin NA

Gene	Size (aa)	Proposed function
nocE	1414	Unknown
nocD	185	Resistance
nat	301	3-Amino-3-carboxypropyl transferase
nocB	1925	(Nonribosomal peptide synthetase) Module 4,5
nocA	3692	(Nonribosomal peptide synthetase) Module 1,2,3
nocF	345	p-Hydroxymandelate synthase
nocG	431	p-Hydroxyphenylglycine
nocH	408	Transport
nocI	74	Unknown
nocJ	327	Unknown
nocK	344	Unknown
nocL	398	Oxime formation
nocR	582	Regulation
nocN	376	p-Hydroxymandelate oxidase

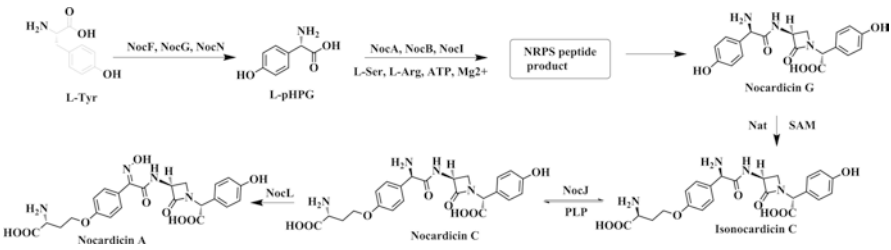

Fig. 3.3 Biosynthetic mechanisms of nocardicin A (Adapted and redrawn from Gaudelli and Townsend 2014). L-tyrosine is first converted to L-pHPG, which in turn condenses with L-Ser and L-Arg to form nonribosomal peptide synthase, peptide product, and eventually nocardicin G. The addition of the homoseryl side chain is catalyzed by *nat,* and subsequent N-oxygenation by NocL leads to formation of nocardicin A. *L-pHPG* L-p-hydroxyphenylglycine, *PLP* pyridoxal 5′-phosphate

3.3.2 Nocobactin

Metal ions such as iron are essential for growth and metabolism in different microorganisms. Generally, under low-iron conditions, the microorganisms usually produce organic compounds with low molecular masses called as "siderophores." Their major function is chelating the ferric ion and making them available for the microbial uses in different physiological and biochemical processes (Ahmed and Holmstrom 2014). *Nocardia* spp. produce structurally diverse siderophores (Table 3.1), for facilitating uptake of iron. *Nocardia* spp. are highly related to

Table 3.3 Biosynthetic gene cluster of nocobactin A

Gene	Size (aa)	Proposed function
nbtS	438	Salicylate synthase
nbtT	536	Salicylate-AMP ligase
nbtG	429	Lysine-N-oxygenase
nbtH	226	Lysine acetyltransferase
nbtA	251	Thioesterase
nbtB	436	Polyketide synthase
nbtC	1028	Polyketide synthase
nbtD	1701	Nonribosomal peptide synthetase
nbtE	1522	Nonribosomal peptide synthetase
nbtF	1167	Nonribosomal peptide synthetase

mycobacteria so they produce numerous mycobactin-like compounds (Patel and Ratledge 1973), which are otherwise called as nocobactin. The basic difference between these compounds is that nocobactin has an oxazole ring, whereas the mycobactin has an oxazoline ring (Ratledge and Snow 1974). Nocobactin A (Fig. 3.2) is mycobactin-like siderophores produced by *Nocardia farcinica* ATCC 3318 (Sakagami et al. 2005).

The analysis of complete genome sequence of *Nocardia farcinica* IFM 10152 (Ishikawa et al. 2004) revealed presence of biosynthetic gene cluster of nocobactin A (Hoshino et al. 2011). The gene cluster included eight genes with significant homologies to biosynthetic genes for mycobactin, as shown in Table 3.3. NbtA is thioesterases, whereas NbtB and NbtC are involved in formation of core polyketide backbone. Both NbtB and NbtC contain required domains as ketoacylsynthase, ketoreductase, acyltransferase, and acyl carrier protein required for polyketide biosynthesis (Staunton and Weissman 2001). NbtDEF are NRPS modules, whereas each of them possesses peptidyl carrier protein, adenylation, and condensation domains. NbtG catalyzes the N6-hydroxylation of lysine, whereas NbtH transfers an acyl chain to the ε-amino group of lysine. Thus based on biochemical and genetic studies, the proposed biosynthesis mechanism for nocobactin A is as illustrated in Fig. 3.4.

3.3.3 Nocardithiocin

Thiopeptides (thiazolyl peptides) are sulfur-rich peptides usually containing a six-membered ring with a central nitrogen (Just-Baringo et al. 2014). The thiopeptide compounds have broad spectrum of biological activities as antimicrobial, antiviral, anticancer, antiplasmodial, etc. Nocardithiocin (Fig. 3.2) is thiopeptide produced by *Nocardia pseudobrasiliensis*, which exhibits prominent activity against acid-fast bacteria including clinical pathogens as rifampicin-resistant *Mycobacterium tuberculosis* (Mukai et al. 2009).

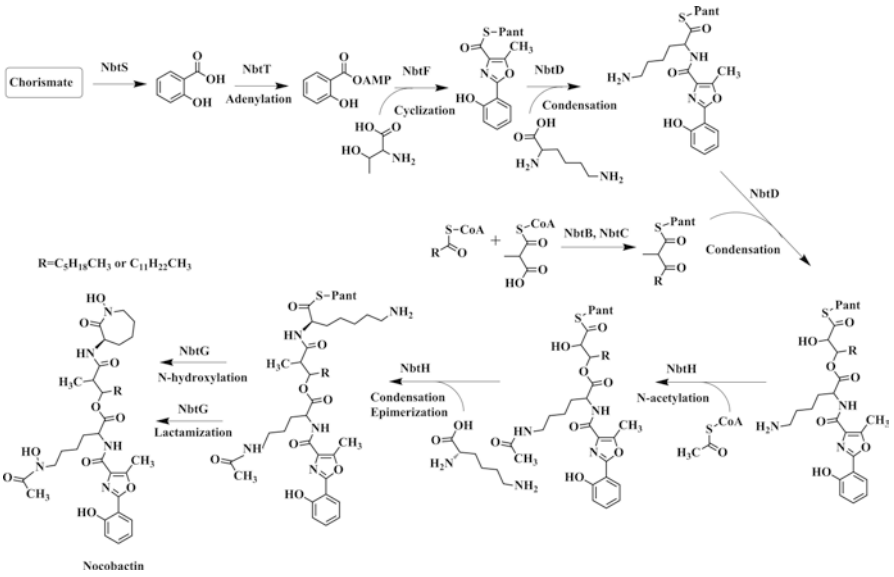

Fig. 3.4 Biosynthetic mechanism of nocobactin A (Adapted and redrawn from Hoshino et al. 2011). The chorismate is converted to salicylate by NbtS. Salicylate is adenylated and cyclilized by NbtT and NbtF, respectively. NbtB and NbtC are involved in formation of core polyketide backbone, and NbtA is thioesterases. NbtG catalyzed the condensation. NbtG catalyzes the N6-hydroxylation of lysine, whereas NbtH transfers an acyl chain to the ε-amino group of lysine

The sequencing of whole genome *N. pseudobrasiliensis* and comparative analysis of biosynthetic gene cluster of thiopeptide compounds in diverse microbes led to identification of the biosynthetic gene cluster of nocardithiocin (Sakai et al. 2015). The gene inactivation and complementation studies of *notL* (the putative cyclodehydratase) were performed to confirm the genomic loci of biosynthetic gene cluster. The comparative transcriptional analysis and analysis of significant changes in the gene expression were performed in nocardithiocin-producing and nonproducing conditions. The biosynthetic gene cluster was predicted based on the catalogue of genes that were only overexpressed in the case of production of compound (Table 3.4). The biosynthetic mechanism was proposed based on the biosynthetic genes, and their putative functions are as shown in Fig. 3.5.

3.3.4 Nargenicin

Nargenicin A1 (Fig. 3.2) which is structurally unique due to the presence of a cis-fused octalin ring system was first reported from cultures of *N. argentinensis* in 1977 (Celmer et al. 1980). Later nargenicin A1 was isolated and characterized from *Nocardia* sp. CS682 (KCTC 11297BP), a soil isolate from Jeonnam, South Korea (Sohng et al. 2008). Nargenicin A1 is significantly effective toward Gram-positive

Table 3.4 Biosynthetic gene cluster of nocardithiocin

Gene	Proposed function
notA	Dehydratase
notB	Cyclodehydratase/peptidase
notC	Methyltransferase
notD	Regulator
notE	Methyltransferase
notF	Cytochrome P450
notG	Precursor protein
notH	Cytochrome P450
notI	Dehydratase
notJ	Dehydratase
notK	Dehydrogenase
notL	Cyclodehydratase

bacteria including methicillin-resistant *Staphylococcus aureus*. It possesses stronger activity and lower cytotoxicity than commonly used macrolides drugs as erythromycin, spiramycin, and vancomycin (Koju et al. 2012; Dhakal et al. 2015). Recently, it was characterized as first identified natural product inhibiting DnaE, a crucial enzyme involved in DNA replication (Painter et al. 2015).

The biosynthetic mechanism for nargenicin has been studied and confirmed through different feeding experiments with different radiolabeled precursors (Cane and Yang 1984). Experimentation with ^{13}C-labeled sodium acetate indicated that the carbon pairs such as C_1-C_2, C_3-C_4, C_5-C_6, C_7-C_8, and C_{11}-C_{12} of nargenicin A1 are derived from acetate. Similarly, analogous studies with ^{13}C-labeled propionate have shown that the C_{17}-C_{18}-C_{19}, C_{15}-C_{16}-C_{20}, C_{13}-C_{14}-C_{21}, and C_9-C_{10}-C_{22} triads of nargenicin A1 are derived from propionate. It was demonstrated that advanced di-, tri-, and tetraketide fatty acid precursors are incorporated directly into the nargenicin A1 (Cane et al. 1993). The labeling studies with ^{18}O-labeled propionate-acetate precursors indicated that oxygen atoms at C_1, C_9, C_{11}, and C_{17} are derived from propionate and acetate. The fermentation studies conducted in an $^{18}O_2$ atmosphere indicate that the C_2 and C_{18} oxygen substituents and the C_8-C_{13} ether bridge of nargenicin A1 originate from molecular oxygen (Cane and Yang 1985). Further, the formation of pyrrole moiety originates from L-proline, which is proposed based on in vitro enzymatic assay (Maharjan et al. 2012). The proposed biosynthetic pathway based on these inferences from different experimental feeding studies is represented in Fig. 3.6.

3.3.5 Biosynthesis of Other Metabolites from Nocardia *spp.*

Most of the bioactive compounds from *Nocardia* spp. have not been characterized and studied properly, and even less molecules are discoursed for their biosynthetic mechanism. The biosynthetic mechanism of few bioactive molecules derived from

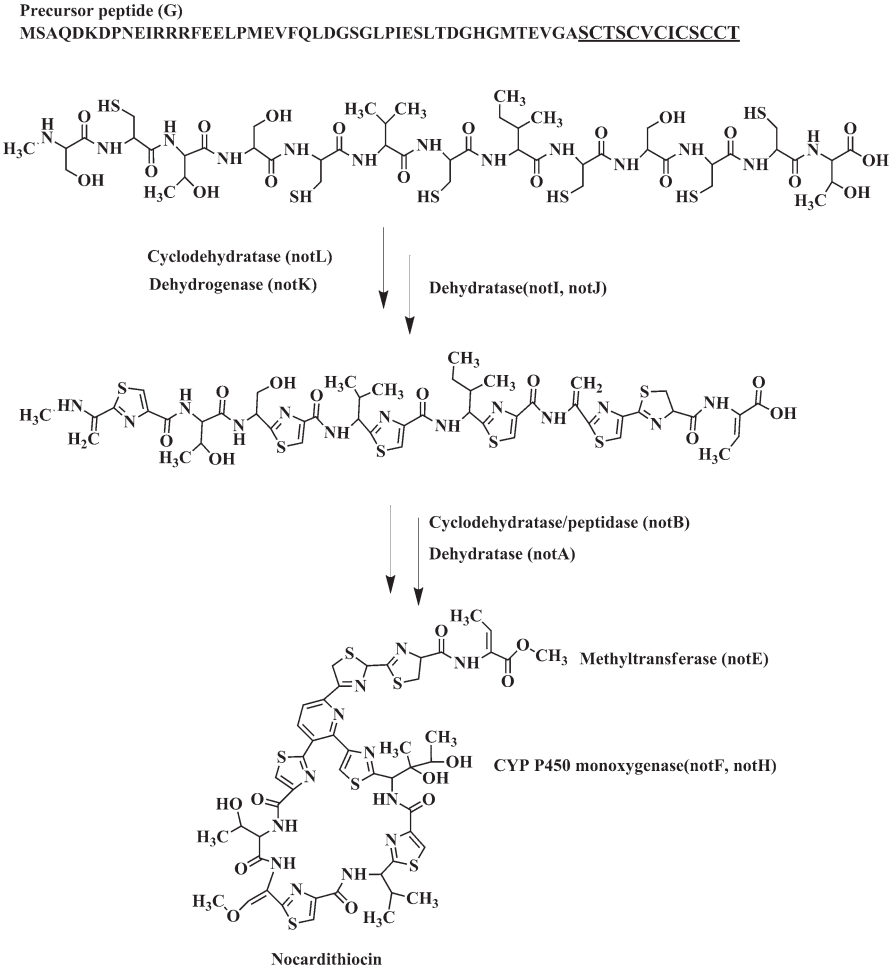

Fig. 3.5 Biosynthetic mechanism of nocardithiocin (Adapted and redrawn from Sakai et al. 2015). Cascades of enzymes resulting in dehydration and dehydrogenation act upon the precursor peptide. Finally, the *notE* and *notH* are responsible for characteristic methylation and hydroxylation reaction, respectively

Nocardia spp. whose biosynthetic steps have been studied in detail has been discussed already. However, few other compounds have been partially studied for their biosynthetic steps. Brasilicardins are reported to be produced by *Nocardia brasiliensis* IFM 0406 (currently refereed as *N. terpenica*). Their unique structure includes diterpene skeleton attached with N-acetylglucosamine, amino acid, L-rhamnose, and 3-hydroxybenzoate moieties. Brasilicardin A (Fig. 3.2) exhibits potent immunosuppressive activities. Shigemori et al. (1999) showed that the perhydrophenanthrene skeleton in the compound is derived from geranylgeranyl phosphate (Shigemori et al. 1999). Further the genes responsible for geranylgeranyl

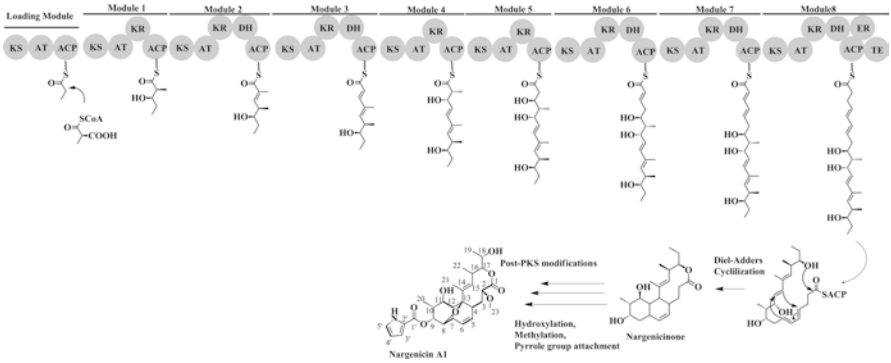

Fig. 3.6 Proposed biosynthetic mechanism of nargenicin A1. The proposed pathway includes three major biosynthetic stages: (1) condensation of short-chain fatty acid precursors to form long-chain polyketides, (2) cyclization to generate macrolide systems, and (3) final post-modifications by oxidations and introduction of the C_{23} methyl and C_9-pyrrole groups of nargenicin A1

diphosphate synthase were cloned and functionally characterized. In vivo gene inactivation in the producer strain helped in determination of the biosynthetic gene cluster (Hayashi et al. 2008).

Formycin is composed of C-ribosides of pyrazolopyrimidine derivatives (Kunimoto et al. 1971). By feeding diverse precursors, the key steps in biosynthesis mechanism of formycin A (Fig. 3.2) produced from *Nocardia interforma* were determined (Ochi et al. 1976; Sawa et al. 1968). Similarly, a novel orphan polyketide synthase was identified by utilizing scrutinized in vitro reaction systems and subsequent feeding of malonyl-CoA, octanoyl-CoA, NADPH, and S-adenosyl methionine. Subsequent studies revealed that the penta modular polyketide synthase system generated unprecedented octaketide and heptaketide products, which were associated with clinical cases of nocardiosis (Kuo et al. 2016).

3.4 Metabolic Engineering for Production of Bioactive Compounds from *Nocardia* spp.

Generally, the natural products derived from plant or microbial sources are the most appropriate starting point in most of the drug discovery processes. These molecules of natural origin are particularly produced by primary or secondary metabolic pathways of producer hosts (Dhakal et al. 2017; Chaudhary et al. 2013). For commercial use, large-scale production by microbial fermentation, chemical synthesis, or semi-synthetic processes is required (Wohlleben et al. 2012; Dhakal and Sohng 2015b). Recently versatile technologies utilizing the coalition of chemical and biological approaches are contributing significantly to drug discovery and development processes (Dhakal and Sohng 2015a, b, 2017). However, natural compounds are preferred over synthetic medicine as synthetic compounds can cause side effects. The

major advantages of biosynthesis over chemical synthesis are its chemical selectivity, environmental friendliness, molecular diversity, and less costly process of scale-ups (Jha et al. 2015; Pokhrel et al. 2015).

For production of expected molecules in significant titer, there is requirement of a stable, well-behaved host, which is genetically tractable (Dhakal and Sohng 2017). However, in case of most of *Nocardia* spp. isolated from nature, they are usually fastidious. They produce only trace amounts of a particular secondary metabolite, which make it difficult for the isolation and production of such compounds for widespread clinical use. Significantly, the genetic studies of these organisms are also hampered by lack of well-characterized genetic manipulation techniques (Dhakal et al. 2016b; Luo et al. 2014). However, recently ample of tools such as plasmids for genetic transfer and techniques for genomic, proteomic, and transcriptomic studies are available (Chiba et al. 2007; Dhakal et al. 2016a, b). These kinds of applications of tools and techniques can utilize for tuning the biosynthetic pathways, precursor engineering, and enzyme engineering for attaining the target natural products in higher titer or functionally/structurally diversified products (Dhakal et al. 2017; Dhakal and Sohng 2017).

The major objective of synthetic biology is to design and construct nonnatural biological systems (e.g., genetic control systems, metabolic pathways, cells) (Dhakal et al. 2016a). Metabolic engineering particularly deals with detailed analysis of metabolic networks or pathways, specifically for finding targets for the reengineering of cell factories (Dhakal and Sohng 2017). The synthetic biology and metabolic engineering can intersect at the level of metabolic pathway construction and process optimization for higher production of targeted compounds/products from engineered microorganism (Nielsen and Moon 2013). There have been very few studies regarding the use of metabolic engineering approaches and synthetic biological tools for rational engineering of *Nocardia* spp.

However, a few applications of metabolic engineering, synthetic biological tools, and enzymatic diversifications have been developed and utilized by our group. This approach has been used for either enhancing production (Fig. 3.7) or structurally modifying key secondary metabolite (Fig. 3.8). The polyketide molecules as nargencin A1 are assembled by a series of decarboxylative condensation of malonyl-CoA and methylmalonyl-CoA. The primary metabolite precursors such as acetate are directed to the pool of malonyl-CoA, and propionate contributes for methylmalonyl-CoA (Stauton and Weissman 2001). Thus, the pool of primary metabolites such as acetate and propionate can be redirected to short-chain fatty acid esters by metabolic engineering approach. These short-chain fatty acid esters can be directly incorporated in polyketide backbone. Thus, nargenicin A1 was successfully enhanced to 3.8-fold by overexpression of acetyl-CoA carboxylase, which is involved in biosynthesis of malonyl-CoA (Maharjan et al. 2012). Further, the level of nargenicin A1 was enhanced to the level of 6.99-fold by using methyl-oleate, sodium propionate, and sodium acetate as sources for enhancing the pool of short fatty acids (Koju et al. 2012), as shown in Fig. 3.7.

Different cheap materials such as ethanol, glucose, glycerol, and propanol as source of fatty acid precursors and L-proline forming pyrrole moiety were used in

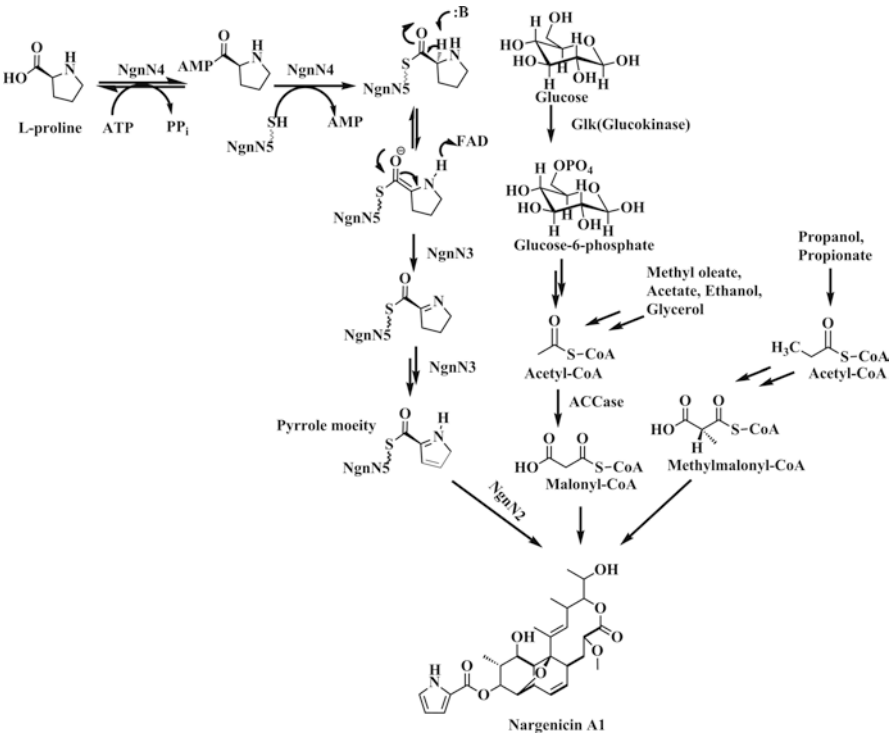

Fig. 3.7 Precursor supplementation and metabolic engineering strategies for enhancement of nargenicin A1 from *Nocardia* sp. CS682. Gene overexpression of *Glf* glucose transporter protein, *Glk* glucokinase, *Ngn2345* pyrrole formation and transfer gene cluster, *ACC* acetyl-CoA carboxylase complex, *Precursor fed* L-proline, glucose, glycerol, ethanol, propanol, methyl-oleate, sodium acetate, sodium propionate, etc

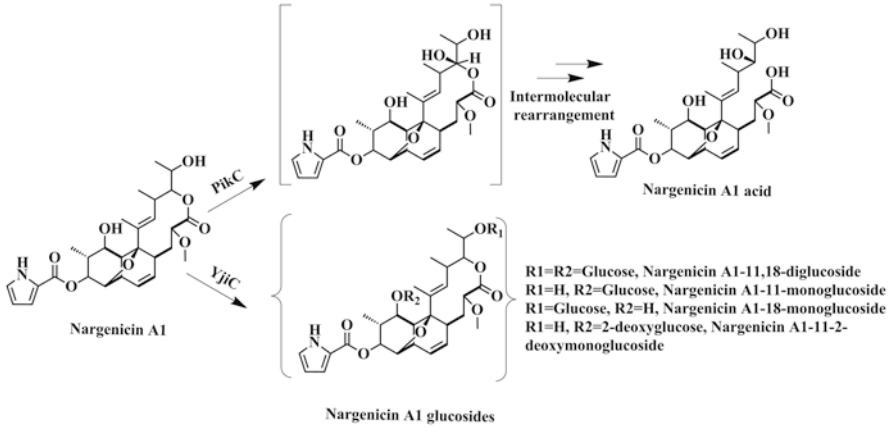

Fig. 3.8 Structural modifications of nargenicin A1 by hydroxylation (in vivo gene overexpression) and glycosylation (in vitro enzymatic reaction)

feeding experiment to *Nocardia* sp. CS682 (Fig. 3.7). Among all of them, the combination of glucose and glycerol exhibited best yield of nargenicin A1. The feeding of such combination to recombinant strains such as *Nocardia* sp. metK1 and *Nocardia* sp. ACC18 and the production titer of nargenicin A1 were enhanced by 6.3-fold and 7.1-fold higher, respectively, in comparison to wild-type *Nocardia* sp. CS682 without feeding (Dhakal et al. 2015). Similarly, the synthetic biology approach was used for developing a multi-monocistronic expression vector for *Nocardia* sp. CS682 (Dhakal et al. 2016a). The precise metabolic engineering approach was used for enhancing the precursor biosynthetic pathways by using combinatorial assembly of genes sets involved in biosynthesis of pyrrole moiety and acyl-CoA esters (Fig. 3.7). Further statistical optimization of precursor feeding time and concentration led to ~24-fold higher production than control strain (Dhakal et al. 2016a). Therefore, this integrated approach of synthetic biology and systematic metabolic engineering was successful model for enhancing the production titer.

Most of the natural products exhibit broad range of pharmacophores with high degree of stereochemistry. Still, there is requirement of new natural products with better uses; hence, these starting natural products are structurally modified to ameliorate their biological or chemical properties for clinical uses (Dhakal and Sohng 2017). Different production host-based biological engineering and chemical synthesis platform can be used for facilitating such structural modification (Dhakal et al. 2016a; Dhakal and Sohng 2017). In an attempt for structural modification by enzymatic glycosylation, a one-pot in vitro system was used, whereas the synthesis of uridine diphosphate (UDP)-α-D-glucose and UDP-α-D-2-deoxyglucose was modified and combined with substrate flexible *Bacillus* glycosyltransferase named YjiC. The novel glucosides generated were nargenicin A_1 11-*O*-β-D-glucopyranoside, nargenicin A_1 18-*O*-β-D-glucopyranoside, nargenicin A_1 11, 18-*O*-β-D-diglucopyranoside, and nargenicin 11-*O*-β-D-2-deoxyglucopyranoside (Fig. 3.8). The assessment of their physiochemical activity exhibited that glycosylated product exhibited higher solubility than parental compound; however, there was a significant loss in antibacterial activities (Dhakal et al. 2015). The lack of 18-OH in 18-deoxynargenicin resulting in the loss of bioactivity has been already reported (Magerlein and Reid 1982). The chances of interaction between water molecule and multiple-OH present in glucose lead to increase in solubility for glucosides (Brown et al. 1970). Hence, the possible reason for loss of bioactivities may be loss of reaction center at 18-OH in glucosides, whereas attachment of glucose provides benefit of higher solubility. Similarly, in another study, nargenicin A1 acid was generated after heterologous expression of PikC (a cytochrome P450 from *Streptomyces venezuelae*) in *Nocardia* sp. CS682 (Fig. 3.8). The compound as well exhibited lower antibacterial activity against *S. aureus* (Dhakal et al., 2016a). The possible reason for loss of activity may be due to loss of lactone ring structure, which may play a vital role in antibacterial activities. In both cases these modification strategies were unable to generate molecule with superior activity, but the feasibility of structural modification by in vitro reaction and whole cell-based in vivo biotransformation was established.

3.5 Future Perspective

The rapid development of genome sequencing has unveiled genetic information of many of the *Nocardia* spp. which give information about their wide metabolic capabilities and biosynthetic pathways (Luo et al. 2014). The "genomic mining" provides connection of genomic sequence (biosynthetic gene cluster) with particular bioactive compound (Zerikly and Challis 2009). Recently, the approaches of introducing the environmental DNA into suitable expression host to create metagenomic library provide exploration of biosynthetic capability for non-cultivable or genetically intractable strains (Dhakal et al. 2017).

In addition diverse approaches on construction-efficient producer strains, optimized precursor and biosynthetic pathways, and enzyme level alteration of biogenesis or transport systems have revolutionized the drug discovery based on natural products (Dhakal and Sohng 2017; Liu et al. 2017). The activation of production of bioactive compounds by co-culture, in situ cultivation, and addition of biological or chemical elucidators has been recent ecological-engineering approach for facilitating production of useful natural products (Dhakal and Sohng 2015b, 2017; Onaka et al. 2011; Seyedsayamdost 2014).

Similarly, pathway engineering using multiplexed genome editing techniques as CRISPER-Cas9 previously utilized for systematic characterization and precise modulation of biosynthetic pathways has been extended for activation of silent biosynthetic gene clusters in various actinomycetes (Zhang et al. 2017; Tong et al. 2015). In addition diverse approaches of enzyme engineering such as domain swapping, targeted mutation, or directed evolution have been supplemented with evolution-inspired biosynthetic enzyme engineering (Metsa-Ketela et al. 2017). Besides the approaches as mutasynthesis, combinatorial biosynthesis and chemobiosynthesis intertwining the chemical reactions with metabolism have been successful for generating diverse natural products with promising pharmaceutical applications (Dhakal and Sohng 2015b, 2017; Pickens et al. 2011).

The advances in knowledge resources have enabled our capacity for evaluating the genomic, proteomic, transcriptomic, and metabolic information of most of the microbial resources (Chaudhary et al. 2013). Concurrently, different tools/techniques based on biology, chemistry, and chemo-biology are available for engineering the producer hosts, modulation of the metabolic pathways, as well as modification of bioactive compounds produced from such microbial hosts (Dhakal and Sohng 2017). Hence, the rational translation of the knowledge to refined platform can embark generation of "super hosts" with capability to produce "super compounds." These kinds of refined platforms provide the next dimension for exploring the biosynthetic ability of *Nocardia* species and generating wonder molecules derived from these biochemically and metabolically refined rare actinobacteria.

3.6 Conclusion

Nocardia species, which are occasionally characterized as pathogen, are revealed as prolific sources of novel and effective bioactive molecules in recent years. The physiological and biochemical features for adaptation to their habitat under harsh condition render them with discrete metabolic features. These intricacies in metabolism direct for high propensity toward biosynthesis of molecules with diverse chemical and biological importance. Even though higher attention is directed on this genus, there is not enough information on the molecular basis of their physiological and biochemical features and biosynthetic capability. However, the availability of genetic information through whole genome analysis and application of precise genetic engineering tools can provide deep insight on the metabolism and biosynthesis mechanism. In addition, the application of advanced tools of genomics, transcriptomics, proteomics, metabolomics, and fluxomics can provide adequate information about cross talks in regulation mechanism in physiology, biochemistry, and metabolism of this fascinating genus. Thus, it is imperative to assume that utilizing all these techniques and tools, more than sufficient information can be easily attained, which can unwire the connection between the genetic architecture and logics of discrete metabolism or biosynthetic ability.

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Chapter 4
Bioactive Metabolites Isolated from Microorganisms for Healthcare: Types and Delivery Routes

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Contents

Abstract Microbial bioactive compounds are one of the most raw forms of chemical metabolites present in nature. Essentially, these compounds play an important role in establishing inter-kingdom interactions. In the last few decades, researchers have explored many types of microbes for bioactive metabolites having pharmacological properties. Microorganisms are known as the potential source for antioxidants, vitamins, antibiotics and enzymes. The number of microbial metabolites being isolated and screened for the treatment of human diseases has increased manifolds. With the development of high-throughput techniques, the quality and quantity of microbial metabolites being tested has also grown rapidly. There are reports suggesting that microbial metabolites are more reliable in terms of efficacy and potential when compared to its chemical counterparts for curing human diseases.

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This chapter discusses microbial isolates having antidiabetic, anticancerous, antibacterial, and antifungal properties. Different strains of marine bacteria and fungi have been used to isolate metabolites exhibiting anticancerous properties. *Micromonospora marina* is one of the extensively studied microbes for isolating anticancerous metabolites. Some microbial metabolites are known for their antifungal properties. In the early 1970s, echinocandin B extracted from *Aspergillus nidulans* was reported to have antifungal property, thereby opening up avenues for the screening of more such metabolites to cure human diseases. Due to the involvement of advanced automated equipments, it is easier to screen a large number of compounds for their pharmacological examination in in vitro conditions. However, the major challenge happens to be the delivery of these bioactive compounds in in vivo conditions. Indeed, the biological barriers in the body restrict the delivery of drugs in in vivo conditions. Since these bioactive compounds are more labile than the chemically synthesized constituents of drugs, it becomes a challenge for these compounds to reach its target site without getting degraded in the metabolic processes. The chapter discusses the three most patient-friendly delivery routes, i.e., oral, intravenous, and intradermal. Since the major share of drugs consumed is in the form of oral dosage, the chemical compounds have been categorized into four classes under the "biopharmaceutical classification system."

4.1 Introduction

Microbes are the tiny microscopic units of life which exists in nature autonomously playing their biological role in the earth's micro flora. They show a symbiotic relationship with the organisms present in their biological vicinity (Webster 2014). The microbial interaction with other class of organisms takes place on a molecular level through secretion of various chemicals which are considered to be as the bioactive compounds (Braga et al. 2016). These bioactive compounds play a significant role in inter-kingdom interactions as they have unique chemical properties, which have attracted the researchers to investigate the true potentials of these compounds (Tarkka et al. 2009; Braga et al. 2016; Kalia 2017). Due to the unique properties of these bioactive compounds, their large-scale applications in different fields of biotechnology such as food industry, drug discovery and development, etc. have been explored. During the past few years, scientists and researchers have explored these bioactive compounds for their use in therapeutics and pharmaceutical industry. These bioactive compounds show great potential and are considered more reliable for their use as chemical substitutes in drugs over the existing ones (Cragg and Newman 2013; Lahlou 2013). Various isolated compounds from bacteria and fungi have also shown their immense ability in curing fatal diseases like cancer, diabetes, Crohn's disease, etc. (Gupta et al. 2014a; Manivasagan et al. 2014; Aftab et al. 2015). Although the bioactive compounds are known for their therapeutic nature, it is imperative to develop efficient delivery strategies which will enable the bioactive

molecules to reach the designated organ and perform its therapeutic action in human body.

In recent years, drug discovery processes for pharmaceutical applications have gained pace due to the advancements and involvement of highly mechanized instrumentations. Scientist and researchers are always in a search of new compounds or molecules that can cure diseases. Highly automated instrumentation technologies which involve robotics, high-throughput screening (HTS) platforms, and high-throughput chemistry have set up an effective trend for evaluation and screening of vast variety of bioactive compounds in a very cost-effective manner. There are numerous commercially available bioassays which can determine the toxicity and effectiveness of newly discovered drugs (Montalvao et al. 2014). The success and failure of the drug discovered can easily be evaluated in in vitro conditions, but the major challenge for the validation of drug comes when the study proceeds for in vivo conditions. For evaluating the drug in in vivo condition, preclinical studies are conducted on mice (*Mus musculus*), zebra fish (*Danio rerio*), etc. due to their genetic similarity to humans (West et al. 2000; Lieschke and Currie 2007; Paulson 2016). In in vivo studies, effectiveness of the drug can only be determined if the drug reaches to its desired site of action in the body. Here comes the concept of targeted drug delivery systems which has always been a major challenge that affects the efficacy of a drug. Since the discovery of nanoparticle and their recognition as an efficient drug delivery vehicle, they have been primarily under investigation for their utilization in developing targeted delivery systems. It is essential that the drug is delivered efficiently to the target site before getting degraded in body. Many biodegradable nanoparticles such as lysosomes, lipids, and prebiotic compounds are being studied for their utilization in targeted delivery systems. This chapter briefly discusses about the various bioactive compounds isolated from microbes having therapeutically important properties, followed by an overview of recent advancements and developments made in the field of drug delivery strategies. The strategies described in the chapter will briefly give an insight on the three major routes of delivery: oral, intravenous, and intradermal.

4.2 Bioactive Metabolites as Therapeutics

The drug discovery processes have always relied on the use of naturally extracted compounds and chemicals which are synthesized from various microbes (Zhang 2005). One of the greatest examples in the history of drug discoveries is the discovery of penicillin by the great Scottish scientist, Alexander Fleming, in the year 1928 (Fleming 1929). Fleming observed the growth of Penicillium molds developed in the *Staphylococcus* culture plates. The molds secreted a bioactive compound which was later named as penicillin (antibiotic). Penicillin led to the lysis of bacterial culture which highlighted its antibacterial properties. Since then the compound has

been taken into account for developing numerous commercially available antibiotics from penicillin and its derivatives such as amoxicillin, ampicillin, and many more (Bruggink et al. 1998). Although the discovery was accidental, it revolutionized the field of therapeutics and pharmaceutical sciences which planted a seed for drug discoveries from naturally synthesized bioactive compounds.

In recent years, researchers and scientists have explored the bioactive compounds from various microbes to analyze their anticancerous, antidiabetic, antifungal, and antibacterial properties (Zhang 2005; Gupta et al. 2014a). Microorganisms produce secondary metabolites which interact with the defective cells and affect their growth. It may hinder the natural metabolic processes of cell to stop its growth or even lead to cellular death (Kalimuthu and Se-Kwon 2013; Lin et al. 2017). The basic components of the secondary bioactive compounds are terpenoids, phenolic acids, steroids, quinones, saponins, tannins, and alkaloids (Gupta et al. 2014a; Gouda et al. 2016). Many marine bacteria and fungi have been identified for producing the secondary bioactives which have shown therapeutic role (Debbab et al. 2010). Many class of marine bacteria and fungi such as *Micromonospora marina* which was isolated from marine corals of Indian Ocean exhibited anticancerous properties. This species produces thiocoraline as a metabolite which has been shown to have anticancerous properties (Romero et al. 1997). Similarly, many such strains of marine micro flora are being examined for analyzing their secondary metabolites having therapeutic properties.

4.2.1 Microbial Metabolites Having Antidiabetic Properties

Diabetes, one of the most common diseases in the world, has been on an upward trend owing to our present lifestyle activities. Due to the increased consumption of sugar intake inside the body, which is a result of improper dietary habits, the body's response toward breakdown of sugars has deteriorated during the past few decades. According to recent reports quoted by WHO, approximately 422 million adults were suffering from diabetes in 2014, which has increased substantially from the past few decades. Compared to the year 1980 when only 180 million adults were affected with the disease, the number has crossed the double figure in the year 2014 (WHO 2016). Obesity and increased blood sugar is not only a cause for diabetes, but it also leads to hypertension, heart attacks, multiple organ failures, organ amputation, and even premature deaths in some cases (Wang and Fan 1990; Long and Dagogo-Jack 2011). Diabetes is generally classified as type I and type II, out of which only type II is considered to be curable (WHO 2016). In type I diabetes mellitus, there is a malfunctioning of the pancreas, i.e., the body is unable to produce the necessary amount of insulin required for the breakdown of glucose produced during the metabolic processes. As a result, the body needs to be supplemented with insulin from outside the body for proper breakdown of these sugars. Type II diabetes is the most common form of diabetes in which the body is unable to use insulin to break down sugar although the amount of insulin produced is sufficient. Another

class of diabetes, type III, has been interlinked with a neurodegenerative disorder that is Alzheimer's disease. Although it is still a clinically controversial aspect that probably modern dietary habits and lifestyle changes contribute largely for the occurrence of the disorder (Steen et al. 2005; Kandimalla et al. 2017). Reports suggest there is an insulin resistance in the body which results in an impaired blood flow to the brain. There have been several evidences which suggest insulin resistance and insulin deficiency as mediators of Alzheimer's disease-type neurodegeneration, but there is no evident cause which validates this hypothesis (De la Monte and Wands 2008).

To treat this disorder, researchers and scientists used to procure insulin from various animal species such as pig, cow, goat, etc. before the recombinant insulin was developed. However, this was met with a very low rate of success as a result of insulin rejection in the human body. The demand for insulin was much more than the supply. The concept of humanized insulin was much under consideration. The year 1978 marked the discovery of recombinant human insulin which was synthesized in genetically modified *E. coli* bacteria. Two American scientists, Arthur Riggs and Keiichi Itakura, collaborated with Herbert Boyer and successfully produced human insulin which was later named humulin. Humulin was found to interact with the insulin receptors in a manner similar to native human insulin (Keefer et al. 1981). Since then microbial abilities have been explored to produce therapeutically important bioactive compounds as preventives and drug development for diabetes.

Various plant microbes and marine species of bacteria and fungi have been investigated to find new antidiabetic bioactive compounds (Dompeipen et al. 2011; Bhattacharjee et al. 2014). Endophytic microbes which reside in root nodules and play a symbiotic relationship with the plant have been explored for their potential in producing antidiabetic bioactive compounds (Dompeipen et al. 2011). The chemical extracts obtained from the microbes were found to act as an inhibitor for α-glucosidase enzyme. This enzyme plays an important role in the absorption of sugars in the blood. The extracts helped in suppressing the activity of these enzymes resulting in reducing the sugar intake getting absorbed in the body (Dompeipen et al. 2011). Similarly, various marine-oriented microbes have also been examined for their extracts having antidiabetic properties. The major resources of bioactive compounds are found from species like sponges, marine bacteria, fungi, algae, and many more. In 1987, Cannell and his team identified around 500 cyanobacteria species from marine water and fresh water microflora which highlighted the prominent inhibitory action of α-glucosidase and α-amylase which were validated using various colorimetric assays. They even found several other compelling cyanobacterial species containing possible glycosidase inhibitory factors. Pandey et al. (2013) discovered bioactive compounds obtained from bacteria and reported their inhibitory effects on β-glucosidase. The key role of the enzyme involves degradation of polysaccharides and the processing of glycoproteins and glycolipids, which can be proven as a good and effective option for curing diabetes and obesity. Some of the other bioactive compounds possessing antidiabetic properties have been listed in Table 4.1.

Table 4.1 Bioactive compounds possessing antidiabetic properties

Source organism	Bioactive compound	Activity/mode of action	References
Cosmospora sp. (marine derived fungus)	Aquastatin A (101)	Inhibits α-glucosidase	Imada (2005)
Cunninghamella echinulata	Madasiatic acid, trihydroxyoic acid and it's derivatives	Inhibition of α-glucosidase	Feng et al. (2017)
Cochlioboluslunatus and *Streptomyces asparaginoviolaceus*	Triterpenes	Inhibition of α-glucosidase	Feng et al. (2014)
Ficus religiosa	Crude extracts	Inhibition of α-amylase, α-glucosidase inhibition	Tiwari et al. (2017)
Streptomyces strain PM0324667	NFAT-133	Antidiabetic activity	Kulkarni-Almeida et al. (2011)

4.2.2 Microbial Metabolites Having Anticancerous Properties

The major area of drug development in the present scenario revolves much around the discovery of microbial metabolites which have anticancerous or antitumor properties. Several metabolites exhibit these properties which hinders the rate of cellular growth. Diacylglycerol, ceramides, sphingosine, NAD^+, arginine, and many such compounds play the role of signaling molecules which help in manipulating the cellular functions (Arakaki et al. 2008). Carcinogens lead to impairment in the cellular mechanism leading to cell proliferation and accumulation of genetic defects which may lead to the tumor formation (Johnstone et al. 2002). One of the preferred methods of curing cancer is chemotherapy, which includes prescription of anticancerous drugs to the patients. These drugs could be either of natural or synthetic origin. A promising class of natural anticancerous drugs is bioactive metabolites isolated from microbes (Kuno et al. 2012). Many strains such as *Micromonospora marina* produce thiocoraline which is an anticancerous chemical compound (Romero et al. 1997). Recently, Rahman et al. (2017) reported *Fagonia indica* to have secondary metabolites having anticancerous properties. Many bioactive compounds of Pseudomonas from marine origin also produce metabolites having anticancerous properties (Romanenko et al. 2008). Microbial fungus *Penicillium* sp. PR19 N-1 which were found in Antarctica deep-sea habitat produces metabolites which showed strong cytotoxic effect on HL-60 cells (leukemia cells) (Lin et al. 2014). A

list of secondary metabolites from microbes having anticancerous properties is listed in Table 4.2.

Another source of bioactive compounds exhibiting anticancerous activity has been isolated from ascidian organisms. One such component known as trabectedin which was extracted from a Caribbean ascidian got its approval as a promising anticancerous agent for soft tissue such as sarcomas and ovarian cancer (Carter and Keam 2007; Gennigens and Jerusalem 2011). Another group of researchers reported a chemical compound named aplidine, which was obtained from the Mediterranean ascidian species *Aplidium albicans*, exhibiting strong anticancerous properties and have great potential for their use in the treatment of prostate, gastric, breast, and colon cancers. This molecule is currently under human clinical trials (Brandon et al. 2007). An experimental study conducted by Broggini and his team (2003) reported the effect of aplidine on the human leukemia cell line MOLT-4 and showed that alpidine inhibited the growth and induced apoptosis in the cell line. The report sug-

Table 4.2 Microbial metabolites having anticancerous properties

Source organism	Bioactive compounds	Activity/mode of action	References
Citricoccus sp.	Nocardamine	Inhibition of colony formation of breast cancer cell lines T-47D and various other tumor cell lines	Kalinovskaya et al. (2011)
Halomonas sulfitobacter from the Red sea	Crude extracts	Significant apoptotic and cytotoxic activities on treated cancer cell lines	Sagar et al. (2013b)
Carteriospongia sp. and *Dysidea* sp.	Scalaranesesterterpenes	Anticancerous activity	Gao et al. (2014)
Halophilic sp., *Chromohalobacter salexigens*, *Halomonas meridian*, *Idiomarina oihiensis*, *Chromohalobacter israelensis*	Crude extracts	Cytotoxic and apoptotic effects against human breast adenocarcinoma (MCF-7), cervical carcinoma (HeLa), and prostate carcinoma (DU145) cell lines	Sagar et al. (2013a)
Aaptos sp.	Aaptamine	Apoptosis in monocytic leukemia cell line (THP-1)	Dyshlovoy et al. (2014)
Penicillium sp.	Penialidin A-C, citromycetin, p-hydroxyphenylglyoxalaldoxime, and brefeldin A	Anticancerous activity against HeLa cells	Jouda et al. (2016)
Garcinia nobilis			

gested that aplidine inhibits some factors some of the necessary growth factors which prevents the cellular growth. For instance, the inhibition of vascular endothelial growth factor (VEGF) secretion was found due to the blockage of receptors VEGF/VEGFR-1 by alpidine. The effects on cancerous cell lines are studied in various assays to validate the anticancerous activity of various metabolites. In vitro analysis explains inhibitory activity of these compounds with various enzymes. It becomes a major challenge to examine the effect of these microbial metabolites in in vivo conditions. The bioavailability of anticancerous drugs has to be enhanced to increase the efficiency. Various encapsulation techniques have been employed to deliver the drug(s) to the target site(s). Intravenous delivery of encapsulated drug systems uses lipid-based carrier, hydrogels, and various other engineered nanoparticles, which will be discussed later in the chapter.

4.2.3 Microbial Metabolite Having Antibacterial Properties

In spite of major advancements and progress made in medicine, infectious bacterial diseases have still been a major challenge to human health especially in developing countries where people are more prone to these bacterial diseases due to the exposure to unhygienic conditions in their day to day life. The issues that have developed due to widespread growth of drug-resistant pathogenic bacteria have resulted in increased sufferings to manyfold and shown prolonged illness (Edmond et al. 1995; Demain and Sanchez 2009; Leclercq 2009). One of the recent WHO reports suggests that approximately >70% of pathogenic bacteria are resistant to at least one existing antibiotic (WHO 2017), which is quite alarming. As a result, there has been a drastic increase in the growth of drug-resistant pathogens putting the onus on scientists and researchers to develop new and effective antimicrobial drugs.

Many marine bacteria have been discovered having antibacterial properties and counter the demands of antibacterial drugs. One such bacterial species found in marine biota of Mediterranean Sea of the Murcia coast was identified as *Marinomonas mediterranea*. Experimental reports of *M. mediterranea* exhibited antibacterial activity *Pseudomonas* sp. and *Staphylococcus aureus* which were otherwise showing resistance against antibiotics ceftazidime and methicillin, respectively (Lucas-Elio et al. 2005). Earlier, in 1993 McEvoy reported a metabolite "bacitracin" which was produced by *Bacillus licheniformis* that showed antibacterial activity against many Gram-positive organisms including anaerobic cocci, but not against Gram-negative bacteria (McEvoy 1993). In 2009, a strain of Pseudomonas was isolated from India which showed its potential for antimicrobial activity against pathogenic microbes (Charyulu et al. 2009). In 2012, *Pseudoalteromonas piscicida* was isolated by Darabpour and coworkers from Iran, and *Pseudomonas aeruginosa* was isolated by Tawiah and coworkers from Ghana which exhibited an eminent amount of antimicrobial activity (Tawiah et al. 2012). A large number of bacterial strains isolated from marine environment have exhibited antimicrobial activity against the most common pathogenic bacteria, methicillin-resistant *Staphylococcus*

Table 4.3 Microbial metabolites having antibacterial properties

Source organism	Bioactive compound	Activity/mode of action	References
Brevibacillus laterosporus	Tauramamide and Tauramamide ethyl ester	Selective activity against Gram-positive human pathogen *Enterococcus* sp.	Desjardine et al. (2007)
Marinispora (strain NPS008920)	2-alkylidene-5-alkyl-4-oxazolidinones, lipoxazolidinone A	Antimicrobial activities similar to those of the commercial drug, Zyvox	Debbab et al. (2010)
Pseudomonas stutzeri	Zafrin, tetrahydrophenanthrenone	The antimicrobial activity of zafrin against *Bacillus subtilis* is higher than ampicillin, vancomycin, and tetracycline	Debbab et al. (2010)
Streptomyces netropsis	Distamycin	Inhibits the transcription and increases the activity of the topoisomerase II	Majumder et al. (2013)
Brevibacterium sp., *Moraxella* sp., *Corynebacterium* sp.	Crude extracts	Antibacterial and antioxidant properties	Al-Zereini (2014)

aureus (MRSA). One such marine bacterium is *Pseudoalteromonas phenolica* isolated by Isnansetyo and Kamei (2003) which was detrimental for MRSA. Radjasa et al. (2007) identified a coral-associated bacterium as *Pseudoalteromonas luteoviolacea* TAB4.2 which showed inhibitory action against several pathogenic as well as many coral bacteria. Holmström and Kjelleberg (1999) have reported that *Pseudoalteromonas* spp. isolated from tunicates exhibits antibacterial activity. Table 4.3 enlists some of the microbial metabolites and their activity against pathogenic bacteria.

4.2.4 Microbial Metabolites Having Antifungal Properties

The major type of fungal infection occurs on the outermost surface of any organ, tissue, or skin it infects. In humans, it mostly occurs on the outermost layers of the skin, for instance, ringworm infection on athlete's foot or on nails (onychomycoses) which might even lead to life-threatening diseases. Some of the human invasive fungal infections are caused by *Cryptococcus neoformans*, *Candida* spp., *Aspergillus* spp., *Pneumocystis carinii*, and *Histoplasma capsulatum*, which are a menace to the human health (Ravikant et al. 2015). During the past few decades, the occurrence of these systemic fungal infections has increased substantially.

In the early 1970s, fungal infections were more overly considered as quite easily treatable infections, and the demand for antifungal drugs were in scarce amount. But in the late 1970s, the perceptive of therapeutics changed due to the introduction of *Candida albicans* or *Aspergillus* which proved to be very infective agent for the

cause of infections in humans which mostly emerged during transplantation processes. *Candida* species proved to be an opportunistic pathogen which infected the host during immunocompromised situations. During transplantation, patient's immunity is suppressed for the acceptance of organ, but the patient is at high risk of getting the fungal infection. So it was essential to counter the challenge of fungal infection and develop new drugs for its prevention. Some of the commercially available antifungal agents such as polyenes, azoles, and Cancidas are present in a very scarce amount which makes it more challenging for the treatment of life-threatening fungal infections. These chemically synthesized compounds have various limitations such as nephrotoxicity (Georgopapadakou and Walsh 1994) and significant cellular toxicity which prevents it from its excessive use as antifungal drugs. These shortcomings have opened up vistas for discovery of new antifungal-based drugs. Naturally synthesized bioactive compounds comparatively show a lower toxicity. The major target of antifungal compounds is to impede the fungal cell wall synthesis which is achieved by inhibition of glucan synthesis required for the formation of cell wall. Many such inhibitory biomolecules have been examined which are extracted from various microbial sources. Nyfeler and Keller-Schierlein (1974) reported Echinocandin B possessing antifungal properties which was extracted from *Aspergillus nidulans.* Similarly, in 2000 Fujie and his team identified a compound named FR901469 which had a composition of macrocyclic lipopeptidolactone containing12 amino acids and a 3-hydroxypalmitoyl moiety. This compound was found to be an inhibitor of glucan synthesis both in in vitro and in vivo conditions. The compound was isolated from an unidentified fungus No.11243. II. The compound isolated from the ethyl acetate extract of *Streptomyces galbus* showed antifungal activities against pathogenic fungi (Paul and Banerjee 1983). In 2011, Sajid and coworkers isolated bioactive compounds, phenylacetic acid and indolyl-3-lactic acid from *Streptomyces malachitofuscus* which showed antifungal activities. Recently, Andayani et al. (2015) reported antifungal properties of isolates from soil microorganisms obtained from Tangkuban Perahu Mountain. Although, there is a scarcity of bioactive compounds having strong antifungal activity, suggested use of Ketoconazole or Miconazole with bioactive compounds can increase their efficacy in combating fungal infection (Fukuda et al. 1996; Zhang 2005). The combination of these compounds showed appreciable antifungal properties. The bio-accessibility of these bioactive compounds can be enhanced if delivered through the intradermal routes. Some of the metabolites having antifungal activities have been enlisted in Table 4.4.

The delivery of all the above discussed bioactive compounds majorly depends upon their mechanism and the site of action inside the human body. For instance, antidiabetic drugs either have to be delivered intravenously or directly to the pancreatic tissue so that it shows its maximal effect in the body. The delivery of these compounds should be administered precisely and efficiently to its target site. Drug delivery processes can be either directly supplied to the target site or should be carried forward with the use of various material-based carriers. The materials used for the delivery should be biocompatible and biodegradable. The drug delivered must overcome the various biological barriers to achieve maximum efficacy. The two fac-

Table 4.4 Microbial metabolites having antifungal properties

Source organism	Bioactive compound	Activity/mode of action	References
Serratia plymuthica A 153	Haterumalides NA, B and NE ZEAMINE	Complete suppression of apothecial formation in sclerotia of *Sclerotinia sclerotiorum* at a concentration of 0.5 µg ml^{-1}	Levenfors et al. (2004)
Streptomyces galbus	Ethyl acetate extract	Antifungal activity against *Microsporum gypseum*	Paul and Banerjee (1983)
Streptomyces malachitofuscus	Phenylacetic acid and indolyl-3-lactic acid	Antifungal activity against *Mucormiehei* and *Candida albicans*	Sajid et al. (2011)
Aspergillus nidulans	Echinocandin B	Antifungal activity	Nyfeler and Keller-Schierlein (1974)
Aspergillus versicolor	Isorhodoptilometrin-1-methyl ether, emodin, 1-methyl emodin, evariquinone, 7-hydroxyemodin 6,8-methyl ether, siderin, arugosin C, and variculanol	Antifungal activity	Sagar et al. (2013b)

tors which essentially govern the bio-accessibility and the bioavailability of a drug depend upon its solubility and permeability inside the human system. On the basis of these factors, the United States Pharmacopeia has established a Biopharmaceutics Classification System. According to this classification system, there are four categories of drug compounds which are characterized on the basis of their permeability and solubility (Cardot et al. 2016). These classifications are also helpful in determining whether the drug needs to be encapsulated for delivery. The delivery route administered for a particular drug largely decides its fate and efficiency as a therapeutic compound. Hence, it is essential to select the most suitable route to showcase the maximum potential of the selected drug.

4.3 Delivery of Bioactive Compounds

The effectiveness of a bioactive molecule can be achieved when it is efficiently delivered into the system. The bioavailability of these compounds can be increased by opting for targeted delivery systems. Since the bioactive compounds are more labile chemical compounds as compared to synthetically synthesized chemicals, it becomes more essential to deliver the drug to the target site before getting degraded during the pathway (Cai et al. 2013). Many nanoparticle encapsulated carrier systems have been utilized for increasing the efficiency of these compounds. Most of the delivery systems are used to deliver molecules of biological origin such as

antioxidants, immunomodulatory compounds, antimicrobials, and prebiotics (Cornara et al. 2017). Lipid-based nanoparticles have been successfully used to deliver bioactives in in vivo experiments as they are associated with lower cellular toxicity in comparison to other types of metallic nanoparticles such as silver nanoparticles and carbon nanotubes (Chuang et al. 2018; Khan et al. 2017). Nanoparticles can be modified for targeted delivery of bioactive metabolites (Watkins et al. 2015). This can be achieved by attaching ligands to the nanoparticles which will help the encapsulated molecule reach its target site. Some of the most commonly used polymeric nanoparticles are derived from poly(lactic-co-glycolic acid) (PLGA), poly-L-lactic acid (PLA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), hyaluronic acid, polycaprolactone (PCL), and chitosan. These polymers have higher biocompatibility and biodegradability and have high proficiency of getting functionalized (Watkins et al. 2015). Probiotic bacteria have been encapsulated in polymers such as alginate, chitosan, gelatin, etc. in the form of nanoparticles. The different methods of encapsulation used are spray drying, nanoemulsions, etc. These biopolymers are reported to protect the encapsulated probiotic bacteria (Huq et al. 2013).

The next section discusses about the three most commonly used delivery routes for bioactives which are oral, intravenous, and intradermal.

4.3.1 Oral Delivery of Bioactive Compounds

The oral route for delivery of drugs has always been considered one of the most patient friendly techniques. In recent years there have been major advancements in techniques to develop an efficient carrier system that may enable the controlled or regulated release of encapsulated compound, hence will provide a better dosing pattern having minimal side effects (Heidarpour et al. 2011). Low bioavailability of many bioactive compounds is a major barrier for developing oral dosage of drugs (Lin et al. 2017). Low chemical stability of compounds under gastrointestinal tract conditions suppresses the overall effectiveness of drug and decreases its bioavailability in plasma concentrations. The major challenge for developing an oral dosage is to ensure that it should be effective enough to bypass the first pass metabolism and reach its target site. Nanocarriers have great potential in protecting the drug to surpass various metabolic degradation which are carried out in the body thereby enhancing the bioavailability of the drug.

The two important factors which majorly govern the bioavailability of a drug depend upon its solubility and permeability, as discussed earlier. These factors are more important for developing oral drug formulations. It is essential that whichever carrier systems are selected, the purpose is to address the problems and challenges faced by the drug to reach its target site (McClements et al. 2009). Class I type comprises of compounds which are characterized with high solubility and permeability. They have a higher bioavailability and essentially do not require a delivery system. However, issues regarding to their chemical stability might be low or even

their solubility may not fit the human food system, eventually they may require encapsulation for better proficiency. The second category of compounds, class II, is the ones which have low solubility and high permeability. These compounds are generally lipophilic in nature, and hence lipid-based nanocarriers are the most suitable delivery carriers that can be utilized to enhance the overall bioavailability and bio-accessibility of the compounds. Molecular interactions between nanomaterial and bile salt takes place with the formation of micelles which eventually governs their uptake rates inside the body. In contrast to the larger particles, nanocarriers increase the solubility of compound. The compounds of class III category are sufficiently soluble under internal biotic conditions, gastrointestinal tract, but they possess comparatively lower permeability. In such conditions, the components of nanocarriers having mucoadhesive properties may help in enhanced bioavailability of the compound by increasing the interaction time of the bioactive compound with the gastrointestinal tract. The opening of the tight junctions in the outermost layer of the gastrointestinal tract helps in diffusion of bioactive compounds which will facilitate to increase the efficiency of the drug. Among all classes, the fourth class of compounds (class IV) is the most challenging to manage as these compounds have low solubility as well as low permeability (e.g., curcumin). This class of compounds requires a carrier that can help in substantial increase of solubility, e.g., a lipid phase or surfactant-rich carrier and a mucoadhesive compound such as chitosan that can help in increasing the interaction time with the gastrointestinal tract. Thus, liposomes can be one such nanocarrier having a mucoadhesive compound coating which could be utilized as a suitable carrier system for delivery of such compounds.

The most commonly used carrier systems are lipid-based or polysaccharide-based compounds which are used for encapsulation of drug. Lipid-based delivery vehicles which are most commonly used for delivering bioactive compounds are liposomes. Liposomes are the nanostructured compounds having phospholipid bilayers (lamellas) which greatly contribute in encapsulation of the proteins or the peptides. The size and shape of liposomes depends on the process by which it is synthesized. On the basis of the number of phospholipid layers, liposomes have been categorized into three categories: large unilayer, small unilayer, and multilayer liposomes. There are clinically approved liposomal-based drugs in the markets such as liposomal amphotericin and liposomal doxorubicin (Sharma and Sharma 1997). Many of the experimental reports suggest that the liposomes protect the encapsulated drug/peptide from oxidation and deamidation. Also, the drug/peptide constrained inside the liposomes is taken up by the lymphatic system which then redirects them into the systemic blood circulation. In doing so, the drug of interest is by passed from the first pass metabolism (Porter and Charman 1997). Some of the advanced techniques that have been reported in the recent years, for administration of oral drug delivery of peptides, have utilized substances such as the liposomes consisting covalently linked carbopol molecule with wheat germ agglutinin. The cytotoxic analysis for these modified liposomes as compared to the unmodified liposomes showed lower toxicity in caco-2 cells. Pharmacological efficiency of non-modified calcitonin-loaded wheat germ agglutinin-carbopol was found to be

20-fold and threefold higher than the non-modified and carbopol-modified liposomes, respectively. These results show the potential of liposomes as an efficient oral peptide delivery system (Makhlof et al. 2011). Bilosomes are other novel colloidal delivery particles wherein the bile salts are incorporated in the lipid bilayer membrane. In 2011, Shukla et al. synthesized bilosomes for oral delivery of diphtheria toxoid using thin-film hydration method. When administered in vivo, these bilosomes gave results comparable to those induced by intramuscular alum-adsorbed diphtheria toxoid. Therefore, these orally deliverable nanobilosomes offer a good alternative compared to intramuscular alum-adsorbed diphtheria toxoid with good patient compliance (Shukla et al. 2011).

Food extracts and molecules are also in trend for use as drug delivery vehicles. The concept of nutraceuticals food has fascinated various researchers for the use of food materials for drug delivery purpose (Gupta et al. 2014b). Prebiotics are such ideal food ingredients which have been utilized for nano-encapsulation of drug for oral delivery due to their inherent ability to protect the encapsulated compound in the upper gastrointestinal tract. Prebiotic encapsulated drugs have been in the recent trends for the controlled delivery of the bioactive compounds. The technique involves entrapment of the bioactive compound so that it can travel through the gut without getting degraded by digestive acids present in it. Prebiotics are food ingredients which are nondigestible but hold beneficial properties which are helpful for selective stimulation for the enhanced growth of gut bacteria in the colon resulting in improvement of host health (Gibson and Roberfroid 1995; Gibson et al. 2004). In context to monogastric animals and humans gut, the major emphasis is on the colon involving a considerable degree of fermentation for the bacterial growth in the upper gastrointestinal tract (Gaskins 2001; Thursby and Juge 2017). In recent years, the focus has been to develop a stable and complex commensal bacterial community which is essential for developing a healthy gut ecosystem (Verstegen and Williams 2002; Konstantinov et al. 2004). Prebiotics include oligosaccharides of fructose and galactose as well as lactulose, which are nondigestible by human digestive enzymes but can be metabolized by colonic bacteria to produce short-chain fatty acids such as butyrate. Some of the naturally occurring and synthetic polysaccharides studied extensively for their prebiotic properties in humans are inulin, guar gum, resistant starch, pectins, chitosan, and lactulose (Awati et al. 2005; Kelly 2008).

4.3.2 Intradermal Delivery of Bioactive Compounds

Transdermal or intradermal drug delivery offers numerous advantages to the patient, as it is not only convenient but also noninvasive in nature. Due to the factors such as ability to avoid the first pass metabolism and gastrointestinal degradation, it is the most reliable route for skin-specific or tissue-specific diseases such as fungal infection, bacterial infection, etc. (Hutin et al. 2003; Sadik and Zillikens 2013; Yellepeddi et al. 2015). For systemic delivery of drugs via transdermal route, microneedle arrays have been designed and tested. In the past few years, numerous drug and

vaccine delivery systems have been designed based on microneedles arrays. These arrays are patient friendly as they are minimally invasive.

Microneedle arrays bypass the skin's protective barrier, stratum corneum barrier, thus accessing the skin microcirculation and achieving systemic delivery by the transdermal route. These arrays consist of several microprojections of different shapes attached to a base support and are generally of 25–2000 μm in height (Donnelly et al. 2012). Microneedle arrays are available in various shapes and materials (silicon, metal, polymers) and are produced using microfabrication techniques (Indermun et al. 2014). The arrays when applied to the skin surface painlessly pierce the epidermis creating microscopic pores through which the drug enters the dermal microcirculation (Donnelly et al. 2014). Microneedles were developed to increase the permeability of the drug. There are reports which suggest that these delivery systems have successfully delivered hydrophilic drugs and macromolecules such as peptides, DNA, etc. (Kim et al. 2012). Microneedle arrays penetrate the skin through the stratum corneum and into the viable epidermis so that they avoid contact with the dermal layer with nerve fibers and blood vessels (Tuan-Mahmood et al. 2013). These arrays have reduced needle insertion pain and tissue trauma in patients. They have been widely used for pain-free intradermal delivery of biomacromolecules.

Solid microneedle arrays are normally employed in the so-called "poke with patch" approach (Prausnitz 2004). These are applied to the skin and then removed, creating transient aqueous micro-channels in the stratum corneum. Subsequently, the drug formulation of choice (transdermal patch, solution, cream, or gel) is applied to the treated area from where the drug permeates via passive diffusion into the dermal tissue through the micro-channels created by solid microneedle arrays. The main limitation of this approach is the requirement for a two-step application process, which may lead to practicality issues for patients. The materials commonly used to produce solid microneedle arrays are silicon, metals, and polymers (Larraneta et al. 2016).

4.3.3 Intravenous Delivery of Bioactive Compounds

Intravenous delivery of drugs is commonly used for the instant distribution of injected drugs from the veins throughout the entire body. The major challenge in intravenous drugs is that it is quickly metabolized and has high chances of being excreted out from the body in very short durations of clinical efficacy. Scientists and researchers are always in search of developing sustainable form of intravenous drug delivery systems. There are various advanced techniques which may be beneficial in increasing the efficacy of drug through sustained release in the body. Nanoparticles are one of the most suitable materials for transporting bioactive compounds in vivo. When injected intravenously, nanoparticles have shown promising results in the bioavailability of the encapsulated compound (Vauthier 2012). Their use in drug delivery has made possible the development of novel methods for treatments using new molecules such as nucleic acids, proteins, and peptides which display a high

therapeutic potential. Success of nanoparticle-based drug delivery systems largely depends on its ability to cross biological barriers and its bio-distribution. The concept of controlled drug delivery or pulsatile drug delivery is essential for the efficiency of intravenous drug delivery. In the human body, many important biological functions are regulated by controlled or transient release of bioactive substances at a specific site for a specific time and duration (Patel and Patel 2015). From a therapeutic perspective, it is important to develop those drug delivery devices which can achieve regulated dose delivery of a bioactive compound at predetermined time intervals. The transient or pulsatile release of bioactive compounds and/or therapeutic agents to a patient has been a major goal in drug delivery research over the last two decades. The plasma peak is obtained at an optimal time by administering different doses of the drug at scheduled time. Based on the studies and research, the number of doses per day can be altered. Different strategies have been developed or formulated to develop systems for pulsatile release of the bioactives. These systems can be broadly categorized into reservoir, capsular, and osmotic devices (Anal 2007).

Pulsatile drug delivery systems can be regulated by varying factors such as osmotic pressure, pH, and regulated temperature. Hydrogels are one such class of compounds which is most commonly used as a pulsatile drug delivery carrier (Kikuchi and Okano 2002; Kushwaha et al. 2012). Hydrogels that undergo reversible volume changes as and when there is a change in temperature are known as thermosensitive or thermo-responsive gels. These gels shrink at a transition temperature known as lower critical solution temperature (LCST) of the linear polymer. This characteristic property of volume change can be utilized to obtain a squeezing hydrogel device by positioning hydrogel within a rigid capsule. The reversible volume change of temperature-sensitive hydrogels accomplishes on-off release of drugs. On and off is governed by swelling/deswelling phases, and it swells below 32 °C temperature and on the other side shrinks above this temperature (James et al. 2014). pH-sensitive hydrogels can be used to deliver drugs to different organs. Anticancerous metabolites can be encapsulated in pH-sensitive nanoparticles and delivered by targeted approach to tumor tissues where the environment is acidic. Depending upon the need, the hydrogels can be customized and used to deliver the microbial metabolites to specific target organs.

4.4 Conclusions

Delivery of bioactive molecules isolated from microbes having therapeutic use is a major challenge for scientists and researchers. The efficacy of bioactive molecules depends largely on the formulation and route of delivery in human body. Formulation of drug depends on the solubility and permeability as defined under Biopharmaceutics Classification System. Since most of the drugs are designed for oral dosage, it is essential to consider their solubility and permeability in the gastrointestinal tract. Accordingly, various delivery systems have been developed, viz., prebiotics,

liposomes, polysaccharide-based systems, etc. Researchers have also reported nanoparticle-based targeted delivery systems for achieving high therapeutic efficiency of bioactive compounds. Microneedles offer almost a painless intradermal delivery route of bioactive molecules. The "poke with patch" approach of microneedles help in overcoming the first pass metabolism resulting in increased efficiency of the delivered bioactive compounds. The intravenous route for bioactive compounds delivery is a major challenge as they undergo fast clearance from systemic circulation. To circumvent this challenge, hydrogels are used as controlled delivery systems. The delivery of bioactive compounds isolated from microbes is in nascent stage and requires a lot of research and clinical trials for its large-scale application in therapeutics. Although few of the metabolites developed or obtained from microbes are in the market, extensive clinical trials have to be carried out for prescribing these microbial metabolites for drug therapy. Also, the route of delivery of these bioactive metabolites will play a significant role in achieving the full potential of these potent drugs in the making.

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Chapter 5
Siderophores: A Novel Approach to Fight Antimicrobial Resistance

Marta Ribeiro and Manuel Simões

Contents

Abstract The increasing bacterial resistance subsequent to antibiotic use has instigated the development of new and effective antimicrobial strategies. Bacterial iron uptake systems are novel therapeutic agents since iron is crucial for the growth and development of microorganisms as well as a main virulence factor during the establishment of an infection. The method commonly used for iron assimilation is based on the production of siderophores, which are low molecular weight iron chelators produced by bacteria, fungi, and plants to facilitate iron uptake and crucial for bacterial pathogenicity. Therefore, in recent year's siderophore iron uptake, systems have received much attention as novel targets for antimicrobial approaches.

Here we review siderophores in the antimicrobial field. We first outline the problematic of bacterial resistance to available marketed antibacterial drugs and, consequently, the current needs to contrast with the emergence of bacterial resistance.

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After, we emphasize the critical role of iron for bacterial growth and development and how pathogens compete with the host for iron. The biosynthesis, regulation, and transport of siderophores are also discussed. Lastly, we review work done with siderophores in the antimicrobial field. Such work has generally been done using three essential approaches: siderophore-mediated drug delivery, inhibition of siderophores biosynthesis, and iron starvation via competitive chelation.

5.1 Introduction

The increased resistance of bacterial pathogens against the available marketed antibacterial agents is contributing for an increasing number of infections as well as a decline in the discovery of new antimicrobials (Fischbach and Walsh 2009; Andersson and Hughes 2011). Therefore, the need for new efficient therapeutics has been urgently demanded, and the iron acquisition mechanisms could be an effective antimicrobial approach. Iron is crucial for the growth and development of microorganisms. Under iron-limited conditions, the microorganisms survive through the production of siderophores which are low molecular weight compounds with high affinity for ferric iron (Schalk et al. 2011; Javvadi et al. 2018). The biosynthesis, regulation, and transport of natural siderophores are fundamental to exploit iron-associated mechanisms (Hider and Kong 2010). Several Gram-positive and Gram-negative bacteria use siderophores to scavenge iron from this restricted environment and then return it to the bacterial cell (Hider and Kong 2010; Schalk et al. 2011). Siderophore-mediated drug delivery, a Trojan Horse strategy, is an ideal strategy to circumvent membrane-associated drug resistance, in which the drug is incapable to cross the bacterial membrane. Inhibition of the biosynthesis of siderophores that are crucial for survival of bacteria under iron-limited conditions represents another promising approach. Depriving pathogenic bacteria from iron is another potential antimicrobial strategy.

This chapter outlines the siderophores biosynthesis as well as the main classes of these compounds. It also presents some recent results for the development of antimicrobial drugs that can disturb the assimilation of iron by bacteria, interfering at different levels with the bacterial iron metabolism that is not targeted by the marketed panel of antibiotics.

5.2 Bacterial Resistance and Current Needs

All living microorganisms struggle to adapt to their environment in order to survive, including bacteria that have shown an outstanding capability to endure and adapt to undesirable environmental conditions, particularly the capacity to grow as sessile bacteria or biofilm. Biofilms are one of the most significant causes of bacterial

resistance to antibacterial agents. A biofilm is a structured consortium of bacteria embedded in a self-produced polymeric matrix consisting mostly of polysaccharide, protein and DNA (Høiby et al. 2010; Simões 2011; Koo et al. 2017). It is nowadays known that most bacterial-related infections, such as respiratory tract and blood, lung, bone, wound, ear, urinary tract, skin and soft tissues, dental caries, cerebrospinal fluid, indwelling devices, and implant-associated infections, are problematic because of biofilms (Simões 2011; Azevedo et al. 2017; Pitts et al. 2017; Thet et al. 2018). Clinically, infections caused by bacterial biofilms are associated to prolonged hospital permanence, continued administration of antibiotics, infection recurrence, and increased mortality compared to those caused by susceptible bacteria, carrying higher healthcare costs (Azevedo et al. 2017; Murray et al. 2017; Cabral et al. 2018). Additionally, around 80% of bacterial infections in humans are associated to the biofilm formation (Blackledge et al. 2013; Singh et al. 2017). Methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* resistant to β-lactams, and *Mycobacterium tuberculosis* belong to the most clinically significant bacteria related to drug-resistant infections (Simões 2011; Cai et al. 2018; Coll et al. 2018; O'Driscoll et al. 2018; Shukla et al. 2018).

Antimicrobial concentrations required to eradicate a biofilm can be up to 10–1000 times higher than their planktonic counterparts, the reason why biofilms have the ability to remain viable after antibiotic treatment and, consequently, a major global healthcare problem (Alanis 2005; Simões 2011; Blackledge et al. 2013; Singh et al. 2017; Ivanova et al. 2018). Besides the severity and required longer and complex treatments related to infections caused by resistant bacteria, they are as well significantly more expensive to diagnose and treat (Simões 2011; Blackledge et al. 2013).

The increase of antibiotic resistance is now recognized as a dynamic process and the abuse of antibiotics has intensified this problematic by inducing microorganisms to modify permeability barriers and drug-target binding locations, develop efflux mechanisms to pump antibiotics out of the target cell, and biosynthesize enzymes that can destroy antibiotics (Ding et al. 2008; Miller et al. 2009). Therefore, in an attempt to overcome this problem, there is a high demand to develop new antimicrobial drugs that target resistant microorganisms.

5.3 Siderophore Iron Uptake System

Iron is a vital component for the growth and development of microorganisms, contributing for the virulence during the establishment of a pathogenic infection (Roosenberg et al. 2000; Ganz 2018). Iron is a transition metal that can exist in two oxidation states, Fe(III) and Fe(II) (Hider and Kong 2010; Saha et al. 2016). This metal has a significant role in some of the most vital enzymatic processes such as oxygen metabolism and DNA and RNA synthesis as well as in the biosynthesis of

porphyrins, antibiotics, toxins, cytochromes, siderophores, aromatic compounds, and nucleic acid (Schalk et al. 2011; Saha et al. 2016). Recently, impact of iron on microbial biofilms has been reported, including in the beginning of the biofilm formation and their maturation into 3-D structures (Saha et al. 2016; Swarupa et al. 2018). Iron is so crucial to cellular mechanisms that the competition for this metal between a host and bacteria is determinant during a bacterial infection. However, in the environment, under the presence of oxygen and at neutral pH, bacteria are faced with the problem of iron-deficient conditions due to fast oxidation of Fe(II) to Fe(III), causing the insolubility of Fe(III). Therefore, to overcome these barriers, many bacteria can produce specific low molecular weight iron (III) chelators named siderophores, to sequester and solubilize iron (Fig. 5.1) (Banin et al. 2005; Chu et al. 2010; Glick et al. 2010; Hider and Kong 2010; Schalk et al. 2011; Chatterjee and O'Brian 2018). Gram-positive bacteria, including *S. aureus*, and Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, *Klebsiella pneumonia*, *A. baumannii*, and acid-fast bacilli, including *Mycobacterium tuberculosis*, all use siderophores, contributing for the infection (Lamont et al. 2002; Smith 2003; Struve and Krogfelt 2004; Cheung et al. 2009; Zimbler et al. 2009).

The siderophore production is commonly detected by a universal and sensitive assay based on the competition for iron between the ferric complex of chrome azurol S and a chelator or siderophore produced by microorganisms, a method developed by Schwyn and Neilands (Schwyn and Neilands 1987). The siderophore production can be detected by using chrome azurol S agar medium as shown in

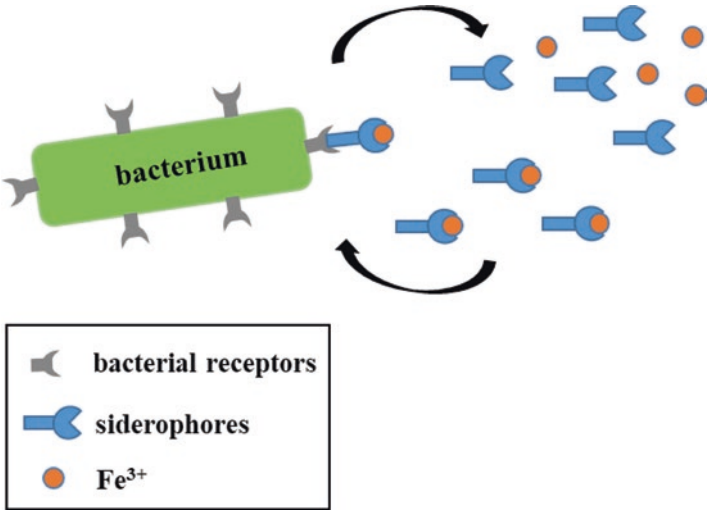

Fig. 5.1 Siderophore-mediated iron uptake by bacteria. High levels of iron are necessary for the biofilm formation and maturation. However, under iron restriction conditions, many bacteria can produce specific low molecular weight iron (III) chelators named siderophores, to sequester and solubilize iron. (Banin et al. 2005; Chu et al. 2010; Glick et al. 2010; Hider and Kong 2010; Schalk et al. 2011).

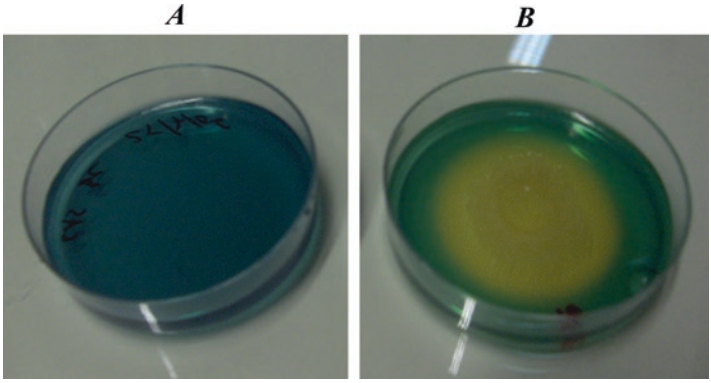

Fig. 5.2 Siderophore production can be detected by using chrome azurol S agar medium (**a**). The development of an orange halo zone against blue background (**b**) was due to the removal of iron from dye complex by *Pseudomonas* spp. siderophore. Thus, it was considered positive for siderophore production (unpublished data)

Fig. 5.2. The development of an orange halo zone against blue background (Fig. 5.2b) was due to the removal iron from dye complex by *Pseudomonas* spp. siderophore. Thus, it was considered positive for siderophore production.

5.3.1 Siderophore Biosynthesis

Siderophores are low molecular weight compounds, 500–1500 Daltons, synthetized by microorganisms under iron-limited conditions, with a high affinity and selectivity for iron (III) (Grobelak and Hiller 2017). The microorganisms use siderophores through tightly mechanisms such as enzymes and transport systems (Miethke and Marahiel 2007). The siderophore biosynthesis occurs via different mechanisms, which is dependent of their chemical nature. Overall, siderophore biosynthesis can be either dependent on or independent of a nonribosomal peptide synthetases (Kohli et al. 2001; Finking and Marahiel 2004). Nonribosomal peptide synthetases are multimodular biocatalysts, which form diverse peptides without the RNA template. Essentially, nonribosomal peptide synthetases consist of adenylation (A) domain, peptidyl carrier protein (PCP) domain, condensation (C) domain, and thioesterase (TE) domain. These four catalytic domain are involved in the formation of hydroxyl, carboxy, and amino acids in several combinations, in a way to generate structural variability (Kohli et al. 2001; Finking and Marahiel 2004; Saha et al. 2013). In general, the iron homeostasis and gene regulation of siderophore is mediated mostly by the ferric uptake repressor or the diphtheria toxin regulator (Miethke and Marahiel 2007). While the ferric uptake repressor is the global iron regulator in several Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, *Klebsiella* spp., *Salmonella enterica*, and low GC content Gram-positive bacteria, including *Bacillus* spp.,

diphtheria toxin regulator achieves a similar function in bacteria with a high GC content, including mycobacteria (Miethke and Marahiel 2007).

5.3.2 Classification of Siderophores

Siderophores have up to six oxygen or nitrogen electron donor atoms that can bind metal cations. They are able to bind effectively with iron by forming an octahedral siderophore-iron complex (Miethke and Marahiel 2007). Siderophores chelate free iron ions and transport them into the cell, requiring flexibility and ability to coordinate Fe(III) (Hider and Kong 2010). The iron complexes can be known by their respective outer membrane receptors, or transport proteins, and therefore be actively transported through the cell membrane into the cell via specific protein channels. Most microorganisms have specific outer membrane proteins that recognize the iron complexes of their native siderophores. Moreover, some microorganisms can develop receptors for siderophore-iron complexes from other microorganisms to assure a competitive growth (Miller et al. 2009; Schalk et al. 2011; Ji et al. 2012; Ahmed and Holmstrom 2014). Over 500 different siderophores are known, of which 270 have been structurally characterized (Hider and Kong 2010; Ahmed and Holmstrom 2014). The siderophores can be classified according to some criteria, for instance, if it is synthetized from plants and microbes; if the backbone is peptide or non-peptides, open chain or cyclic; and the constitution of the chelating group. Siderophores are hexadentate ligands that can be classified into three groups according the chemical nature of the moieties that donate oxygen ligands for Fe(III) coordination, which can be hydroxamates, catecholates, or carboxylates (Table 5.1) (Roosenberg et al. 2000; Miller et al. 2009; Schalk et al. 2011; Ji et al. 2012; Ahmed and Holmstrom 2014).

Microorganisms use different Fe-siderophore transport systems (Ahmed and Holmstrom 2014). Commonly, most of the facultative anaerobic and aerobic bacteria synthetize siderophore under iron stress condition. It has been shown that both Gram-positive and Gram-negative bacteria produce siderophore under iron-deprived conditions (Neilands 1995; Krewulak and Vogel 2008; Chu et al. 2010). Fe(III)-siderophore complexes bind to highly specific receptor proteins. Then, they are transported into the cytoplasm, which is different for Gram-positive and Gram-negative bacteria (Fukushima et al. 2013).

5.3.3 Siderophores in Gram-Positive Versus Gram-Negative Bacteria

Siderophore trafficking is different between Gram-positive and Gram-negative bacteria. While Gram-positive bacteria just have a single membrane, Gram-negative bacteria have inner and outer membranes separated by a periplasmic space as shown in Fig. 5.3 (Ahmed and Holmstrom 2014). In Gram-positive bacteria, due to the

Table 5.1 Classification of siderophores, with the respective mechanism of binding to iron, and example of bacteria responsible for their production

Type of siderophores	Mechanism of binding to iron	Siderophore-producing bacteria	References
Hydroxamate	There is a formation of a bidentate ligand between the two oxygen molecules of each hydroxamate group and the iron and, consequently, each siderophore forms a hexadentate octahedral complex with iron	*E. coli*	Matzanke et al. (2004), Meneely and Lamb (2007), Lamont et al. (2009), Schalk et al. (2011), and Saha et al. (2016)
		Pseudomonas spp.	
		Bacillus spp.	
Catecholate	Formation of a hexadentate octahedral complex between each catecholate group, through two oxygen molecules, and iron	*E. coli*	May et al. (2001), Wilson et al. (2006), Garenaux et al. (2011), Peek et al. (2012), and Saha et al. (2016)
		Pseudomonas spp.	
		Bacillus spp.	
		Klebsiella spp.	
		Salmonella spp.	
Carboxylate	Binding of the carboxylate group to iron by the carboxyl and hydroxyl groups	*Rhizobium* spp.	Drechsel et al. (1995), Dave et al. (2006), and Beasley et al. (2011)
		Staphylococcus spp.	

absence of a periplasmic space, the Fe(III)-siderophore complexes are bound by periplasmic siderophore-binding proteins, which are anchored to the cell membrane (Krewulak and Vogel 2008; Chu et al. 2010; Fukushima et al. 2013; Ellermann and Arthur 2017). Then, the complex is transported through ATP-dependent transporters to the cytoplasm according to the ATP-binding cassette transport systems (Fig. 5.3) (Chu et al. 2010; Fukushima et al. 2013; Ellermann and Arthur 2017).

In Gram-negative bacteria, the process in the cytoplasmic membrane is similar. However, iron uptake in these microorganisms is more complicated since there is an additional lipid bilayer, called outer membrane (Ahmed and Holmstrom 2014). This outer membrane is essential to provide an improved resistance to antibiotics, detergents, degradative enzymes, and host-defense proteins (Faraldo-Gomez and Sansom 2003). Nevertheless, this outer membrane forms a permeable barrier for large nutrients, preventing their transport into the cell cytoplasm. The transport of small nutrients, like amino acids or glucose, across the membrane is typically mediated by small pores present in the outer membranes (Faraldo-Gomez and Sansom 2003; Krewulak and Vogel 2008; Chu et al. 2010). Nevertheless, for ferric-siderophores and other large nutrients, these transport systems are insufficient, requiring their own outer membrane transporters (Krewulak and Vogel 2008; Chu et al. 2010).

Gram-negative bacteria have TonB-dependent outer membrane receptors able to identify the Fe(III)-siderophore complexes (Krewulak and Vogel 2008; Ahmed and Holmstrom 2014). After the binding of the Fe(III)-siderophore complex to the outer membrane receptor, it crosses the membrane by an energy-dependent system, which comprises the outer membrane receptor proteins, periplasmic binding proteins, and inner membrane transport proteins (Faraldo-Gomez and Sansom 2003; Krewulak

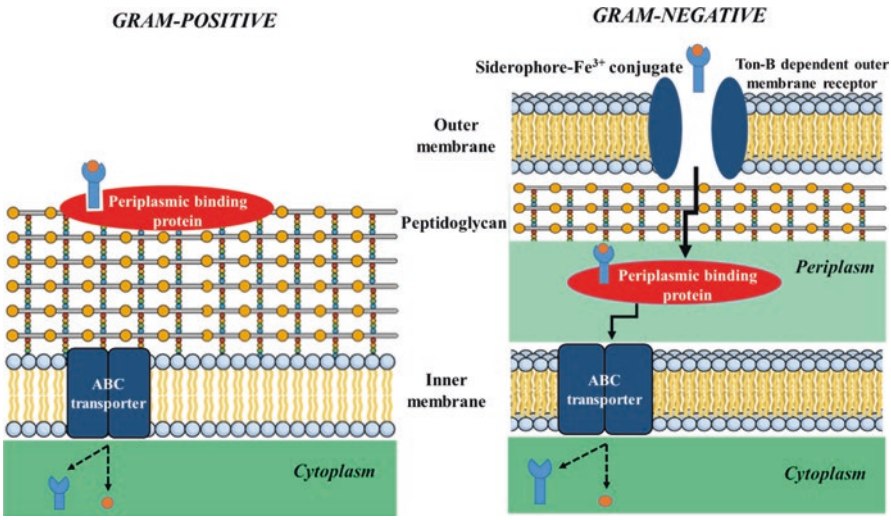

Fig. 5.3 Siderophore trafficking in Gram-positive and Gram-negative bacteria. In Gram-positive bacteria, due to the absence of a periplasmic space, the Fe(III)-siderophore complexes are bound by periplasmic siderophore-binding proteins, which are anchored to the cell membrane. Then, the complex is transported through ATP-dependent transporters to the cytoplasm according to the ATP-binding cassette transport systems. (Krewulak and Vogel 2008; Chu et al. 2010; Fukushima et al. 2013). In Gram-negative bacteria, the process in the cytoplasmic membrane is similar; however, iron uptake in these microorganisms is more complicated since there is an outer membrane. Gram-negative bacteria have TonB-dependent outer membrane receptors able to identify the Fe(III)-siderophore complexes. After the binding of the Fe(III)-siderophore complex to the outer membrane receptor, it crosses the membrane by an energy-dependent system, which comprises the outer membrane receptor proteins, periplasmic binding proteins, and inner membrane transport proteins. Subsequently, this complex, which is bound by a high-affinity periplasmic binding protein, is released into the periplasmic space, transported across the cytoplasmic membrane through an ATP-binding cassette transport system, and finally it reaches the cytoplasm. (Faraldo-Gomez and Sansom 2003; Krewulak and Vogel 2008; Noinaj et al. 2010; Ahmed and Holmstrom 2014)

and Vogel 2008). Subsequently, this complex, which is bound by a high-affinity periplasmic binding protein, is released into the periplasmic space, transported across the cytoplasmic membrane through an ATP-binding cassette transport system, and finally it reaches the cytoplasm (Fig. 5.3) (Faraldo-Gomez and Sansom 2003; Krewulak and Vogel 2008; Noinaj et al. 2010; Ahmed and Holmstrom 2014).

5.3.4 Siderophores as Virulence Determinants

Bacterial pathogens when faced with iron-deprived conditions of the human body produce siderophores to acquire the iron necessary for growth and development. Besides iron chelation, siderophores are taking a critical role in virulence mechanisms, as they act as signals to host defense by inducing cytokine production,

mitophagy, and hypoxic responses (Wilson et al. 2016). Some siderophores have the capacity to trigger cytokine expression such as desferrioxamine, a nonpathogenic siderophore that has been shown to induce IL-8 secretion through p38 mitogen-activated protein kinase signaling in a lung carcinoma and intestinal epithelial cell lines. Desferrioxamine is also involved in the stabilization of the master transcription factor hypoxia inducible factor 1. Hypoxia inducible factor 1 controls the expression of several genes families, namely, genes associated with glycolysis, angiogenesis, iron metabolism, and inflammation, and it is regulated by the availability of both oxygen and iron starvation within a cell (Holden et al. 2014, 2016; Behnsen and Raffatellu 2016; Wilson et al. 2016). Therefore, siderophores assume an important role in inducing host defense pathways that are essential for host survival.

5.4 Antimicrobial Applications of Siderophores

Siderophore iron uptake systems have been recognized as a promising antimicrobial approach due to their crucial role for the survival of pathogenic microorganisms (Miethke and Marahiel 2007). One of the most important applications of these systems is selective drug delivery, a Trojan Horse strategy, mainly focused in antibiotic resistant bacteria. Several factors can dramatically upsurge the bacterial resistance, including the outer membrane permeability barrier, target alteration, and drug inactivating enzymes (Ferreras et al. 2005; Miller et al. 2009). Thus, this can be a strategy to overcome this membrane-mediated resistance, in which siderophores can act as transport vectors of drugs through to the inner parts of the cell. In addition, other different approaches such as inhibition of siderophore biosynthesis and iron starvation via competitive chelation were also explored (Ferreras et al. 2005; Miller et al. 2009).

5.4.1 Siderophore-Mediated Drug Delivery

Siderophores are a newer antimicrobial strategy especially valuable to circumvent membrane-associated drug resistance, using their iron transport capacity to bring drugs into cells by conjugates of siderophores and antimicrobials, by the Trojan Horse approach (Miller et al. 2009; Mollmann et al. 2009). The drug that is incapable to cross the bacterial membrane barrier is linked to a siderophore. Then, the Fe(III)-siderophore complex is recognized by the cognate receptor and transported across the outer membrane with the drug attached (Fig. 5.2) (Roosenberg et al. 2000; Miller et al. 2009; Mollmann et al. 2009). Therefore, siderophore-drug conjugates are constituted by four components consisting of iron, a siderophore for binding the iron, a linker for attaching the drug to the siderophore, and the drug, as shown in Fig. 5.4. All the components serve a vital function. When the siderophore-drug conjugate reaches the cytoplasm, the microorganism may be killed by several

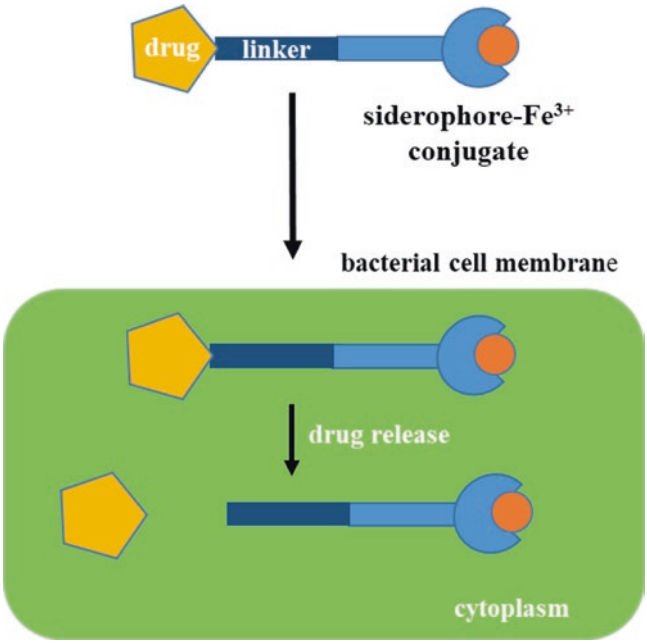

Fig. 5.4 Trojan Horse strategy. Siderophore-drug conjugates are constituted by four components consisting of iron, a siderophore for binding the iron, a linker for attaching the drug to the siderophore, and the drug. When the siderophore-drug conjugate reaches the cytoplasm, the microorganism may be killed by several reasons, including release of the drug, act as a whole antibacterial agent, or block more iron assimilation (Ding et al. 2008; Miller et al. 2009; Mollmann et al. 2009)

reasons, including release of the drug, act as a whole antibacterial agent, or block more iron assimilation (Ding et al. 2008; Miller et al. 2009; Mollmann et al. 2009).

There are bacteria that have been developed their own natural Trojan Horse conjugates, recognized as sideromycins, which include the salmycins, ferrimycin, and albomycins, for instance, albomycin d2. As a first approach, natural siderophores coupled to antibiotics have been used as proof of concept. It has been reported that albomycin exhibit in vitro and in vivo antibacterial action against both Gram-positive and Gram-negative bacteria (Braun et al. 2009). Albomycin synthesized by *Actinomyces subtropicus* is constituted by a hydroxamate siderophore, similar to ferrichrome, linked to a thioribosyl pyrimidine antibiotic through a serine spacer, and it is recognized by the outer membrane ferrichrome receptor protein FhuA. When albomycin reaches the bacterial cell, the release of the antibiotic by enzymatic cleavage and, consequently, the cell death occurs (Ferguson et al. 2000; Braun et al. 2009; Page 2013). They have been shown to be very effective in mouse infection models (Braun et al. 2009). Likewise, salimycins have a trihydroxamate siderophore linked to an aminoglycoside antibiotic through a dicarboxylic acid (Braun et al. 2009). Salimycins have shown strong and selective antibacterial activity against Gram-positive bacteria, even in antibiotic resistant strains (Braun et al.

2009). Like the albomycins, this potent antibacterial effect is due to active transport of the salmycins through hydroxamate siderophore membrane transport proteins (Bunet et al. 2006). Ferrimycin, another naturally occurring sideromycins, has attached a moiety with antibiotic activity to ferrioxamine B by an amide link (Pramanik and Braun 2006). The occurrence of natural siderophore antibiotic has opened the way to produce synthetic Trojan Horses.

A series of synthetic siderophore-drug conjugates have been designed, synthesized, and biologically evaluated, and the results are encouraging, indicating that the rational design and synthesis of effective conjugates between a siderophore and an antibacterial agent are possible. A conjugate consisting of a mixed ligand catechol-hydroxamate siderophore linked to Loracarbef, a syntheticβ-lactam antibiotic of the carbacephem class for oral administration, presented a 2000-fold increase in the antibacterial action against *Acinetobacter* comparatively to the control drug (Ghosh et al. 1996). Recently, in another study, functionalized enterobactin-β-lactam conjugates were synthetized and exhibited a1000-fold increase in the antibacterial activity against *E. coli* bacteria comparatively to the control β-lactam drug (Zheng and Nolan 2014). A conjugate composed by a hydroxypyridinone-based iron chelator and a monosulfactam antimicrobial, currently undergoing clinical trials, has presented powerful action against several Gram-negative bacteria, including multidrug-resistant *P. aeruginosa* and multidrug-resistant *Acinetobacter* spp. (Page et al. 2010; Russo et al. 2011). Therefore, the data presented here showed that these conjugates can be used to successfully transport antibacterial agents across the outer membrane permeability barrier of Gram-negative bacteria and be an efficient approach to fight multidrug-resistant infections. Although mostly catecholate and hydroxamate siderophores have been used as antimicrobials delivery vehicles, carboxylate siderophores, as for instance, staphyloferrin A, can be an effective choice in topical applications since this siderophore type exhibited improved iron chelating properties in acidic environments when compared to catecholate and hydroxamate siderophores (Milner et al. 2013; Saha et al. 2016). The hydrophilic characteristic of staphyloferrin A enhances the water solubility of its conjugates and improves the transport rate of the drug to the bacterial cell (Milner et al. 2013; Saha et al. 2016). *S. aureus* has the ability to colonize human skin and the gut, and, besides that, some of the most difficult healthcare-related infections, such as lower respiratory tract infections, bacteraemias, and surgical site infections, are due to methicillin-resistant *S. aureus*, and these bacteria are known to secrete staphyloferrin siderophores (Milner et al. 2013). Several staphyloferrin-based Trojan Horse conjugates have been synthetized as well as the evaluation of the antimicrobial properties of those conjugates on *S. aureus*. These conjugates have the capacity to target staphylococci, including *S. aureus*, because they are known to produce staphyloferrin-type siderophores. It was reported that a conjugate based on staphyloferrin A was found to present antimicrobial activity (Milner et al. 2013).

5.4.1.1 Siderophore-Trojan Horse Systems for Medical Diagnostic Imaging

Early and accurate diagnosis is a crucial factor for successful therapy, underlining the pressing need for precise and sensitive diagnostic tools. Nevertheless, the existent diagnostic methods, like computer tomography and laboratory tests, possess some limitations, particularly concerning to sensitivity and specificity (Signore and Glaudemans 2011; Auletta et al. 2016; Mills et al. 2016; Petrik et al. 2017). Molecular imaging procedures can offer a more robust, noninvasive, selective, and sensitive diagnosis of infections, improving the clinical decisions as well as the patient management with better healthcare outcomes (Signore and Glaudemans 2011; Auletta et al. 2016; Mills et al. 2016; Petrik et al. 2017).

In this context, siderophores in Trojan Horse systems can be used for diagnosis purposes, and, for that, the siderophores are not meant to target and kill bacteria, but instead they are only used as a vehicle (Noël et al. 2011; Szebesczyk et al. 2016). A siderophore-diagnostic probe conjugate is constituted by a siderophore extracted from bacteria or a synthetic analogue of a siderophore, which will be responsible for the transport of diagnosis probe to target the bacterial cell without affecting mammalian cells. This is because the biosynthesis and uptake systems in mammalian cells are absent, and, consequently, there is not interaction between these compounds and human cells (Szebesczyk et al. 2016). Then, the conjugation of antibacterial agents to siderophores may improve their specificity to the bacterial cell (Noël et al. 2011; Szebesczyk et al. 2016). The preparation of labelled siderophores can be or by the insertion of the radiometal to the natural iron-siderophore complex through the exchange of iron or artificially by the alteration of natural siderophore with a chromophore appropriate for optical imaging (Ouchetto et al. 2005; Petrik et al. 2017). Radiolabelled siderophores may be highly specific means for infection imaging with the advantage that these siderophores are not used by mammals (Szebesczyk et al. 2016; Petrik et al. 2017).

5.4.2 Inhibition of Siderophore Biosynthesis

The siderophore biosynthesis may be inhibited through compounds that have the capacity to prevent bacterial growth in iron-limiting conditions (Cornelis 2010; Youard et al. 2011; Lamb 2015). The bacteria that have acquired numerous iron acquisition routes may be more resistant to antimicrobial agents developed against a single siderophore system for ion scavenging. *P. aeruginosa* is an example of a bacterium that possesses molecular mechanisms to synthesize two siderophores: pyoverdine, extremely effective nonetheless metabolically expensive, and pyochelin, not efficient however metabolically inexpensive (Cornelis 2010; Youard et al. 2011; Dumas et al. 2013; Lamb 2015).

P. aeruginosa is one of the most relevant nosocomial bacteria responsible for human opportunistic infections, being a leading pathogen in chronic lung infection in patients with cystic fibrosis. Furthermore, multidrug-resistant *P. aeruginosa* has been identified as a main pathogen in healthcare sceneries (Driscoll et al. 2007; Page and Heim 2009; Juan et al. 2017). Pyoverdine, a siderophore secreted by *P. aeruginosa*, may be an interesting target for antivirulence compounds, since besides their fundamental role during *P. aeruginosa* biofilm formation, it is also responsible to control virulence gene expression (Lamont et al. 2002; Visca et al. 2007; Kang and Kirienko 2017). The factor PvdS is responsible for expression of the pyoverdine biosynthesis genes when iron is limited and also promotes the expression of key virulence factors, including exoproteases and exotoxin A (Imperi et al. 2013; Granato and Kümmerli 2017). The compound flucytosine, a synthetic fluorinated pyrimidine, was shown to have an essential role in the inhibition of *pvdS* gene expression, thus triggering downregulation of virulence genes like these involved in pyoverdine biosynthesis. It was found that the compound flucytosine suppressed the pathogenicity of *P. aeruginosa* in a mouse model of lung infection (Imperi et al. 2013).

S. aureus is recognized as one of the major nosocomial human pathogen, and it has been developed resistance to methicillin as well as to several other antibiotics. Therefore, there is a high demand for the development of novel antimicrobial strategies to fight the infections caused by this pathogen. *S. aureus* produce two citrate-based siderophores, designated staphyloferrin A and staphyloferrin B. However, staphyloferrin B appear to have a more critical role for survival under iron-depriving conditions (Dale et al. 2004; Cheung et al. 2009; Cheung et al. 2012; Miao et al. 2017), and the inactivation of this siderophore biosynthesis showed a reduction in the virulence of *S. aureus* in a mouse infection model (Dale et al. 2004).

Additionally, baulamycin A and baulamycin B, two molecules extracted from *Streptomyces tempisquensis*, presented a high inhibition of siderophore biosynthesis pathways in numerous bacteria, such as *E. coli* and methicillin-resistant *S. aureus*. It was shown that they were effective in vitro as well as capable to penetrate the bacterial barriers to prevent bacterial growth, both Gram-positive and Gram-negative bacteria, proposing that they may be used as broad-spectrum antibiotics (Tripathi et al. 2014).

5.4.3 Iron Starvation via Competitive Chelation

The biofilm formation and development are strongly dependent on iron availability, and, for this reason, iron chelation may be an encouraging and novel approach to control biofilms. The antibacterial activity may be achieved by the ability of non-metabolizable iron chelators to diminish iron that could otherwise be used for bacterial replication (Raymond et al. 2003; Wandersman and Delepelaire 2004; Moreau-Marquis et al. 2009). Through host proteins, including transferrin, lactoferrin,

or ferritin, which act as iron chelators, the natural host defenses maintain a low level of existing circulating iron inside microorganisms (Raymond et al. 2003; Wandersman and Delepelaire 2004; Moreau-Marquis et al. 2009).

Very effective chelators may deprive pathogenic microorganisms of the iron essential for growth. Thus, iron chelators can be used as therapeutic compounds. Numerous compounds have been subject of study to evaluate their capability to avoid biofilm formation or disrupt established biofilms on abiotic surfaces. The iron-binding protein lactoferrin, present in airway secretions, was able to inhibit biofilm formation by preventing *P. aeruginosa* from adhering on surfaces (Singh et al. 2002). The metal chelator EDTA, disodium salt of ethylenediaminetetraacetic acid, is recognized to have antimicrobial action against biofilms of Gram-positive bacteria, including *S. aureus* and *S. epidermidis* (Raad et al. 2003), being also a potent biofilm disruptor of *P. aeruginosa* (Banin et al. 2006). It was also observed that the combination of tobramycin, the main antibiotic used to treat cystic fibrosis lung infections, with deferoxamine or deferasirox, two FDA-approved iron chelators, reduced established biofilm biomass as well as prevented the biofilm formation of *P. aeruginosa* on cystic fibrosis airway cells. Furthermore, it was observed that none of these three compounds alone presented such a pronounced effect, suggesting that the combination of tobramycin and deferoxamine or deferasirox may be a good strategy to treat patients with cystic fibrosis as well as other lung diseases (Moreau-Marquis et al. 2009).

It has also been known that gallium, due to its chemical similarities to iron, can replace iron in several biological systems and disrupt iron-dependent processes, since unlike Fe(III), Ga(III) cannot be reduced. Therefore, disrupting bacterial iron metabolism by gallium can lead to destruction of biofilms (Kaneko et al. 2007; Banin et al. 2008; Bonchi et al. 2014). It was found that gallium inhibited the biofilm formation of *P. aeruginosa* and killed planktonic and sessile bacteria in vitro. The action mode of gallium is based on the decrease of bacterial iron uptake and the interference with iron signaling by the *pvdS* (Kaneko et al. 2007). Antimicrobial effects have been also observed against multidrug-resistant isolates of *Acinetobacter baumannii*, which is considered one of the most resistant pathogens among Gram-negative bacteria (Peleg and Hooper 2010; Antunes et al. 2012; de Leseleuc et al. 2012). It was demonstrated that gallium (III) exerted a strong in vitro and in vivo activity against *A. baumannii*, suppressing the growth of genotypically diverse multidrug-resistant *A. baumannii* strains. The inhibitory effect of gallium (III) was counteracted by iron, showing that in fact the gallium (III) in *A. baumannii* acts in the disruption of iron metabolism (Antunes et al. 2012).

The similar properties between gallium and iron allow it to bind to determined iron-binding proteins. This competition of gallium with iron in biologic systems is relevant for antimicrobial purposes. Nevertheless, it is important to take in account its toxic effects on mammalian cells (Chitambar 2016). Qiu et al. (2014) evaluated the cytotoxicity of titanium-gallium alloys containing 10% of gallium on L929 fibroblasts and MG63 osteosarcoma cells and observed no significant toxic effects.

The cells were capable to adhere and proliferate (Qiu et al. 2014). A study based on the development of a nanocomposite consisting of hydroxyapatite and gallium nanoparticles showed strong antibacterial properties against *P. aeruginosa* and low in vitro cytotoxicity for human lung fibroblasts IMR-90 and L929 fibroblasts (Kurtjak et al. 2016). Furthermore, it has also been reported that gallium shows anti-inflammatory, immunosuppressive, and antitumor properties (Eby 2005; Verron et al. 2012; Chitambar 2017; Wang et al. 2017).

5.5 Conclusion

The bacteria mostly live as biofilms, complex communities adhered on surfaces, which are associated to numerous health problems and contribute to more than 80% of human infections. In the fight of these pathogenic biofilms, the increase of antibiotic resistance is an emerging public health concern. Current treatments are being commonly less efficient, which makes crucial development of novel antimicrobial compounds and strategies. One approach that has been exploited to overcome the drug resistance is to target the iron metabolism of bacteria. This metal is essential for the growth and survival of bacteria, being in some cases a main virulence factor throughout the establishment of a bacterial infection. To date, three main strategies have been evaluated to interfere with several levels of the bacterial iron metabolism. The Trojan Horse approach by using siderophores has the capacity to deliver antibacterial drugs inside the cells, overcoming permeability-related resistance through the delivery of the drug via the iron uptake systems. The use of inhibitors of siderophore biosynthesis also holds considerable potential as an antimicrobial approach, preventing the bacterial growth in iron-limited conditions. The use of iron chelators to decrease iron availability may be another effective strategy. Collectively, the promising data presented in this chapter might expand the lineup of siderophores for new and innovative broad-spectrum antimicrobial drugs, which can attend as significant lead to develop powerful anti-infective agents.

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Chapter 6
An Update on Bioactive Natural Products from Endophytic Fungi of Medicinal Plants

Nisha Sharma, Vishal Sharma, Vidushi Abrol, Anil Panghal, and Sundeep Jaglan

Contents

Abstract Pathogens are developing resistance against the current regime of drugs, which urge the need of novel drugs. This has led scientists to explore natural sources that are safe as well as potent. Microbes have been explored for over decades for natural products and have been the vast reservoir of secondary metabolites of drugs

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potential. Because of their huge diversity and particular habituation, they can act as good resource to obtain bioactive secondary metabolites. Endophytes have been exploited to get drug-like molecules that have antibacterial, antifungal, anticancer, antioxidant, antidiabetic, antileishmaniasis, and antiviral activities. Here we review endophytic fungi as storehouse of naturally occurring bioactive secondary metabolites.

6.1 Introduction

De Bary in 1866 coined the term "endophyte," and according to him, "Endophytes are the microorganisms, which reside inside the plant tissues and are significantly different from those found on the plant surface." Since then a large number of definition were given by various researchers; each definition collectively says "Endophytes are the microbes that reside intra or intercellular in the tissues of host plants and there they do not give rise to any apparent deleterious effects." The endophytes have been found to be an excellent producer of bioactive metabolites of pharmaceutical importance (Bascom-Slack et al. 2009; Pimentel et al. 2011; Brader et al. 2014). Today most of the world population depends on natural products for treating various diseases because of its least side effects. Endophytes are propitious source of bioactive compounds with pharmaceutical importance, and many secondary metabolites of microbial origin are under clinical trials. The natural products were either derived from plants or microorganisms, and among microorganisms, endophytes have the major contribution. Since both plants and microbes have largely been explored for the isolation of compounds, bioprospection of microbes from unique niches would serve to gain the source of new metabolites to combat the upcoming challenges like resistance against the available drugs in the market (Kudo et al. 1998; Zuck 1998; Qin et al. 2010).

Due to extensive diversity of plants, it is expected that microbes residing inside them will act as an excellent source of novel compounds of various bioactivities. An endophytic fungus associates specifically with their host in a facultative or obligate or symbiotic relationship and mediates various signals of mutual relationship (Nair and Padmavathy 2014). These signals depend on the environment which the microbe faces inside the host plant, resulting in the specificity with their host (Dudeja et al. 2012). The endophytes face biotic and abiotic stresses inside the host plant which they overcome through interaction with each other and also with their host plant, resulting to produce diverse secondary metabolites (Liu et al. 2010; Shimizu et al. 2001) (Fig. 6.1). The majority of the bioactive compounds produced by endophytic fungi belong to the different structures and classes, such as quinolones, flavonoids, steroids, phenols, xanthones, terpenoids, etc. (Tan and Zou 2001) exhibiting diverse bioactive potentials including antibacterial, antifungal, antioxidants, antiviral, anticancer, immunosuppressive, etc. (Gunatilaka 2006; Qin et al. 2008). Being a source of structurally diverse and novel metabolites, the endophytic fungi would be

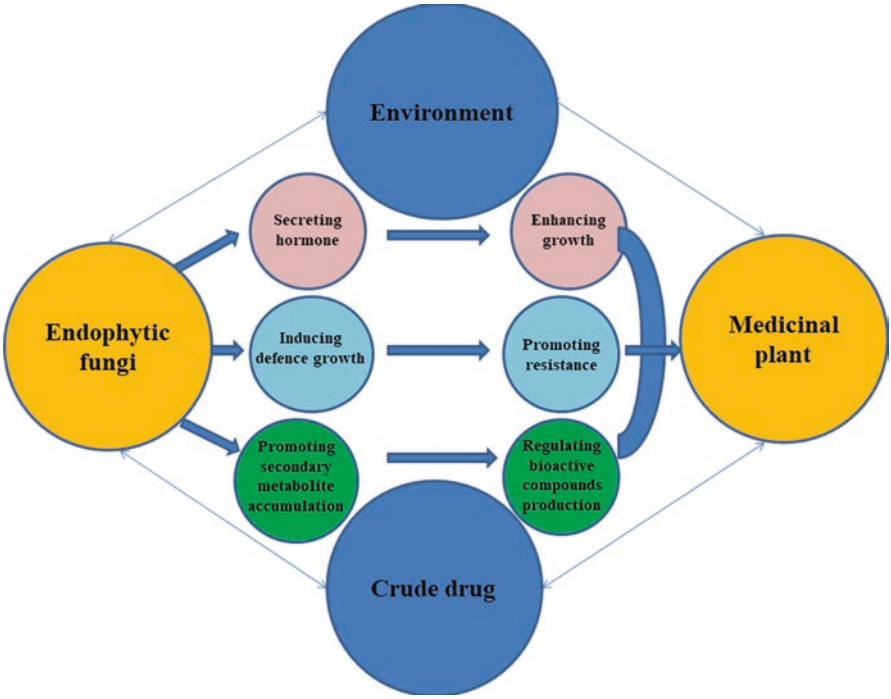


Fig. 6.1 Relationship of endophytic fungi with their host, enhancing growth, promoting resistance, and regulating the production and accumulation of secondary metabolites

promising to obtain the novel compounds if unique niches may explored. A schematic representation to exploit the endophytic fungi for bioactive metabolites has been given in Fig. 6.2.

6.2 Endophytic Fungi as a Storehouse of Bioactive Compounds

Endophytic fungi remain rich sources of many important therapeutic agents. Since decade endophytic fungi have been extensively explored for bioactive natural products. This appears to be emerging area of research which could give a boost to the drug discovery programs. Here we have reviewed and compiled the bioactive compounds reported from the endophytic fungi since the last 5 years. The secondary metabolites of antimicrobial, anticancer, antioxidant, antidiabetic, antileishmaniasis, and antiviral potential obtained from endophytic fungi of medicinal plants are listed and discussed below in the following sections.

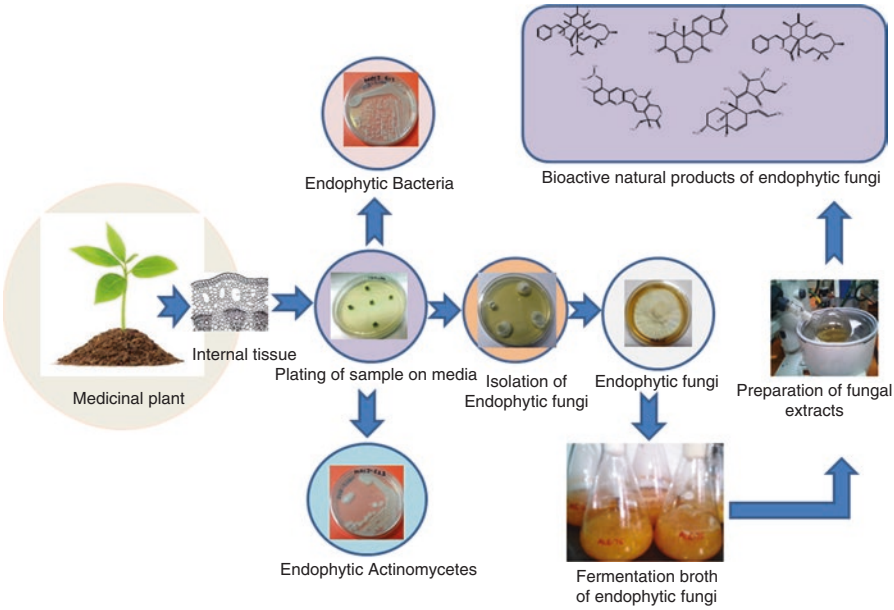


Fig. 6.2 Production of bioactive metabolites in endophytic fungi

6.2.1 Antibacterial Compounds from Endophytic Fungi

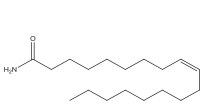
Antibacterial compounds can be defined as the molecules which have potential to kill or inhibit the growth of other microorganisms. Since two decades there has been an extensive hike of penicillin-resistant *Enterococcus faecium*, *Staphylococcus pneumonia*, and methicillin-resistant *Staphylococcus aureus* (MRSA). In view of resistance and inadequate number of drugs against the pathogens, there is a need for the hunt of new and diverse drugs that can confront with the upcoming challenges of variety of infections although a huge number of diverse and novel compounds with varied bioactivities have been reported from endophytic fungi but still many more to be discovered. The reviewed literature clearly indicates that the secondary metabolites produced by endophytic fungi belonging to diverse class of compounds such as flavonoids, xanthones, terpenoids, alkaloids, phenols, steroids, and quinines have antibacterial activities (Gao et al. 2018); the recent findings have been enlisted in Table 6.1 and represented in Fig. 6.3.

An endophytic fungus, *Colletotrichum gloeosporioides* of *Lannea coromandelica*, displayed considerable antimicrobial activity. One of the metabolite showed highest zone of inhibition with 25 mm zone formation against *Staphylococcus aureus*. The main compounds responsible for antimicrobial activity were 9-octadecenamide **(1)**, hexadecanamide **(2)**, diethyl phthalate **(3)**, 2-methyl-3-methyl-3-hexene **(4)**, and 3-ethyl-2, 4-dimethyipentane **(5)** (Tayung et al. 2011). In one study, the Di-n-octyl phthalate **(6)** was reported from *Aspergillus terreus* MP15,

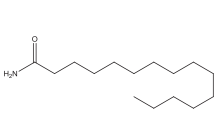
Table 6.1 Antibacterial compounds derived from endophytic fungi

Compound	Endophytic fungi	Host plant	References
9-octadecenamide **(1)**, hexadecanamide **(2)**, diethyl phthalate **(3)**, 2-methyl-3-methyl-3-hexene **(4)**, and 3-ethyl-2,4-dimethyi-pentane **(5)**	*Colletotrichum gloeosporioides*	*Lannea coromandelica*	Tayung et al. (2011)
Di-n-octyl phthalate **(6)**	*Aspergillus terreus*	*Swietenia macrophylla*	Yin et al. (2015)
Ergosterol **(7)** and cerevisterol **(8)**	2L-5	*Ocimum basilicum*	Haque et al. (2005)
Fatty acid and phenolic compound	*Diaporthe phaseolorum*	*Tectona grandis L.f*	Kumala et al. (2015)
Tannins, phenols, and amino acid	*Stemphylium radicinum*	Calyptus	Hussain et al. (2014)
Colletotrichones A **(9)**, colletotrichones B **(10)**, and colletotrichones C **(11)**	*Colletotrichum* sp. BS4	*Buxus sinica*	Wang et al. (2016)
Equisetin **(12)**	*Fusarium* sp.	*Opuntia dillenii*	Ratnaweera et al. (2015)
Asperamide A **(13)** and cerebroside C **(14)**	*Aspergillus niger*	*Ipomoea batatas*	Shaaban et al. (2013)
Fusarubin methyl ether **(15)**	*Cladosporium* sp.	*Rauwolfia serpentina*	Khan et al. (2016)
Viriditoxin **(16)**	*Paecilomyces variotii*	*Laguncularia racemosa*	Silva et al. (2013)
Purpureone **(17)**	*Purpureocillium lilacinum*	*Rauvolfia macrophylla*	Lenta et al. (2016)
Alternariol 9-methyl ether **(18)**	*Alternaria* sp.	*Salvia miltiorrhiza*	Lou et al. (2016)
18-ethoxycytochalasin J **(19)**, cytochalasins H **(20)**, and cytochalasins J **(21)**	*Phomopsis* sp.	*Garcinia kola*	Jouda et al. (2016)
Phomosine A **(22)** and Phomosine C **(23)**	*Diaporthe* sp.	*Siparuna gesnerioides*	Sousa et al. (2016)
Terrein **(24)**	*Aspergillus terreus*	*Achyranthes aspera*	Goutam et al. (2017)
(22*E*,24*R*)-stigmasta-5,7,22-trien-3-β-ol **(25)**	*Aspergillus terreus*	*Carthamus lanatus*	Elkhayat et al. (2016)
Beauvericin **(26)**	*Epicoccum nigrum*	*Entada abyssinica*	Dzoyem et al. (2017)
Alternariol **(27)** and 3,7-dihydroxy-9-methoxy-2-methyl-6Hbenzo[c]chromen-6-one **(28)**	*Alternaria alternata*	*Grewia asiatica*	Deshidi et al. (2017)

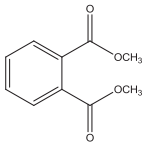
a fungal endophyte of *Swietenia macrophylla*. The Di-n-octyl phthalate was found to be used as food preservative along with antibacterial potential against foodborne pathogenic bacteria that are *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus spizizenii*, and *Bacillus subtilis* (Yin et al. 2015). Antibacterial compounds can also be used as a food preservative, which acts to control the food spoilage that is the main concern for the problems created in the food chain (Liu and Xu 2008).



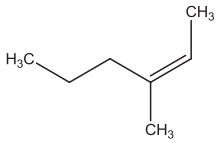
9-octadecenamide **(1)**



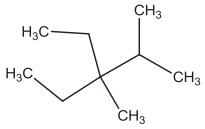
Hexadecanamide **(2)**



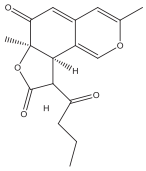
Diethyl pythalate **(3)**



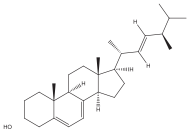
2-methyl-3-methyl-3-hexene **(4)**



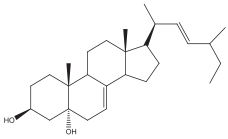
3-ethyl-2,4-dimethyi-pentane **(5)**



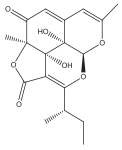
Di-n-octyl phthalate **(6)**



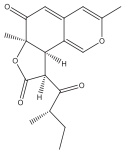
Ergosterol **(7)**



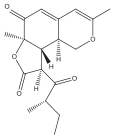
Cerevisterol **(8)**



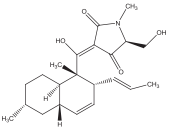
Colletotrichones A **(9)**



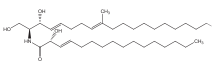
Colletotrichones B **(10)**



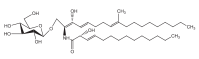
Colletotrichones C **(11)**



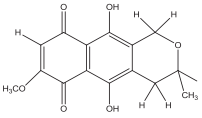
Equisetin **(12)**



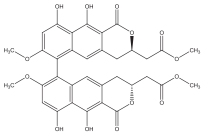
Asperamide A **(13)**



Cerebroside C **(14)**



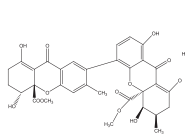
Fusarubin-methyl ether **(15)**



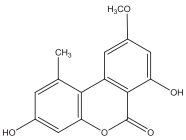
Viriditoxin **(16)**

Fig. 6.3 Antibacterial compounds (**1-28**) from endophytic fungi

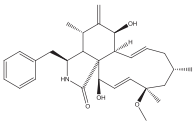
Similarly, *Ocimum basilicum* was explored, resulting to obtain 23 endophytic fungi from healthy leaf, stem, and roots. One of the endophytic fungi among them has been reported to produce two steroidal compounds, ergosterol **(7)** and cerevisterol **(8)**. The compounds were found to have antibacterial potential against *Bacillus cereus* and *Staphylococcus aureus* (Haque et al. 2005). Jati tree (*Tectona grandis L.f*) is an herbal plant that acts as host of an endophytic fungus *Diaporthe phaseolorum*. Four fractions were obtained from this fungus by using column chromatography;



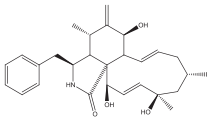
Purpureone **(17)**



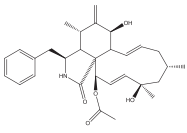
Alternariol 9-methyl ether **(18)**



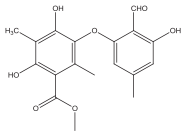
18-methoxy cytochalasin J **(19)**



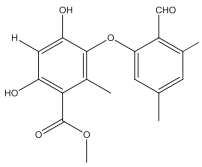
Cytochalasins H **(20)**



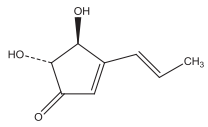
Cytochalasins J **(21)**



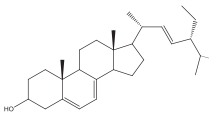
Phomosine A **(22)**



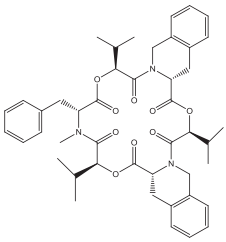
Phomosine C **(23)**



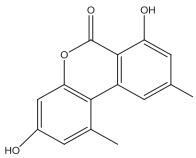
Terrein **(24)**



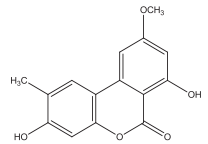
(22*E*,24*R*) -stigmasta-5,7,22-trien-3-*β*-ol **(25)**



Beauvericin **(26)**



Alternariol **(27)**



3,7-dihydroxy-9-methoxy-2-methyl-6Hbenzo[c] chromen-6-one **(28)**

Fig. 6.3 (continued)

consequently the fraction IV was found to have potent antibacterial activity with a zone of inhibition at 11.10 mm when tested against *Staphylococcus aureus*. Further, a fatty acid and a phenolic compound have been reported via GCMS in the bioactive fraction (Kumala et al. 2015).

Stemphylium radicinum an endophytic fungus was reported from the *Calyptus* roots collected from Misan city of Iraq. The extract of the fungus was found to have tannins, phenols, and amino acid as active secondary metabolites and shows potent activity against *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Streptococcus pyogenes*. The antibacterial activity was performed using the disc diffusion technique, and the zones of inhibition were reported in the range of 22.5–35.5 mm with MIC of 25.0–100 μg/mL (Hussain et al. 2014).

Besides a reported compound chermesinone B, three novel, colletotrichones A−C **(9–11)** were isolated from the fungus *Colletotrichum* sp. BS4. The fungus was isolated from the leaves of traditional medicinal plant *Buxus sinica* of China. The compounds were evaluated for antibacterial potential tested with *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Colletotrichone A **(9)** was having MIC of 0.1 and 1.0 μg/mL against *B. subtilis* and *E.coli*, respectively, whereas the compound **10** and **11** shows activity with MIC value of 5.0 μg/mL against *S. aureus* and *E. coli*, respectively (Wang et al. 2013).

In one study, eight fungi were isolated from *Opuntia dillenii* and reported first time, among them the *Fusarium* sp. and *Aspergillus niger* were found to be the most bioactive. The fractionation of the extract of *Fusarium* sp., followed by bioactivity, led to the isolation of tetramic acid derivative, named equisetin **(12)**. Equisetin exhibited MIC value of 8 μg/mL against *Bacillus subtilis* and 16 μg/mL against MRSA and *Staphylococcus aureus*. The research article also concluded that the endophytic fungi isolated from rough and combative environment can also act as a source for diverse and novel bioactive compounds (Ratnaweera et al. 2015).

Aspergillus niger isolated from the leaves of *Ipomoea batatas* (sweet potato) was found to produce eight diverse bioactive compounds. The isolated compounds were characterized as ergosta-7,22-dien-3β,5α,6α-triol, aurasperone B, aurasperone C, aurasperone F, asperamide A **(13)**, cerebroside C **(14)**, linoleic acid, and R(-)-glycerol monolinoleate. Compounds **13** and **14** exhibited weak to moderate antibacterial activities (11–17 mm), among them cerebroside C showed higher activity than those of the non-glycosylated asperamide A. Cytotoxic examination of the isolated compounds against the brine shrimp confirmed their activities to range between moderate (28–35%) and weak (9–11%) (Naureen et al. 2015).

Two naphthoquinones, namely, anhydrofusarubin and methyl ether of fusarubin **(15)**, were isolated from an endophytic fungus, *Cladosporium* sp., isolated from the leaves of *Rauwolfia serpentina*. Fusarubin methyl ester at a concentration of 40 μg/disc exhibited prominent antibacterial activities against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus megaterium* with zone of inhibition of 27 mm, 25 mm, 24 mm, and 22 mm, respectively (Khan et al. 2016).

Viriditoxin **(16)** was isolated using column chromatography from *Paecilomyces variotii*, an endophytic fungus isolated from the healthy leaves of *Laguncularia racemosa*. Viriditoxin showed antibacterial activity against *Staphylococcus aureus* and *Enterococcus* sp. with MIC of 0.5 and 2 μg/mL, respectively (Silva et al. 2013). A new ergochromone derivative purpureone **(17)** was reported from an endophytic fungus *Purpureocillium lilacinum*, isolated from the roots of *Rauvolfia macrophylla*. Purpureone exhibited antibacterial activity against *Bacillus cereus*, *Escherichia coli*, and *Providencia stuartii* with MIC values below 62.6 μg/mL (Lenta et al. 2016).

Alternariol 9-methyl ether **(18)** isolated from the extract of *Alternaria* sp., an endophytic fungus from the roots of *Salvia miltiorrhiza Bunge*, inhibited the growth of six bacteria, i.e., *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Pseudomonas lachrymans*, *Ralstonia solanacearum*, *Staphylococcus haemolyticus*, and *Xanthomonas vesicatoria* with MIC values ranging from 25 to 75 μg/mL (Lou et al. 2016).

Three reported cytochalasins that are 18-methoxycytochalasin J **(19)**, cytochalasin H **(20)**, and cytochalasin J **(21)** along with alternariol were reported from *Phomopsis* sp., an endophytic fungus obtained from the nuts of *Garcinia kola*. The cytochalasins exhibited strong activity against *Shigella flexneri*. The antibiotic ampicillin at concentration of 512 μg/mL was not able to show any inhibition against *Vibrio cholerae* NB2, *Vibrio cholerae* PC2, and *Shigella flexneri*, but interestingly *Shigella flexneri* was sensitive to the cytochalasins (Jouda et al. 2016).

Six compounds, 4H-1-benzopyra-4-one-2,3-dihydro-5-hydroxy-2,8-dimetyl, 4H-1-benzopyran-4-one-2,3-dihydro-5-hydroxy-8-(hydroxy-lmethyl)-2-methyl, 4H-1-benzopyra-4-one-2,3-dihydro-5-methoxyl-2,8-dimetyl, phomosine A **(22)**, phomosine C **(23)**, and phomosine D, were isolated from endophytic fungus *Diaporthe* sp. F2934 of *Siparuna gesnerioides*. Compound **22** at concentration 4 μg/mL showed 20% higher inhibition zone diameter (IZD) than vancomycin, standard against *Staphylococcus aureus*. It also exhibited activity against both Gram (+ve) and (−ve) bacteria, with respective IZD of 77–90% and 83–92%. Phomosine C showed an IZD against *S. aureus* (90%), *Micrococcus luteus* (60%), and *Streptococcus oralis* (61%) (Sousa et al. 2016). Terrein **(24)** exhibited IC_{50} of 20 μg/mL against *Enterococcus faecalis* and ˃20 μg/mL against *Staphylococcus aureus* and *Aeromonas hydrophila* (Goutam et al. 2017).

(22*E*,24*R*)-stigmasta-5,7,22-trien-3-*β*-ol **(25)** reported from the *Aspergillus terreus* an endophytic fungus isolated from *Carthamus lanatus* roots exhibited a potent activity against MRSA and *Cryptococcus neoformans* with respective IC_{50} values of 2.29 and 10.68 μM (Elkhayat et al. 2016).

In one study, beauvericin **(26)** isolated from *Epicoccum nigrum*, an endophytic fungus of *Entada abyssinica*, had significant antibacterial activity against *Bacillus cereus* and *Salmonella typhimurium* with MIC values of 3.12 and 6.25 μg/mL, respectively (Hewage et al. 2014).

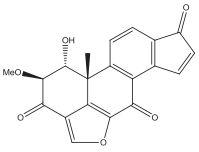
Recently, alternariol **(27)**, alternariol 9-methyl ether, and 3,7-dihydroxy-9-methoxy-2-methyl-6Hbenzo[c]chromen-6-one **(28)** has been reported from an endophytic fungus *Alternaria alternata,* isolated from a well-known folk medicinal plant *Grewia asiatica*. This was the first report for the isolation of *Alternaria alternata* from this plant. Compounds were detected and quantified by a new multimode LC-ESI-MS/MS method using multiple reaction monitoring. Both **27** and **28** exhibited antibacterial activity against *S. aureus* and vancomycin-resistant *Enterococci* (VRE) with MIC values of 32 and 128 μg/mL, respectively, whereas **27** exhibited promising activity against methicillin-resistant *S. aureus* (MRSA)-15187 with MIC value of 8 μg/mL comparable to ciprofloxacin in contrast to 64 μg/mL of **28** (Deshidi et al. 2017).

6.2.2 Antifungal Compounds from Endophytic Fungi

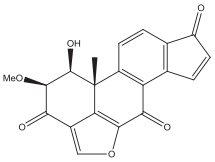
The rate of fungal infection increases during graft transfer, allogeneic transplantation of the bone marrow, and chemotherapy in cancer patients. Still, a very less number of antifungal drugs are there in the market for treating lethal diseases like

aspergillosis, candidiasis, meningitis, fungal eye infection, ringworm, valley fever caused by *Cryptococcus gattii*, histoplasmosis, and sporotrichosis. Due to the evolving pathogenicity and resistance among the fungal pathogens, there is an immediate need of diverse and novel compounds to combat.

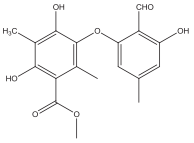
Isolation of microbial secondary metabolites has been a natural alternative for various therapeutic applications. Secondary metabolites of endophytic fungi have a great potential in various agricultural, industrial, and medical fields. There are many antifungal compounds that have been isolated from endophytic fungi (Fig. 6.4) which are listed in Table 6.2 and discussed in the following text.



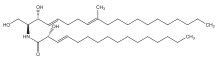
α-viridin **(29)**



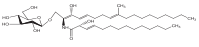
β-Viridin **(30)**



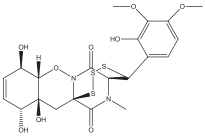
adenosyl 9a-D-arabinofuranoside (31)



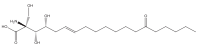
Asperamide A **(32)**



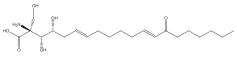
Cerebroside C **(33)**



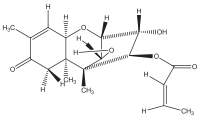
Outovirin C **(34)**



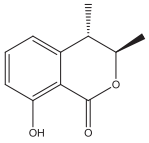
(2S,3R,4R)-(E)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxoeicos-6,12-dienoic acid **(35)**



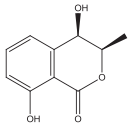
Myriocin **(36)**



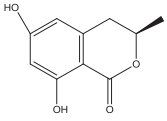
Trichothecinol-A **(37)**



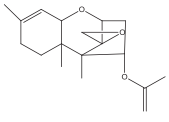
4-hydroxymellein **(38)**



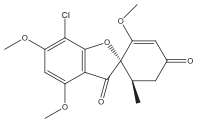
6-hydroxymellein **(39)**



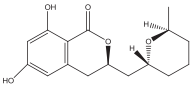
Tyrosol **(40)**



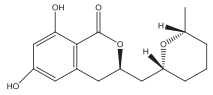
Trichodermin **(41)**



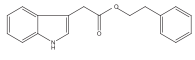
Griseofulvin **(42)**



Cladosporin **(43)**



Iso-Cladosporin **(44)**



2-phenylethyl 1*H*-indol-3-yl-acetate **(45)**

Fig. 6.4 Structures of antifungal compounds (**29–45**) from endophytic fungi

Table 6.2 Antifungal compounds derived from endophytic fungi

Compound	Endophytic fungi	Host plant	References
Terrein **(24)**	*Aspergillus terreus*	*Achyranthes aspera*	Goutam et al. (2017)
α-viridin **(29)**, β-viridin **(30)**, and adenosyl 9a-D-arabinofuranoside **(31)**	*Trichoderma* sp.	*Centaurea stoebe*	Abdou and Abdelhady (2015)
Asperamide A **(32)** and cerebroside C **(33)**	*Aspergillus niger*	*Ipomoea batatas*	Shaaban et al. (2013)
Outovirin C **(34)**	*Penicillium raciborskii*	*Rhododendron tomentosum*	Kajula et al. (2016)
(2S,3R,4R)-(E)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxoeicos-6,12-dienoic acid **(35)** and myriocin **(36)**	*Mycosphaerella* sp.	*Eugenia bimarginata*	Pereira et al. (2015)
Trichothecinol-A **(37)**	*Trichothecium* sp.	*Phyllanthus amarus*	Taware et al. (2014)
4-hydroxymellein **(38)** and 6-hydroxymellein **(39)**, tyrosol **(40)**, cervesterol **(8)**	*Penicillium* sp.	*Senecio flavus*	Elkhayat and Goda (2017)
Trichodermin **(41)**	*Trichoderma brevicompactum*	*Allium sativum*	Shentu et al. (2014)
Griseofulvin **(42)**	*Nigrospora oryzae*	*Emblica officinalis*	Rathod et al. (2014)
Cladosporin **(43)** and isocladosporin **(44)**	Cladosporium cladosporioides	–	Wang et al. (2013)
2-phenylethyl 1*H*-indol-3-yl-acetate **(45)**	*Colletotrichum gloeosporioides*	*Michelia champaca*	Chapla et al. (2014)

Secondary metabolites α-viridin **(29)**, β-viridin **(30)**, and adenosyl 9a-D-arabinofuranoside **(31)** were reported from an endophytic fungus *Trichoderma* sp. cultured from the stem of the medicinal plant *Centaurea stoebe*. These fungal metabolites **(29–31)** were tested for antifungal potential which shows MIC of 7.81, 15.63, and 8.52 μg/mL against *Aspergillus terreus*, respectively, while against *Fusarium oxysporum*, these compounds have MIC of 57.50, 75.00, and 63.30 μg/mL, respectively (Abdou and Abdelhady 2015).

In one study, asperamide A **(32)** and cerebroside C **(33)** displayed moderate to high activities against *Candida albicans* (13–15 mm) and *Mucor miehi* (16–18 mm) and the microalgae *Chlorella vulgaris* (13–14 mm), *Chlorella sorokiniana* (12–14 mm), and *Scenedesmus subspicatus* (13–15 mm) (Naureen et al. 2015).

Three novel epithiodiketopiperazine compounds outovirin A, outovirin B, and outovirin C **(34)** have been reported from the extracts of endophytic fungi *Penicillium raciborskii*, isolated from *Rhododendron tomentosum*. The essential oil and crude extracts of this plant have a number of properties like anti-insecticidal, antioxidant, and antimicrobial; leaves and flower extracts have traditionally been used in the primitive time for the treatment of various infections. Outovirin A is the first reported epimonothiodiketopiperazine, and outovirin C represents first most reported trisul-

fide gliovirin-like compound. Outovirin C inhibited growth of *Fusarium oxysporum*, *Botrytis cinerea*, *and Verticillium dahliae* at a mild concentration of 0.38 mM (207 μg/mL), but a more significant inhibition of growth was observed at a higher concentration of 0.76 mM (413 μg/mL) (Kajula et al. 2016).

Similarly, two eicosanoic acid compounds, (2S,3R,4R)-(E)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxoeicos-6,12-dienoic acid **(35)** and myriocin **(36)**, were isolated using bioassay-guided fractionation of crude extract by semi-prep HPLC and liquid-liquid partitioning of an endophytic fungus *Mycosphaerella* sp. UFMGCB 2032 cultured from *Eugenia bimarginata* DC. Both the compounds exhibited strong antifungal activity against *Cryptococcus neoformans* and *Cryptococcus gattii* and showed MIC values in the range of 1.3–2.50 μg/mL and 0.5 μg/mL, respectively (Pereira et al. 2015).

In one study trichothecinol-A **(37)** a sesquiterpene was reported for the very first time. The compound was isolated from an endophytic fungus *Trichothecium* sp. cultured from *Phyllanthus amarus*. Also, it exhibited moderate inhibition of *Cryptococcus albidus* var. *diffluens* (NCIM 3371 and 3372) with MIC values of 36 and 20 μg/mL, respectively, and against *Penicillium expansum* (NCIM 939) with MIC of 36 μg/mL (Taware et al. 2014). Recently, four compounds were reported from an endophytic fungus *Penicillium* sp. and characterized as 4-methylmellein, 4-hydroxymellein **(38)**, 6-hydroxymellein **(39)**, and tyrosol **(40)**. These compounds had shown activity against *Candida albicans*, *Fusarium oxysporum*, and *Aspergillus flavus* at the concentration of 50 μg per disc, with zones of inhibition in the range of 6–8 mm (Elkhayat and Goda 2017).

Trichodermin **(41)** was characterized from *Trichoderma brevicompactum*, an endophytic fungus strain 0248, reported from garlic. Bioactivity-guided fractionation was used to isolate this compound and identified by spectral analysis and mass spectrometry. Trichodermin showed potent antifungal activity against *Rhizoctonia solani* and *Botrytis cinerea* with an EC_{50} of 0.25 μg/mL and 2.02 μg/mL, respectively (Shentu et al. 2014).

Similarly, the compound griseofulvin **(42)** was reported from *Nigrospora oryzae*, an endophytic fungus isolated from *Emblica officinalis*. Griseofulvin exhibited antifungal activity against *Fusarium oxysporum*, *Trichophyton mentagrophytes*, and *Microsporum canis* (Rathod et al. 2014).

The extract of *Fusarium proliferatum* an endophytic fungus cultured from *Syzygium cordatum* showed 100% cytotoxicity at 100 μg/mL against the brine shrimp *Artemia salina*. Upon fractionation seven biologically active colored compounds were isolated, namely, ergosta-5,7,22-trien-3β-ol, nectriafurone-8-methyl ether, 9-*O*-methyl fusarubin, bostrycoidin, bostrycoidin-9-methyl ether, and 8-hydroxy-5,6-dimethoxy-2-methyl-3-(2-oxo-propyl)-1,4-naphthoquinone. Fraction BS749_AR was having structural similarity to nectriafurone-8-methyl ether and labeled as compound 7. Due to the small amount of fraction, confirmation of the proposed structure and measuring its 2D NMR spectra was not possible. This fraction inhibited *Staphylococcus aureus* with zone diameter of 11 mm at 40 μg/paper disc. It also exhibited antifungal activity against *Rhizoctonia solani*

and *Aphanomyces cochlioides* with zone diameter of 11 and 10 mm, respectively (Dame et al. 2016).

Cladosporin **(43)**, isocladosporin **(44)**, 5′-hydroxyasperentin, and cladosporin-8-methyl ether were reported from crude extract of an endophytic fungus *Cladosporium cladosporioides* using bioassay-guided fractionation, and acetylation of 5′-hydroxyasperentin was used to synthesize a novel compound 5′,6-diacetylcladosporin. Compound **43** at 30 μM showed 92.7, 90.1, 95.4, and 79.9% growth inhibition of plant pathogens *Colletotrichum acutatum*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, and *Plasmopara viticola*, respectively, while **44** at the same concentration exhibited 50.4, 60.2, and 83.0% growth inhibition. This was the first report of 5-hydroxyasperentin and cladosporin-8-methyl ether from *Cladosporium cladosporioides* (Wang et al. 2013). 2-phenylethyl 1*H*-indol-3-yl-acetate **(45)** reported from an endophytic fungus *Colletotrichum gloeosporioides* cultured from *Michelia champaca* showed propitious antifungal activity against *Cladosporium cladosporioides* and *Cladosporium sphaerospermum* as same as that of the positive standard nystatin (Chapla et al. 2014).

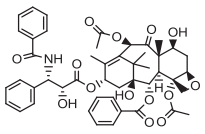
Terrein **(24)** was recently isolated and characterized from an endophytic fungus *Aspergillus terreus* JAS-2 cultured from *Achyranthes aspera* traditional medicinal plant. Analysis of NMR data (^{1}H proton and ^{13}C) and Fourier-transform infrared spectroscopy confirmed and characterized the product as 4,5-Dihydroxy-3-(1-propenyl)-2-cyclopenten-1-one (Goutam et al. 2017). Ten microgram/microliter concentration of terrein had shown inhibition of *Bipolaris sorokiniana* (57.14%), *Aspergillus flavus* (52.5%), and *Alternaria alternata* (91.25%) as compared to positive standard; 1 μg/μL was found sufficient for 100% growth inhibition of *Phytophthora drechsleri*. Terrein had also shown antioxidant potential by DPPH scavenging with IC_{50} value of 112 μg/mL.

6.2.3 Anticancer Compounds from Endophytic Fungi

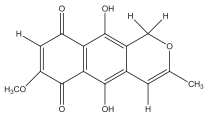
Through literature it is evident that the search for anticancer natural molecules always remains on priority in bioprospection of fungal endophytes, and there are many new compounds (Fig. 6.5) which were reported in recent 5 years, as listed in Table 6.3.

Taxol is a well-known diterpenoid for its anticancer activity (Wani and Taylor 1971); it was initially reported from the bark of western yew plant, *Taxus brevifolia*. The main function of taxol is to inhibit tubulin depolymerization during cell division. Taxol production from fungal endophytes using large-scale fermentation process is cost-effective and safe (Page et al. 2000). It has been reported from many endophytic fungi of genera *Alternaria*, *Fusarium*, *Monochaetia*, *Pestalotia*, *Pestalotiopsis*, *Pithomyces*, and *Taxomyces* (Strobel et al. 1996), and more than 20 fungal genera were identified for the biosynthesis of anticancer drug paclitaxel and its derivatives (Zhao et al. 2010).

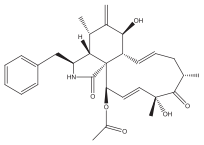
Taxol **(46)** was also isolated from the endophytic fungus that is *Phomopsis longicolla* of *Aliyar* plant, and approximately 381 μg/mL amount of it was detected



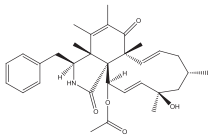
Taxol **(46)**



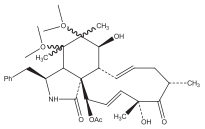
Anhydrofusarubin **(47)**



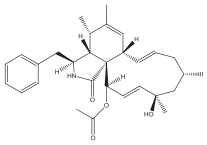
Cytochalasin D **(48)**



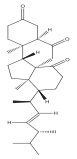
Phomocytochalasin **(49)**



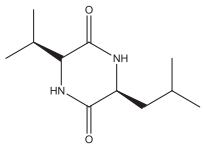
Cytochalasin N **(50)**



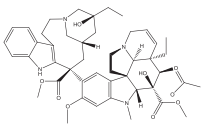
RKS-1778 **(51)**



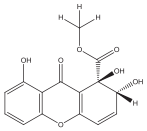
Dankasterone B **(52)**



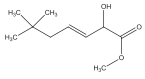
Cyclo(L-Ile-L-Leu) **(53)**



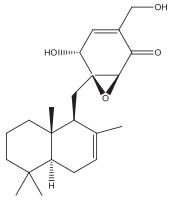
Vincristine **(54)**



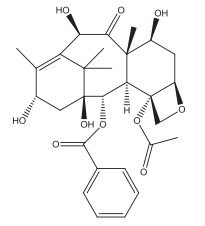
methyl 9-dihydro-8-trihydroxy-9-oxo-Hxanthene-1-carboxylate **(55)**



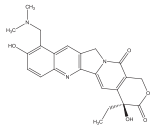
(E)-methyl 2-hydroxy-6, 6-dimethyl hept-3-enoate **(56)**



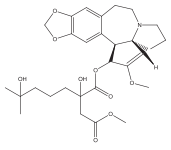
11-dehydroxy epoxyphomalin A **(57)**



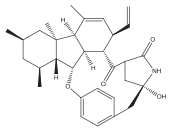
10-deacetybaccatin **(58)**



Camptothecin **(59)**



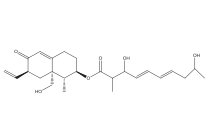
Homoharringtonine **(60)**



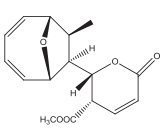
Pyrrocidine A **(61)**

Fig. 6.5 Structures of anticancer compounds (**46–77**) from endophytic fungi

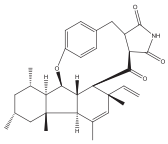
using HPLC. Podophyllotoxin is the precursor for the three clinically approved anticancer drugs, Etoposide™, Etopophos™, and Teniposide™. Occurrence of this compound is very less in nature that cannot meet the requirements for the production of these well-known antineoplastic compounds in the medical industry. The search for the natural production of this compound was started, and this was reported from the endophytic fungus *Alternaria tenuissima* cultured from roots of *Sinopodophyllum*



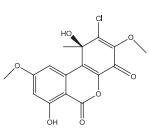
Eremofortin F **(62)**



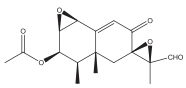
Mycoepoxydiene **(63)**



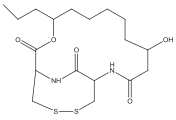
GKK1032B **(64)**



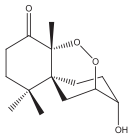
TMC-264 **(65)**



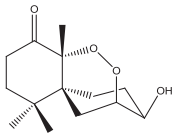
PR-toxin **(66)**



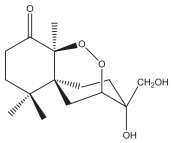
Depsipeptide (PM181110) **(67)**



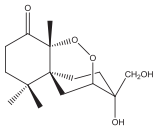
3-*epi*-steperoxide A **(68)**



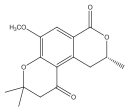
Steperoxide A **(69)**



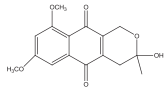
Merulin B **(70)**



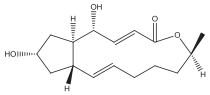
Merulin C **(71)**



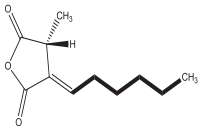
Versicoumarin A **(72)**



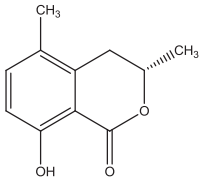
Naphthoquinone (Herbarin) **(73)**



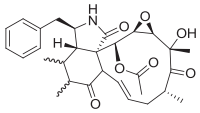
Brefeldin A **(74)**



Xylarione A **(75)**



(-) 5-methylmellein **(76)**



Jammosporin A **(77)**

Fig. 6.5 (continued)

emodi (Wall.). HPLC was used for the isolation and quantification purposes and confirmed by using available authentic standards (Liang et al. 2015). This report concluded that using fermentation, podophyllotoxin can be isolated on large scale to fulfill clinical demands and also confirmed that endophytes isolated from the plant can produce the same compound because of its coevolution with their host due to the uptake of host genomes (Stierle et al. 1993).

Similarly, anhydrofusarubin **(47)** and methyl ether of fusarubin **(15)** isolated from the endophytic fungus *Cladosporium* sp. cultured from *Rauwolfia serpentina* leaves showed potent cytotoxicity against human leukemia cells (K-562) with IC_{50} values of 3.97 μg/mL and 3.58 μg/mL, respectively (Khan et al. 2016).

Table 6.3 Anticancer compounds derived from endophytic fungi

Compounds	Endophytic fungus	Host plant	References
18-methoxycytochalasin J **(19)**, cytochalasin H **(20)**, cytochalasin J **(21)**, and alternariol **(27)**	*Phomopsis* sp.	*Garcinia kola*	Jouda et al. (2016)
Terrein **(24)**	*Aspergillus terreus*	*Achyranthes aspera*	Goutam et al. (2017)
α-viridin **(29)** and β-viridin **(30)**	*Trichoderma* species	*Centaurea stoebe*	Abdou and Abdelhady (2015)
Trichothecinol-A **(37)**	*Trichothecium* sp.	*Phyllanthus amarus*	Taware et al. (2014)
4-hydroxymellein **(38)** and 6-hydroxymellein **(39)**	*Penicillium* sp.	*Senecio flavus*	Elkhayat and Goda 2017
Anhydrofusarubin **(47)** and fusarubin methyl ether **(15)**	*Cladosporium* sp.	*Rauwolfia serpentina*	Khan et al. (2016)
Cytochalasin D **(48)**	*Xylaria* sp.	Seaweed	de Felício et al. (2015)
Phomocytochalasin **(49)**, cytochalasin H **(19)**, cytochalasin N **(50)**, RKS-1778 **(51)**, dankasterone B **(52)**, and Cyclo(L-Ile-L-Leu) **(53)**	*Phomopsis theicola*	*Litsea hypophaea*	Hsiao et al. (2016)
Vinblastine and vincristine **(54)**	*Eutypella* sp.	*Catharanthus roseus*	Hani and Eman (2015)
11-dehydroxy epoxyphomalin A **(57)**	*Peyronellaea coffeae-arabicae*	*Pritchardia lowreyana*	Li et al. (2015b)
10-deacetylbaccatin III **(58)**	*Trichoderma* sp.	*Taxus wallichiana*	Li et al. (2015b)
Camptothecin **(59)**	*Fusarium oxysporum*	*Nothapodytes foetida*	Musavi et al. (2015)
Homoharringtonine **(60)**	*Alternaria tenuissima*	*Cephalotaxus hainanensis* Li	Hu et al. (2016)
Pyrrocidine A **(61)**	*Neonectria ramulariae*	*Cylindrocarpon* sp. and *Acremonium zeae*	Uesugi et al. (2016)
Eremofortin F **(62)** and mycoepoxydiene **(63)**	*Sabicea cinerea*	*Rubiaceae* sp.	Mandavid et al. (2015)
GKK1032B **(64)**	Penicillium citrinum	G. mangostana	Qader et al. (2015)
TMC-264 **(65)** and PR-toxin **(66)**	*Penicillium Chermesinum*	*Heritiera Littoralis*	Darsih et al. (2015)
Depsipeptide (PM181110) **(67)**	*Phomopsis glabrae*	*Pongamia pinnata*	Verekar et al. (2014)
3-*epi*-steperoxide A **(68)**, steperoxide A **(69)**, merulin B **(70)**, and merulin C **(71)**	*Pseudolagarobasidium acaciicola*	*Bruguiera gymnorrhiza*	Wibowo et al. (2014)

(continued)

Table 6.3 (continued)

Compounds	Endophytic fungus	Host plant	References
Versicoumarin A **(72)**	*Aspergillus versicolor*	*Paris marmorata*	Ye et al. (2014)
Naphthoquinone (herbarin) **(73)**	*Dendryphion nanum*	*Ficus religiosa*	Mishra et al. (2013)
Brefeldin A **(74)**	*Penicillium* sp.	*Panax notoginseng*	Xie et al. (2017)
Xylarione A **(75)** and (-) 5-methylmellein **(76)**	*Xylaria psidii*	*Aegle marmelos*	Arora et al. (2016)
Jammosporin A **(77)**	*Rosellinia sanctae-cruciana*	*Albizia lebbeck*	Sharma et al. (2018)
E2.2	*Colletotrichum gloeosporioides*	*Phaleria macrocarpa*	Gasong and Tjandrawinata (2016)
α-Pyrone derivative	*Pestalotiopsis microspore*	*Taxus chinensis*	Li et al. (2015a)

In one study an endophytic fungus, *Colletotrichum gloeosporioides* of *Phaleria macrocarpa*, was reported to yield E2.2 compound. This compound is responsible for its anticancer activity toward MDA-MB-231 and MCF-7 human breast adenocarcinoma cell lines. Phloroglucinol was found to better enhance the production of this compound. Production was found high when incubated in PDB medium, pH 5.0, and kept for 15 days in incubation (Gasong and Tjandrawinata 2016).

A well-known anticancer and antibiotic, cytochalasin D **(48)**, was isolated from *Xylaria* sp. This was the first report for the isolation of cytochalasin from marine seaweed endophyte (de Felício et al. 2015). Recently, three reported cytochalasins, 18-methoxycytochalasin J **(19)**, cytochalasin H **(20)**, and cytochalasin J **(21)**, along with one more compound named alternariol **(27)** characterized from an endophytic fungus *Phomopsis* sp. showed promising cytotoxic activity against human cancer cells with IC_{50} in the range of 3.66–35.69 μg/mL and without any toxicity against normal cell lines (Jouda et al. 2016). A new cytochalasin along with five reported cytochalasins has been characterized from the solid-state fermentation of *Phomopsis theicola* BCRC 09F0213, an endophytic fungus isolated from the leaves of *Litsea hypophaea* Hayata. In one study, a new cytochalasin named as Phomocytochalasin **(49)** and five others were reported as cytochalasin H **(20)**, cytochalasin N **(50)**, RKS-1778 **(51)**, dankasterone B **(52)**, and cyclo(L-Ile-L-Leu) **(53)**. Among the isolated compounds, cytochalasin N showed nitric oxide inhibitory activity with IC_{50} values of 77.8 μM, whereas the other cytochalasins have shown IC_{50} values >100 μM. All the isolates were also tested for interleukin-6 (IL-6) inhibitory activity and showed IC_{50} values of >100 μM. The cytochalasin H showed progesterone receptor antagonism with IC_{50} values of 1.42 μM compared with positive standard RU486 with IC_{50} of 0.063 nM (Hsiao et al. 2016).

Catharanthus roseus is a well-known herbal plant for the synthesis of two valuable anticancer compounds, vincristine and vinblastine. Recently, the production of vincristine **(54)** was reported from an endophytic fungus *Eutypella* sp. CrP14 isolated from the stem of *Catharanthus roseus*. The presence of vincristine was confirmed using chromatographic and spectroscopic analysis. Vincristine showed potent cytotoxic activity in the MTT assay against human squamous carcinoma cells – A431. It was also able to induce apoptosis and causes loss of mitochondrial membrane potential, DNA fragmentation, and generation of reactive oxygen species (Kuriakose et al. 2016).

In one study methyl 9-dihydro-8-trihydroxy-9-oxo-Hxanthene-1-carboxylate **(55)** and (E)-methyl 2-hydroxy-6, 6-dimethyl hept-3-enoate **(56)** were reported from the *Chaetomium globosum*, which suppress the growth of Michigan Cancer Foundation-7 (MCF-7) breast cancer cell line and human liver carcinoma cancer cell line (HepG-2) (Hani and Eman 2015).

The fungus *Peyronellaea coffeae-arabicae* isolated from *Pritchardia lowreyana* was explored, from which peyronellins A–C and 11-dehydroxy epoxyphomalin A **(57)** were obtained. The compound **57** was found to inhibit STAT3 at 5 μm along with potent anticancer activity (IC_{50} 0.5 μM) against ovarian cancer cell lines (OVCAR3) (Li et al. 2016).

Taxol is produced in a semisynthetic method using two precursors baccatin III and 10-deacetylbaccatin III; vast literature is available where these two precursors are produced along with taxol. Endophytic fungi add special references in the production of 10-deacetylbaccatin III **(58)** by *Trichoderma* sp. isolated from *Taxus wallichiana* (Li et al. 2015b).

Camptothecin (CPT) is a topoisomerase I-DNA inhibitor and was first reported from *Camptotheca acuminata* Decne (Nyssaceae), a deciduous tree of South China, which has attained inordinate attention due to its potential antitumor activities in experimental studies. In the last decade, camptothecin (CPT) **(59)** along with its analogues such as 9-methoxycamptothecin (MCPT) and 10-hydroxycamptothecin (HCPT) was found to be produced by many endophytic fungi isolated from plant *Camptotheca acuminata* and many plants belonging to the family *Icacinaceae* (Musavi et al. 2015). There are a number of problems regarding production of these compounds by fermentation, as in many endophytic fungal strains like *Fusarium solani* (Kusari et al. 2011) and *Aspergillus* sp. (Pu et al. 2013); after subsequent subculturing the loss of the biosynthetic ability was observed. Recently, Venugopalan and Srivastava (2015) highlighted the increased production of CPT by endophytic fungi *F. solani* isolated from *Camptotheca acuminata*.

α-Viridin **(29)**, β-viridin **(30)**, and adenosyl 9a-D-arabinofuranoside **(31)** were reported from an endophytic *Trichoderma* sp. cultured from the stems of the medicinal plant *Centaurea stoebe*. **29–31** shows promising cytotoxic and antitumor effects on different cancer cell lines such as HUVEC, K-562, and HeLa. β-Viridin shows GI_{50} value of 0.5 and 0.25 μg/mL against HUVEC and K-562 cell lines, respectively, whereas α-viridin shows GI_{50} of 0.02 and 0.01 μg/mL, respectively. α-Viridin and β-viridin show potent cytotoxicity with CC_{50} (HeLa) of 29.09 and 11.43 μg/mL, respectively. A novel α-pyrone derivative, together with four reported metabolites,

was isolated from *Pestalotiopsis microspora*, an endophytic fungus cultured from the stem of *Taxus chinensis* on the solid-state media. Both antimicrobial and anticancer activities were evaluated, but this compound did not have any significant activity (Li et al. 2015b).

Homoharringtonine (HHT) **(60)** is a natural alkaloid obtained from the bark of *Cephalotaxus hainanensis* and is widely used for treating human chronic myeloid leukemia. Due to slow growing and scarcity of the plant, there is a need for some other alternative that can fulfill the rising requirement of the drug in the market. An endophytic fungus *Alternaria tenuissima* isolated from this plant was found to produce the compound homoharringtonine; this supports the theory that endophytes during its coevolution with plants had gained the ability to synthesize the same product as host plant. The extract of endophytic fungi exhibited strong antiproliferative activity against K562, NB4, and HL-60 cancer cell lines with the IC_{50} values 67.25 ± 4.26, 65.02 ± 4.75, and 99.23 ± 4.26 μg/mL, respectively. Activity may be due to additional compounds in the crude extract that shows synergetic effect. Screening of HHT production was carried out using HPLC analysis and comparing the mass with that of standard (Hu et al. 2016).

Pyrrocidines A **(61)** is a natural metabolite of alkaloid class and reported from endophytic fungi (*Cylindrocarpon* sp. and *Acremonium zeae*), and it is well known for its antimicrobial activity. This compound is also capable of inducing shrinkage of nuclear DNA and its breakage into small fragments and activation of caspase activity in HL60 cancer cell lines. Its action of inducing apoptosis is different from other clinically approved drugs in the market, and it is due to the presence of unique α,β-unsaturated carbonyl moiety in the molecule (Uesugi et al. 2016).

In one study four metabolites, altiloxin A, enamidin, eremofortin F **(62)**, and mycoepoxydiene **(63)**, were reported from the *Sabicea cinerea*, an endophytic fungus closely related to *Diaporthe pseudomangiferae* obtained from the leaves of a plant of Rubiaceae family. Mycoepoxydiene and altiloxin A are well-known metabolites, whereas enamidin and eremofortin F were reported first time in the literature. Mycoepoxydiene showed promising cytotoxic activity with IC_{50} values of 7.5, 17.7, and 15.8 μM against KB, MDA-MB-435, and MRC5 cancer cell lines, respectively. Altiloxin A and enamidin showed no effect ($IC_{50} > 30$ μM) on all tested cell lines, and eremofortin F was cytotoxic on KB and MRC5 cells ($IC_{50} = 13.9$ and 12.2 μM, respectively) (Mandavid et al. 2015).

An endophytic fungus *Penicillium citrinum* isolated from the pericarp surface of *Garcinia mangostana* was reported to produce GKK1032B **(64)**, citrinin, and ergosterol. Citrinin is a nephrotoxin mycotoxin that is repeatedly synthesized by fungus *P. citrinum* (Malmstrøm et al. 2000). GKK1032B an antitumor antibiotic was first reported from unidentified species of *Penicillium* in 2001 along with two more metabolites GKK1032A1 and GKK1032A2 (Koizumi et al. 2001). Pastre et al. in 2007 reported reisolation of the compound from an endophytic fungi *Penicillium* sp. of *Melia azedarach* and *Murraya paniculata*. This was the third time to report isolation of GKK1032B from *Penicillium citrinum*. Since this class of metabolites has not been isolated from any other species of *Penicillium*, it was concluded that the

two unidentified *Penicillium* strains in the previous papers can be *P. citrinum* (Qader et al. 2015).

Similarly, new fungal metabolites, penicilliumolide A, penicilliumolide B, penicilliumolide C, and penicilliumolide D, a derivative of PR-toxin, along with three reported compounds, TMC-264 **(65)**, PR-toxin **(66)**, and a sesquiterpene, were isolated from *Penicillium chermesinum*, an endophytic fungus of a mangrove tree, *Heritiera littoralis*. The TMC-264 and PR-toxin showed effective as well as selective cytotoxicity against some cancer cell lines. TMC-264 also showed similar cytotoxic activity as of standard doxorubicin against T47D and MDA-MB231 cancer cell lines, and its cytotoxicity against HepG2 cell line was higher than etoposide. Derivatives of TMC-264 did not possess the activity; therefore, it is concluded that the cytotoxicity of TMC-264 was probably because of the presence of β-chloro-substituted α,β-unsaturated ketone in its structure (Darsih et al. 2015).

A new depsipeptide (PM181110) **(67)** was isolated from *Phomopsis glabrae*, an endophytic fungus cultured from the leaves of *Pongamia pinnata*. The compound was tested for its cytotoxicity against a panel of 40 human tumor cell lines out of which it showed activity in a concentration-dependent manner against all cell lines of bladder, colon, gastric, head and neck, liver, lung (non-small-cell lung carcinoma), mammary, ovarian, pancreatic, prostate, renal, and uterus cancer, as well as melanoma, pleural mesothelioma, and sarcoma. Pancreatic cancer cell line PAXF 546L (IC_{50}, 0.016 μM) and the lung cancer cell line LXFA 526 L (IC_{50}, 0.021 μM) were the most sensitive cell lines. The compound also exhibited ex vivo efficiency toward all human tumor xenografts (mean IC_{50} 0.245 μM) (Verekar et al. 2014).

In one study, two new norsesquiterpenes citicoline A and spiroacaciicolide A, a new nor-chamigrane 3-*epi*-steperoxide A **(68)**, along with three known compounds steperoxide A **(69)**, merulin B **(70)**, and merulin C **(71)**, were reported from an endophytic fungus *Pseudolagarobasidium acaciicola*, cultured from a mangrove tree *Bruguiera gymnorrhiza*. The compounds **68–71** showed cytotoxic activity toward many cancer cell lines. Among them compounds **70** and **71** displayed promising cytotoxic activity against MOLT-3, HuCCA-1, A549, HepG2, HL-60, MDA-MB-231, T47D, and HeLa cancer cell lines with IC_{50} in the ranges of 0.68–3.71 and 0.67–5.25 μg/mL, respectively. Compound **70** exhibited weak cytotoxic activity with an IC_{50} range of 11.94–49.08 μg/mL, whereas compound **71** exhibited the most potent cytotoxic activity against HL-60 cancer cells, with an IC_{50} value of 0.08 μg/mL, and in addition displayed IC_{50} value in the range of 0.19–3.75 μg/mL against other tested cell lines (Wibowo et al. 2014).

Versicoumarin A **(72)** was reported from an endophytic fungus *Aspergillus versicolor* cultured from the rhizomes of Paris marmorata plant. This compound exhibited strong cytotoxicities against A549 and MCF7cancer cells with IC_{50} values of 3.8 and 4.0 mM, respectively (Ye et al. 2014).

Two naphthoquinones were isolated from *Dendryphionnanum*, an endophytic fungus of *Ficus religiosa*. This was the first report for the isolation of naphthoquinone compounds from this fungus. Naphthoquinone antibiotic herbarin **(73)** exhibited potent inhibition of TNF-α and IL-6 cytokine production in human mononuclear

cell line (THP-1) induced with LPS with IC_{50} of 0.60 ± 0.100 μM and 0.06 ± 0.009 μM, respectively (Mishra et al. 2013).

Recently, Brefeldin A **(74)** produced from *Penicillium* sp., an endophytic fungus isolated from the healthy root of *Panax notoginseng*, showed strong anticancer activity against 293 (human renal epithelial cell line), HepG2, Huh (human hepatocellular carcinoma cell line), and KB (human oral epidermoid carcinoma cell line) with ID_{50} value of 0.45 ± 0.008, 0.0024 ± 0.002, 0.035 ± 0.005, and 0.06 ± 0.009 μM, respectively (Xie et al. 2017).

One new compound xylarione A **(75)** along with (-) 5-methylmellein **(76)** has been reported from an endophytic fungus *Xylaria psidii* cultured from the leaves of a well-known traditional medicinal plant, *Aegle marmelos*, also called "Bael" in Hindi. Both the compounds were active against pancreatic cancer cell line (MIA-Pa-Ca-2) and exhibited cytotoxicity with IC_{50} values of 16.0 and 19.0 μM, respectively. Compounds were capable of cell arrest at the sub-G1 phase, exhibited different signs of apoptotic mechanism, and also cause loss of MMP in a dose-dependent manner when analyzed using flow cytometry. This result concludes that these metabolites can be modified for making its derivatives and for making more potent drugs (Arora et al. 2016).

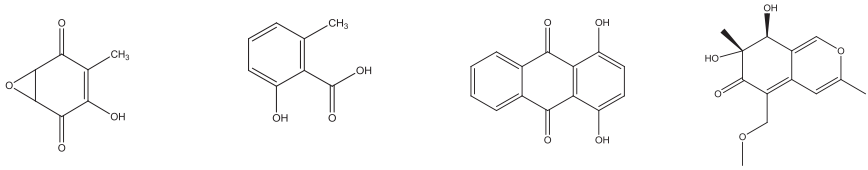
One new cytochalasin, named jammosporin A (**77**), and four known compounds 19,20-epoxycytochalasin D, cytochalasin D, 19,20-epoxycytochalasin C, and cytochalasin C, were isolated from the endophytic fungus *Rosellinia sanctae-cruciana*, cultured from the leaves of medicinal plant *Albizia lebbeck*. Compound **77** showed considerable cytotoxic potential against the human leukemia cancer cell line (MOLT-4) with IC_{50} values of 20.0 μM. This was the first report for the isolation of this class of secondary metabolites in *Rosellinia sanctae-cruciana* fungus from this plant (Sharma et al. 2018).

As discussed in the earlier section about the compounds 4-hydroxymellein **(38)** and 6-hydroxymellein **(39)** having antifungal activity, both the compounds also exhibited cytotoxic activity toward MCF-7 cancer cell lines with ED_{50} value of 6.1 and 8.3 μg/mL, respectively (Elkhayat and Goda 2017). Similarly, the compound **35** at concentration of 500 nM exhibits 50% cell death in HeLa and B16F10 cells and 25% in MDA-MB-231 cells (Taware et al. 2014).

6.2.4 Antioxidant Compounds from Endophytic Fungi

Antioxidants of natural origin are the compounds that are commonly found in medicinal plants, fruits, and vegetables. However, it is reported that a large number of secondary metabolites from fungal endophytes are having potent antioxidant activity, and some of the recent compounds (Fig. 6.6) are listed in Table 6.4.

Recently, two antioxidant compounds, terreic acid **(78)** and 6-methylsalicylic acid **(79)**, were reported from *Pestalotiopsis* sp. EST 02, an endophytic fungus of plant *Elaeocarpus sylvestris*. Both the compounds have shown potent DPPH radical



Terreic acid **(78)** | 6-methylsalicylic acid **(79)** | Quinizarin **(80)** | Calbistrin H **(81)**

Fig. 6.6 Structures of antioxidant compounds (**78–81**) from endophytic fungi

Table 6.4 Antioxidant compounds derived from endophytic fungi

Compounds	Endophytic fungi	Host plant	References
Terreic acid **(78)** and 6-methylsalicylic acid **(79)**	*Pestalotiopsis* sp.	*Elaeocarpus sylvestris*	Prihantini and Tachibana (2017)
Anthraquinone quinizarin **(80)**	*Epicoccum nigrum*	*Entada abyssinica*	Dzoyem et al. (2017)
Calbistrin H **(81)**	*Dothideomycete* sp.	–	Hewage et al. (2014)

scavenging activity with IC_{50} value of 0.22 ± 0.02 mmol/L and 3.87 ± 0.27 mmol/L, respectively. The compounds also showed good activities from the reducing power and β-carotene bleaching assays (Prihantini and Tachibana 2017).

The four compounds beauvericin, parahydroxybenzaldehyde, indole-3-carboxylic acid, and quinizarin were isolated from *Epicoccum nigrum*, an endophytic fungus of *Entada abyssinica*. Anthraquinone quinizarin **(80)** exhibited excellent scavenging activity having IC_{50} values of 10.86 and 11.36 μg/mL in the ABTS and DPPH assays, respectively (Dzoyem et al. 2017).

Calbistrin H **(81)** was reported from an endophytic fungus *Dothideomycete* sp. CRI7 on PDB medium using one strain many compounds (OSMAC) approach. It shows radical scavenging potential with an IC_{50} value of 21.7 μM (Hewage et al. 2014).

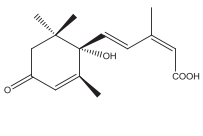
6.2.5 Antidiabetic Compounds from Endophytic Fungi

Diabetes mellitus is a metabolic disease caused by a defect in secretion of insulin and its action. Since diabetes prevalence has been rising, urge to find natural products of antidiabetic potential. The recent findings have been listed in Table 6.5 and represented in Fig. 6.7.

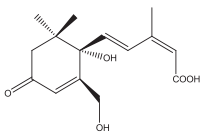
An endophytic fungus *Dendryphion nanum* (Nees) S. Hughes was isolated from the leaves of *Ficus religiosa* from which a naphthoquinone compound herbarin **(73)**

Table 6.5 Antidiabetic compounds derived from endophytic fungi

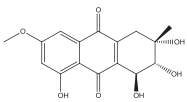
Compound	Endophytic fungi	Host plant	References
Naphthoquinone (herbarin) **(73)**	*Dendryphion nanum*	*Ficus religiosa*	Mishra et al. (2013)
(*S*)-(+)-2-*cis*-4-*trans*-abscisic acid **(82)**, 7′-hydroxy-abscisic acid **(83)**, and 4-des-hydroxyl altersolanol A **(84)**	*Nigrospora oryzae*	*Combretum dolichopetalum*	Uzor et al. (2017)
Thielavins A **(85)**, J **(86)**, and K **(87)**	MEXU 27095	*Hintonia latiflora*	Rivera-Chávez et al. (2013)



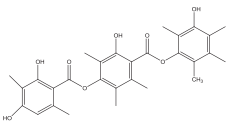
(*S*)-(+)-2-*cis*-4-*trans*-abscisic acid **(82)**



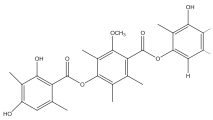
7'-hydroxy-abscisic acid **(83)**



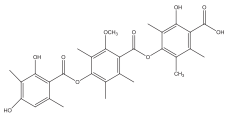
4-des-hydroxyl altersolanol A **(84)**



Thielavins A **(85)**



Thielavins J **(86)**



Thielavins K **(87)**

Fig. 6.7 Structures of antidiabetic compounds (**82–87**) from endophytic fungi

was isolated. The herbarin was found to induce the glucose uptake in rat skeleton muscle with IC_{50} of 0.80 ± 0.090 μM in the presence of insulin (Mishra et al. 2013).

A peptide was obtained via semi-prep HPLC from an endophytic fungus *Aspergillus awamori* of *Acacia nilotica*. The peptide was further resolved using SDS-PAGE to determine its molecular weight, which was found to be of 22 kDa size. The peptide resulted in a mixed type of inhibition against both α-amylase and α- glucosidase and showed IC_{50} values of 3.75 and 5.625 μg/mL, respectively (Singh and Kaur 2016).

Three metabolites (*S*)-(+)-2-*cis*-4-*trans*-abscisic acid **(82)**, 7′-hydroxy-abscisic acid **(83)**, and 4-des-hydroxyl altersolanol A **(84)** were isolated from *Nigrospora oryzae*, an endophytic fungus of *Combretum dolichopetalum*. The crude extract of the fungus reduced the fasting blood sugar by 30.79% in 3 h; simultaneously the compounds also showed reduction of 46.37% (3 h), 44.96% (9 h), and 43.70% (9 h), respectively. Comparable activity of extract and compounds was found with that of standard glibenclamide (reduction of 48.72% in 9 h) (Uzor et al. 2017).

MEXU 27095, an endophytic fungus, was isolated from the Mexican medicinal plant *Hintonia latiflora*, and the bioassay-guided fractionation of the bioactive organic extract resulted in the separation of three tridepsides, namely, thielavins A, J, and K **(85–87)**. Compounds **85–87** were capable of inhibiting α-glucosidase (α-GHY) *Saccharomyces cerevisiae* in a concentration-dependent manner and exhibited IC_{50} values of 23.8, 15.8, and 22.1 μM, respectively. Inhibitory action of the compounds was higher than that of the standard acarbose (IC_{50} = 545 μM), used as a positive control for the analysis (Rivera-Chávez et al. 2013).

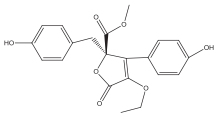
6.2.6 *Antileishmaniasis Compounds from Endophytic Fungi*

Leishmaniasis is a serious health problem worldwide, mainly in tropical and subtropical areas where the protozoan has developed resistance to current available drugs in the market. In the absence of effective vaccines, still now chemotherapy plays an important role in fighting this disease. Therefore, the search for novel, effective, and safe drugs is essential for the treatment, control, and prevention of leishmaniasis. Natural products derived from various endophytic fungi associated with medicinal plants have shown promise as a potential source for antiprotozoal drugs (Table 6.6) (Fig. 6.8).

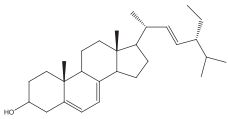
Terrenolide S **(88)**, a novel derivative of butenolide, along with six already reported compounds, (22*E*,24*R*)-stigmasta-5,7,22-trien-3-*β*-ol **(89)**, stigmast-4-ene-3-one **(90)**, stigmasta-4,6,8(14),22-tetraen-3-one, terretonin A, terretonin, and butyrolactone VI, had recently been isolated from *Aspergillus terreus*, an endophytic fungus cultured from the roots of *Carthamus lanatus.* Compounds **88–90** showed antileishmaniasis against *Leishmania donovani* and exhibited IC_{50} values of 11.24, 15.32, and 27.27 μM, respectively (Elkhayat et al. 2016).

Table 6.6 Antileishmaniasis compounds derived from endophytic fungi

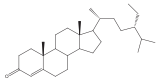
Compound	Endophytic fungi	Host plant	References
Purpureone **(17)**	*Purpureocillium lilacinum*	*Rauvolfia macrophylla*	Lenta et al. (2016)
Alternariol 9-methyl ether **(18)**	*Alternaria* sp.	*Salvia miltiorrhiza*	Lou et al. (2016)
Terrenolide S **(88),** (22*E*,24*R*)-stigmasta-5,7,22-trien-3-*β*-ol **(89)** and stigmast-4-ene-3-one **(90)**	*Aspergillus terreus*	*Carthamus lanatus*	Elkhayat et al. (2016)
(6-S)-3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione **(91)** and (6-R)-3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione **(92)**	*Trichosporum* sp.	*Trigonella foenum-graecum*	Metwaly et al. (2015)



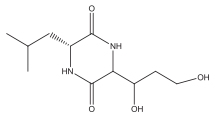
Terrenolide S **(88)**



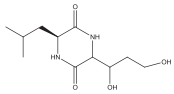
(22*E*,24*R*)-stigmasta-5,7,22-trien-3-*β*-ol **(89)**



stigmast-4-ene-3-one **(90)**



(6-R)-3-(1,3-dihydroxy propyl)-6-(2-methylpropyl) piperazine-2,5-dione **(91)**



(6-S)-3-(1,3-dihydroxy propyl)-6-(2-methylpropyl) piperazine-2,5-dione **(92)**

Fig. 6.8 Structures of antileishmaniasis compounds (**88–92**) from endophytic fungi

Two new diketopiperazine alkaloid isomers (6-S)-3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione **(91)** and (6-R)-3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione **(92)** were isolated from an endophytic fungus *Trichosporum* sp. cultured from the seeds of *Trigonella foenum-graecum*. Three chiral centers in both these compounds were not identified due to very low in amount. The compounds **91–92** exhibited moderate antileishmanial activities with IC_{50} values of 96.3 and 82.5 μg/mL, respectively (Metwaly et al. 2015).

Enniatins (ENs) are a group of antibiotics having six-membered cyclic depsipeptides formed by the combination of three molecules of D-α-hydroxyisovaleric acid and three N-methyl-L-amino acids commonly reported in the literature being produced by *Fusarium* sp. Six enniatins, namely, ENs A, A1, B, B1, B2, and Q, were reported via LCMS from the methanolic extract of *Fusarium tricinctum*, an endophytic fungus cultured from the fruits of *Hordeum sativum* cultivated on rice medium. The methanolic extract of this fungus exhibited mild antibacterial and antileishmanial activity against *L. donovani* ATTC 39930D with an IC_{50} value of 16.96 μg/mL. Moreover the extract at 100 μg/mL was also capable of inhibiting the activity of thioredoxin reductase enzyme present in *Plasmodium falciparum* by a factor of 95% (Zaher et al. 2015).

The antibacterial compound **17** also displayed excellent antileishmanial activity against *L. donovani* with an IC_{50} value of 0.63 μg/mL (0.87 μm) and with good selectivity (SI = 49.5) against the L6 cell line (Lenta et al. 2016). Similarly, the compound **18** inhibited spore germination in *Magnaporthe oryzae* with an IC_{50} value of 87.18 μg/mL. The compound also exhibited antinematodal activity against *Caenorhabditis elegans* and *Bursaphelenchus xylophilus* with an IC_{50} value of 74.62 μg/mL and 98.17 μg/mL, respectively (Lou et al. 2016).

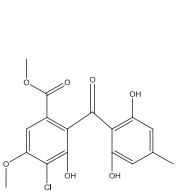
6.2.7 *Antiviral Compounds from Endophytic Fungi*

These days the attack of various viruses causing dreadful effects on human lives is very common. In view of evolving new strains of dreadful viruses, there is a requirement of the search for cheaper, safer, and more robust drug alternative from natural sources like endophytic fungi. Few antiviral compounds have been reported from various endophytic fungi (Fig. 6.9), as listed in Table 6.7.

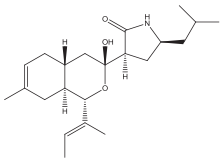
Three novel isocoumarins, versicoumarins A–C, with four reported isocoumarins, were characterized from an endophytic fungus *Aspergillus versicolor* cultured from *Paris marmorata* plant rhizomes. Versicoumarin A **(72)** exhibited significant anti-TMV activity with 28.6%, inhibition that was comparable to that of positive standard ningnanmycin (31.5%) (Ye et al. 2014). Brefeldin A **(74)** reported from *Penicillium* sp., which is discussed as anticancer compound, also exhibited potent antiviral activity against HCV (hepatitis C virus) and HBV (hepatitis B virus) with an ID_{50} value of 0.022 and >0.008 μM, respectively (Xie et al. 2017).

The fungus *Pestalotiopsis thea*, isolated from *Fagara zanthoxyloides*, yielded three known compounds chloroisosulochrin **(93)**, ficipyrone A, and pestheic acid. Compound **93** exhibited potent inhibition with an IC_{50} value of 4.22 ± 1.03 μM comparable with that of the standard ribavirin, having IC_{50} value of 4.91 ± 1.85 μM. The rest of the compounds exhibited mild inhibition with IC_{50} values in the range of 45.00 ± 0.98–259.23 ± 2.36 μM (Uzor et al. 2016).

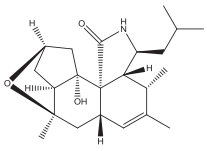
The fungus *Periconia* sp. F-31, isolated from *Annona muricata*, was explored and yielded pericoannosin A **(94)**, periconiasin D, periconiasin E, and periconiasin F **(95)**. Compounds **94** and **95** showed considerable anti-HIV activity with IC_{50} of 69.6 and 29.2 μM, respectively (Zhang et al. 2015).



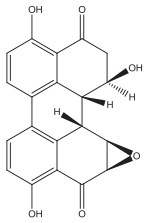
Chloroisosulochrin **(93)**



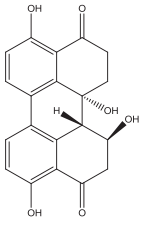
Pericoannosin A **(94)**



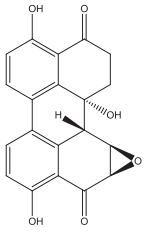
Periconiasin D **(95)**



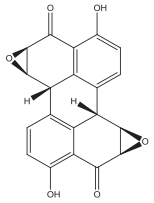
Altertoxin V **(96)**



Altertoxin I **(97)**



Altertoxin II **(98)**



Altertoxin II **(99)**

Fig. 6.9 Structures of antiviral compounds (**93–99**) from endophytic fungi

Table 6.7 Antiviral compounds derived from endophytic fungi

Compounds	Endophytic fungi	Host plant	References
Versicoumarin A **(72)**	*Aspergillus versicolor*	*Paris marmorata*	Ye et al. (2014)
Brefeldin A **(74)**	*Penicillium* sp.	*Panax notoginseng*	Xie et al. (2017)
Chloroisosulochrin **(93)**	*Pestalotiopsis thea*	*Fagara zanthoxyloides*	Uzor et al. (2016)
Pericoannosin A (**94**)	*Periconia* sp.	*Annona muricata*	Zhang et al. (2015)
Pericoannosin D (**95**)			
Altertoxin V (**96**)	*Alternaria tenuissima*	*Quercus emoryi*	Bashyal et al. (2014)
Altertoxin I (**97**)			
Altertoxin II (**98**)			
Altertoxin III (**99**)			

During the bioprospection of endophytic fungi of Sonoran Desert plants, *Alternaria tenuissima* was isolated from *Quercus emoryi*. In screening of natural product extract library and bioactivity-guided fractionation, altertoxin V **(96)**, altertoxin I **(97)**, altertoxin II **(98)**, altertoxin III **(99)**, and altertoxin IV were isolated and characterized. Compounds **96, 97, 98**, **and 99** have completely inhibited the replication of the HIV-1 virus at a very lower concentration with 0.50, 2.20, 0.30, and 1.50 μM, respectively (Bashyal et al. 2014).

6.3 Conclusion

Endophytes stay in the internal tissues of the host plants in symbiotic relationship. They are considered to be a rich source for the production of diverse bioactive metabolites and responsible to cause physiological modification in the host which results in the production of compounds required for tolerating biotic and abiotic stresses by plant. The extensive ability of endophytic fungi to synthesize products of pharmaceutical importance led the researchers and scientists to do the sampling from unique niches so that structurally diverse compounds can be isolated. In cases the endophyte gains the ability to produce either the same or different compounds from the host, the production of the same metabolite as of host plant is due to the long coevolution with their host. In the past two decades, researchers have focused on studying fungal diversity, better understanding of their relationship with the host, isolation of novel metabolites from fungus, and different ways to increase the yield of isolated bioactive compounds using genetic engineering and media engineering, improving culture conditions, and using one strain many compounds (OSMAC) approach. Endophytic fungi are considered as a boon for the society due to their ability to produce diverse metabolites of broad applications in various fields like agriculture, horticulture, medical, and industries. Instantly in favor of mankind, there is a need for the hunt of novel and diverse compounds to combat the upcoming challenges of drug resistance among pathogens.

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Chapter 7
Microbes, Their Metabolites, and Effector Molecules: A Pharmacological Perspective for Host-Microbiota Interaction

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Abstract The human microbiota and probiotics are key players to modulate human health. Microbial cells, as a whole, have either been known to physically react with intestinal surfaces or to produce some enzymes and metabolites to impart a positive or negative impact on human health. Moreover, their specialized metabolites have a profound role in the generation of multivariate clinical responses in humans, with an influence on hosts' metabolism and immunity. Gut microbiota and probiotics are known for their influence on hosts' physiology.

We review clinical trials based on microbiota composition to correlate health status of humans with their gut microbiota, along with few examples of effects of microbial perturbation on health and disease. The chapter also explains the roles of metabolites of human microbiota, in addition to their impacts on hosts' physiology. Besides the positive influences of microbes on humans, negative effects of the microbial metabolism, such as inactivating the pharmacological activity of drugs are also discussed, Selected examples for the roles of gut microbiota in human metabolism, using their enzymatic repertoire for degradation of otherwise indigestible dietary components, are reviewed. We present the mode of action of newly identified effector molecules, polysaccharides, outer membrane proteins, pili, muropeptides, and CpG-rich DNA, both for human microbiota and probiotics (*Lactobacillus* and *Bifidobacterium* strains). Besides effector molecules, clinical outcomes of probiotics (as whole live cells) are also discussed. Moreover, health-improving probiotic metabolites, including vitamins, bacteriocins, and bioactive peptides, are reviewed. In the end, a new perspective of developing a microbial global positioning system (mGPS) for segregation of human population on the basis of their microbiota is discussed.

7.1 Introduction

Roger J. Williams, in 1956, proposed the enlarged inter-individual variations, both chemical and biochemical, as the main reason why a common definition of healthy human could not be established (Williams 1956). That concept is still prevailing in today's pharmacological era, advocating the individual-specific medicinal interventions with high efficacy and minimized side effects (Patterson and Turnbaugh 2014). Two significant factors, host genetics and nutritional status, have been considered as the key players for this variation, although often fail to fully explain the inter-individual disparity in drug response (Ma and Lu 2011). As per recently developed hypothesis based on research findings, human microbiome (or microbiota) has been considered as the third and equally impeccable factor behind biochemical individuality of humans (Patterson and Turnbaugh 2014). Moreover, most of the individuals

have been categorized on the basis of their gut microbiota (enterotypes), individuals harboring either of the dominant genera such as *Bacteroides*, *Prevotella*, or *Ruminococcus* in their gut (Arumugam et al. 2011).

The majority of the human microbiota is found in the large intestine and outnumbers eukaryotic cells by almost 10 to 20-folds (Suau et al. 1999). In quantitative terms, several hundred microbial species add several hundred grams to the weight of healthy human adult (Guarner and Malagelada 2003). Moreover, almost 10% of the circulating metabolites in human system are thought to be derived from healthy gut microbiota (Wikoff et al. 2009). However, before it reaches to mature configuration in host adulthood, the microbiota experiences a progressive compositional change that initiates at birth and evolves in cohabitation with its host (Dicks et al. 2017). This sequential development of early gut microbiota has been known to exert a lifelong impact on host health. Keeping in mind the beneficial effects of gut microbiota, including anti-pathogenic, immunomodulatory, and metabolism-supportive, it has been pictured as a "microbial organ" within the intestine (Jia et al. 2008). The healthy human microbiota has been catalogued by some extraordinary efforts (Human Microbiome Project Consortium 2012 and Metagenomics of the Human Intestine) (Joice et al. 2014) and now been well recognized that the intact gut microbiota benefits the metabolism and physiology of host by participating in a number of chemical, physiological, and immunological activities (Cani and de Vos 2017). And normal physiology of human system can be disturbed as a result of an altered gut microbiota composition, acknowledged as dysbiosis (Thursby and Juge 2017). Gut microbiota has also been known for biotransformation of varied number of chemicals, including foreign compounds (xenobiotics), dietary components, and pharmaceuticals (Koppel et al. 2017). Such chemical modifications irrespective of tested organism (humans or animals or microbes) appeared on surface way back (in 1950) and confirmed after studying the effect of xenobiotics on microbiota free research models, i.e., germ-free animals, or upon microbial perturbation, i.e., antibiotic treatment or dietary modulation (Danielsson and Gustafsson 1959; Sousa et al. 2008). The relation and interdependency of microbiota and pharmaceuticals is very old, keeping in mind the microbial origin of most fundamental antimicrobial agents (penicillin and tetracycline) and recent antitumor therapies (Berdy 2005). The sequential progress in advancement of culture-independent assays have identified a range of new biologically active molecules, with a direct role in modern therapeutics or indirect action in further drug discovery, e.g., by competitive binding or inhibition (Lemon et al. 2012). Some new biosynthetic genes and biochemical pathways have been identified in bacterial genome sequences, and also their chemical nature has been successfully predicted using high-throughput genome mining strategies. This has led to the automated biosynthetic gene cluster identification and unexpected discovery of numerous biosynthetic gene clusters in genomes of human microbiota (Donia and Fischbach 2015).

This chapter has been focused on the roles of gut microbiota, probiotics, and their specialized molecules in human health and disease.

7.2 Host's Microbiota in Health and Disease

Humans are evolved in a close association with microorganisms and during that process a varied number of microbes have chosen different human body sites as their favorable ecological niches (Ley et al. 2006). This co-survival benefits both the domains of life in a number of ways, depending upon the healthy physiological state of the host, diet, and host genetics and epigenetics. The concept of "mammalian holobiont," generated after a revolutionary understanding of host-microbiota interaction, has been set on the basis of two paradigms: (a) discovery of pattern recognition receptors in the late 1990s (Thaiss et al. 2016) and (b) microbiome characterization using culture-independent techniques (Eckburg et al. 2005). However, knowledge regarding relative impact of such factors on human health is still in infancy (Buddington and Sangild 2011; Cerf–Bensussan and Gaboriau–Routhiau 2010; Hansen et al. 2010; Musso et al. 2010).

Ultimately, depending upon the functionality of microbiome, host receives a number of benefits; hence these symbiotic organisms may be considered as health promoting. It has now been well recognized (Clemente et al. 2012) that disturbed microbiota is a cause of acute infections like *Clostridium difficile* and of chronic diseases like cancer, autoimmunity, obesity, and heart-related ailments.

For practical reasons, comparison among microbiota of healthy and diseased state of hosts have been, presently, the major criteria for studying their roles in health and disease. However, the exact composition of human microbiota in any physiological state of body is still not defined due to inter- and intraindividual variations. Hence, it is difficult to assess their possible role in human welfare. Still, the metabolic disorders like obesity, inflammatory bowel disease and irritability bowel syndrome are extensively studied for the impeccable role of microbiota perturbation in human health and disease (Gerritsen et al. 2011).

Multiple factors, including sex, age, and diet, have been found responsible behind the compositional change in intestinal microbiota (Fan et al. 2017).

Inflammatory bowel disease, an assembly of disorders, can be described by a chronic and reversible inflammation of the gastrointestinal tract and is common in Western world (Loftus 2004). Ulcerative colitis and Crohn's disease are the two most frequent forms of inflammatory bowel disease. The structural changes in microbiota composition and resultant altered-microbe-mediated hyper-activation of immune system in susceptible or genetically predisposed individuals are the two main causative reasons of inflammatory bowel disease. Gut microbiota, in Crohn's disease especially, has been highly suspected to act in starting and triggering the immune system, leading to distinctive inflammation (Seksik et al. 2006).

Although a set of bacteria, as microbial signature, has been considered as a major causative agent for onset of inflammatory bowel disease, no single species has been

convincingly considered as the only causative agent. The compositional imbalance between protective and harmful bacteria, which is termed as dysbiosis, was suggested as the major causative event. Collectively, culture-dependent in addition to independent methodologies claimed for an increase in bacterial concentrations and a decrease in bacterial diversity as a common phenomenon for onset of inflammatory bowel disease (Seksik et al. 2006; Sokol et al. 2008; Chassaing and Darfeuille-Michaud 2011). The positive and negative correlations of gut microbiota composition have been reviewed in Table 7.1.

7.3 Role of Microbial Enzymatic Pool in Host's Metabolism

In the mammalian nutrition system, main processes involved in food absorption take place in the gastrointestinal tract, where the commensal bacteria and their metabolites play a significant role (LeBlanc et al. 2017). Certain regions, especially colon, in the human gut are densely populated, microbial count ~10^{11}, by microbes (Flint et al. 2008).

7.3.1 Metabolism of Carbohydrates

These microbes play a major role in not only metabolizing some complex carbohydrates (resistant starch, maltotriose, amylopectin, and maltodextrin) in the mammalian gut but also harvest energy and nutrition from these food-derived glycans (Milani et al. 2015). Almost 10,000 bacterial enzymes have been recognized to be involved in sugar degradation (El Kaoutari et al. 2013). A higher degree of polymerization, ~200 to 6000, and complexity of these dietary glycans make them inaccessible by the host, as human genetic repertoire is far less efficient in metabolizing such complex plant-based food sources. Although the human colonic microbiota (HCM) acquire a significantly lesser amount (10%) of its host energy requirements from indigestible or partially digestible food sources in comparison to rumen microbiota (70%), it plays an impeccable role in generation of few metabolites showing health-promoting or health-degrading pharmaceutical effects (Flint et al. 2008). The amount and type of host-inaccessible dietary components, like fibers and glycans, decide not only the ecological abundance of human colonic microbiota but also the human colonic microbiota-mediated generation of health-promoting metabolites (Duncan et al. 2007). Hence, in order to get microbe-mediated and targeted health-promoting effects, specialized carbohydrates have been added in the diets to modulate the human colonic microbiota and, in turn, host metabolism (Kruse et al. 1999). Importantly, successful implementation of such strategies relies majorly on the substrate preferences and competitiveness of the colonized species. Hence, knowing the microbial functionality in vitro is a prior requirement before approaching toward animal- or human-based studies (Bhushan et al. 2017).

Table 7.1 Relationships of gut microbiota composition with the physiological state of human body

Associated microbiota			
Physiological state of human body	Abundance of microbiota	Function of microbiota	References
Growth improvement with weight gain	↑ *Lactobacillus* spp.	Modest improvement in growth of children	Agustina et al. (2013)
	↑ *Lactobacillus* spp.	Transplanted healthy infant's microbiota improved growth of malnourished newborn mice	Schwarzer et al. (2016)
	↑ *Weissella* spp.	An association between the gut microbiota composition and weight gain in preterm infants at early life	Arboleya et al. (2017)
	↓ *Staphylococcus*		
	↓ *Enterococcus*		
Weight loss	↑ *Akkermansia muciniphila*	Weight reduction or a lean phenotype in humans	Clarke et al. (2014), Le Chatelier et al. (2013), Remely et al. (2015), and Shen et al. (2017)
	↑ *Lactobacillus* spp.	Associated with weight loss	Karlsson et al. (2013), Omar et al. (2013), and Sanchez et al. (2014)
	↑ *Faecalibacterium prausnitzi*	Associated with weight loss	Clarke et al. (2014), Le Chatelier et al. (2013), and Remely et al. (2015)
	↑ *Methanobrevibacter smithii*	Weight loss	Goodrich et al. (2014)
	↑ *Christensenellaceae*	Weight loss	Goodrich et al. (2014)
	Akkermansia muciniphila (live or pasteurized)	↓ Weight	Anhe and Marette (2017)
		↓Inflammation	
		↓Glucose tolerance	
IBS	↓*Prevotella*	Increase in occurrence and severity of IBS	Tap et al. (2017)
	↓*Methanobacteriales*		
	↑*Firmicutes*		Jafferey et al. (2012)
	↓*Bacteroidetes*		
	↑*Dorea*, ↑*Ruminococcus*, ↑*Clostridium* spp.		Rajilić–Stojanović et al. (2011)
	↓*Bacteroidetes*,		
	↓*Bifidobacterium*		
	↓*Faecalibacterium* spp.		
	↓Microbial diversity		Sundin et al. (2015)

(continued)

Table 7.1 (continued)

Associated microbiota		Function of microbiota	References
Physiological state of human body	Abundance of microbiota		
CD	↑*Veillonellaceae*	A strong association was observed among traced microbiota species and diseased state	Gevers et al. (2014)
	↑*Pasteurellaceae*		
	↑*Enterobacteriaceae*	Intestinal colonization with these oral bacteria leads in children with new-onset Crohn's disease	
	↑*Fusobacteriaceae*		
	And		
	↓*Clostridiales*		
	↓*Bacteroidales*		
	↓*Erysipelotrichales*		
	↓*Faecalibacterium prausnitzii*	Reduction of *F. prausnitzii* is associated with a higher risk of postoperative recurrence of ileal CD. Bacterial metabolites blocked the NF-ƙB activation and IL-8 production	Sokol et al. (2008)
UC	↓*Roseburia hominis* ↓ *Faecalibacterium prausnitzii*	A reduction of *F. prausnitzii* and *R. hominis* defines UC dysbiosis. Their restoration might improve the microbiota balance and symptoms in diseased individuals	Machiels et al. (2013)
Immune homeostasis	Complex microbiota in adults	Microbial signal-mediated colony-stimulating factor 2 production by RORγt$^+$ ILC3 cells promotes myeloid lineage differentiation in gut	Mortha et al. (2014)
	Microbiota perturbation using antibiotics	Microbiota depletion reduced circulatory neutrophils and inhibits neutrophil extracellular traps formation	Zhang et al. (2015a)
	Segmented filamentous bacteria, *Mucispirillum*, *Clostridium scindens*, and *Akkermansia muciniphila*	The T-cell-dependent pathway for activation, level, and diversity of IgA in lumen	Honda and Littman (2016)
	Bacteroides thetaiotaomicron,	In the absence of IgA, this bacterium expresses high levels of pro-inflammatory signals in the host	Peterson et al. (2007)

(continued)

Table 7.1 (continued)

Associated microbiota		Function of microbiota	References
Physiological state of human body	Abundance of microbiota		
	Segmented filamentous bacteria	Its colonization on distal ileum surfaces elicits the unique gene expression profile for increased levels of two isoforms of the protein serum amyloid A and simultaneously activates group 3 innate lymphoid cells to produce IL-22	Sano et al. (2015)
	Clostridium spp. (clusters IV, XIVa and XVIII)	Strongly provoke the gathering of Treg cells in the colon and stimulate the expression of IL-10 and CTLA-4	Atarashi et al. (2011, 2013)
	Lactobacillus reuteri and *Lactobacillus murinus*	These members of human gut microbiota raise the level of Treg cells	Tang et al. (2015a)
	Mixed microbiota	Toll-like receptors, proper aging, and number of neutrophils, basophils, and macrophages Myelopoiesis and cellular differentiation	Balmer et al. (2014), Zhang et al. (2015a, b), Hill et al. (2012), and Erny et al. (2015)
Cancer immunotherapy	*Bacteroides fragilis* and *Bacteroides thetaiotaomicron*	Disruption of these bacteria compromised the antitumor response with a subsequent result of CTLA-4 blockade, while therapy, anti-CTLA-4, favors their dominance and resultant anticancer response through TH1 cells	Vétizouet al. (2015)
	Bifidobacterium spp.	Elevation in the antitumor T-cell-dependent response by increasing the Treg cells and blocking the programmed death 1 pathway with a systemic anti-inflammatory response	Atarashi et al. (2011, 2013)

(continued)

Table 7.1 (continued)

Associated microbiota		Function of microbiota	References
Physiological state of human body	Abundance of microbiota		
Allergic diseases	↓*Faecalibacterium* ↓*Lachnospira*,	Lowered prevalence of these microbial species in children rendered them prone to developing atopy and asthma	Arrieta et al. (2015)
	↓*Veillonella*		
	↓*Rothia*		
	L. reuteri	Increase in T_{reg} discouraged the inflammatory cell arrival to the airway and reduced hyper-responsiveness	Karimi et al. (2009)
Rheumatoid arthritis	↑*Prevotella*	Increased prevalence of this organism in oral and gut microbiota predispose an individual to this autoimmune disorder	Zhang et al. (2015b)

Note that gut microbiota dysbiosis has strongly been correlated with the onset of many acute and chronic physiological disorders
↓ decrease, ↑ increase, *IgG* immunoglobulin G, *IgA* immunoglobulin A, *IBS* irritable bowel syndrome, *IBD* inflammatory bowel disease, *CD* Crohn's disease, *IL* interleukin, *NF-Kb* nuclear factor kappa-B, *UC* ulcerative colitis, *Treg* regulatory T cells, *TH* helper T cells, *CTLA* cytotoxic T-lymphocyte-associated protein, *RORɣ* retinoid-related orphan receptor gamma, *ILC3* innate lymphoid cells

One such study described the bifidobacterial saccharolytic features after evaluating some human isolates, using in vitro fermentative, metagenomic, and transcriptomic techniques, for species-specific glycans preferences and cross-feeding patterns supposed to be happened in human gut (Milani et al. 2015). The genus *Bifidobacterium*, along with *Bacteroidales* and *Clostridiales* families, was also recognized as the most versatile species expressing the largest pool of glycosyl hydrolases, with respect to usual enzymatic store of the gut microbiome. A human trial assessed the prebiotic potential of novel galacto-oligosaccharides generated after the action of β galactosidases from *Bifidobacterium bifidum* and reported their bifidogenic effects in 59 healthy human volunteers (Depeint et al. 2008). In a recent report (Chang et al. 2014), gut microbiota have been known for utilization of dietary fibers for butyrate production, which epigenetically regulated the immunological status of host with downregulation of lipopolysaccharide-induced pro-inflammatory mediators, including nitric oxide, IL-6, and IL-12, but did not affect levels of tumor necrosis factor-α or monocyte chemotactic protein-1.

Lactobacilli representing a small but important part of the gut microbiota and being acid and bile tolerant, they can modulate the host metabolism through participating in degradation of lipids and sugars in the small intestine and might play a role in energy expenditure and storage (Drissi et al. 2017). Lactobacilli are well known for their strain-dependent traits enabling them for utilizing different sugar sources by the virtue of their enzymatic repertoire. Depending upon their habitation, they

acquire or excise out the genes responsible for carbohydrates utilization and sugar transportation inside the cells (Makarova et al. 2006). Several studies reported the strain-specific sugar-fermenting capabilities of lactobacilli (Douillard et al. 2013), and these phenotypes decide the fate of sugar metabolism and weight management in host (Drissi et al. 2017).

Although metagenomic approaches, like human microbiome project, helped a lot in exploring the microbiome and related functionalities, still almost 50% of genes could not be attributed to their respective functions using presently available bioinformatics' tools (Human Microbiome Project Consortium 2012; Joice et al. 2014).

7.3.2 Metabolism of Other Dietary Components

Besides the abovementioned detailed description of sugar metabolism by gut microbiota and their enzymatic repertoire, metabolism of other food components has been described in Table 7.2. Although not characterized at the fullest, below mentioned gut microbiota-mediated xenobiotic metabolism highlights the role of human-associated microbes in human health and disease (Koppel et al. 2017).

Gut microbiota has also been known for their bile-degrading capabilities, through the action of enzyme bile salt hydrolase, leading to increased fat metabolism (Wang et al. 2012; Panicker et al. 2015). In addition, gut commensals increase the absorption of fatty acids in intestinal cells (Bäckhed et al. 2004).

Although kynurenine pathway is the principal route for tryptophan catabolism in both humans and mice (Krishnan et al. 2015), other lines of microbiota-mediated mechanisms of tryptophan metabolism exist (Fig. 7.1).

In terms of application, human studies describing the role of gut microbiota in digestion are hard to extrapolate in a specified way. Most of the microbiota-related human trials are dependent on microbial profiling from fecal samples, which is not a comprehensive approach to understand the exact role of gut microbiota in human digestion and metabolism, as many of the lactobacilli spp. are predominant members of human upper gastrointestinal tract (Raoult and Henrissat 2014; Walsh et al. 2008). In addition, the colon cannot be considered as a sole organ with all essential metabolic reactions as subjects with a total colectomy are also seen with normal metabolism and not transform into lean or obese (Christl and Scheppach 1997). Moreover, no sugars have been found in ileostomy effluent; hence ileostomy can be considered inert regarding a significant role in sugar metabolism. Conclusively, more attention should be paid on whole gut microbiota, including proximal region, while designing any digestion study with a role of microbiota (Raoult and Henrissat 2014).

Table 7.2 Microbial metabolism of dietary components and its influence on human health

Dietary component	Microbiota community involved	Impact on human health	References
Protein, gluten	*Lactobacillus* or *Pseudomonas*	*Lactobacillus*-specific gluten-derived peptides reduced the celiac disease-specific gluten-mediated inflammatory response in comparison to *Pseudomonas*-specific disease-prone metabolism	Caminero et al. (2016)
Amino acid, L-tryptophan	Mixed microbiota	Microbiota-mediated indole production leads to generation of uremic toxin indoxyl sulfate, after hydroxylation and sulfation by hepatic enzymes	Devlin et al. (2016)
Amino acid, L-carnitine	Mixed microbiota	Conversion of L-carnitine, a trimethylamine, to produce trimethylamine N-oxide, thereby promoting atherosclerosis	Koeth et al. (2013)
Lipids	*Eubacterium coprostanoligenes*	Cholesterol reduction and coprostanol synthesis with subsequent oxidation steps with resultant ketone reduction	Ren et al. (1996)
Vitamin B12	*Propionibacterium acnes*	B12 supplements modulate the *P. acnes* transcriptome; upregulation of porphyrin synthesis and downregulation of B12 synthesis. Hence this bacterium promotes acne pathogenesis via porphyrin production	Kang et al. (2015)
Soy isoflavones, lignans from seeds, flavonoids like gallate esters and catechins, ellagic acid	Mixed microbiota	Microbiota help humans to absorb these phytochemicals, using some microbe-specific dehydroxylation, demethylation, and ring cleavage reactions for higher bioavailability, raised bioactivity, and reduced ailment risk	Atkinson et al. (2005) and Tang et al. (2015b, c)
Artificial sweeteners, preservatives and emulsifiers	Mixed gut microbiota	Microbial transformation of stevioside and xylitol	Krishnan et al. (1980) and Renwick and Tarka (2008)
2-amino-3-methylimidazo[4,5-f] quinoline (IQ)	*Escherichia coli*	Microbial enzyme β-glucuronidase synthesize unconjugated IQ with mutagenic property in colon	Humblot et al. (2007)

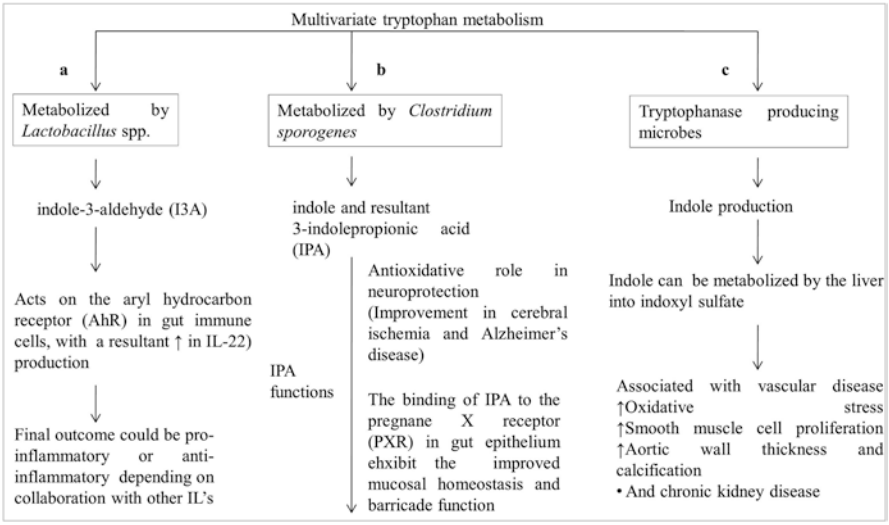

Fig. 7.1 Comparative flowchart for metabolic fate of dietary tryptophan in presence of different set of gut microbiota. Tryptophan metabolisms by *Lactobacillus* spp. (**a**) and *Clostridium sporogenes* (**b**) have been considered beneficial, in comparison to metabolism by tryptophanase-producing microbes (**c**), which cause many vascular and kidney diseases (Wikoff et al. 2009; Zhang et al. 2016). *IL* interleukin

7.4 Microbiota and Their Metabolites for Pharmacological and Clinical Outcomes

Pharmacology, the study of chemicals or drugs, and toxicology, the study of undesirable systemic effects, are few of the matured streams (older than 4.5 centuries) of biological sciences (Frank and Ottoboni 2011), while microbiota and microbiome research are still at infancy stage. However, some pioneering research methodologies, like ribosomal RNA gene sequencing, and advanced research scheme, like metagenomics, have equipped us with a handful knowledge regarding the complex human microbiota (Lane et al. 1985; Schloss and Handelsman 2003). Moreover, they established a basement for assessing microbial role in pharmacology. Besides the cataloguing of microbial species in terms of healthy and diseased states of body (Gerritsen et al. 2011), the comprehensive details of microbial metabolome is lagging behind that of the host (Schloss and Handelsman 2003). It has always been a hard task to deduce metabolism of human microbiota. Majority of influential work in the field relied on the following pathway(s): (1) recognition of biochemical transformation of interest, (2) replication of chemical reaction in vitro, (3) identification and isolation of enzyme(s) responsible, and (4) learning each reaction at organ and subcellular location (Murphy 2001). Most current searches for new drugs are focused on identifying a pharmacologically effective agent that specifically interacts with one molecular target in the eukaryotic host cell to regulate biochemical

alterations in a diseased state and reinstate healthy-state biochemistry (Nicholson et al. 2004). During the past 30 years, such an approach has generated highly successful medicines. However, it does not completely exploit the complex regulatory network that has been engineered through evolutionary processes in humans, who could be viewed as "super-organisms" with an indispensable internal ecosystem: the gut microbiota.

7.4.1 Microbiota in Drug Metabolism, Efficacy, and Toxicity

More than 40 drugs are reported to be modified by human gut microbiota, with significant inference for medicine efficacy and toxicity, the responsible microbe and the enzymatic repertoire involved is still remaining unearthed for majority of these drugs (Haiser and Turnbaugh 2013). Though, few enzymatic systems have been recognized and even linked to few taxonomic groups (Haiser and Turnbaugh 2013). But these enzymes have been scarcely explored for phylogenetic alignment, their activity at cellular level, and scope for horizontal gene transfer. Besides the enzymatic repertoire, a wide gap of knowledge still prevails regarding the actual site of action (mucus or luminal) of such microbes (Patterson and Turnbaugh 2014). Still, some recent studies have extrapolated, on the basis of activity, bioavailability, and toxicity of therapeutic drugs, the clinical relevance of microbial biotransformations of drugs.

Few of the examples of microbial transformation/interaction of drugs and their effects on human health have been given in Table 7.3.

Cardiac glycosides, including digoxin, have been playing the lead role in treatment of heart failure and arrhythmias since 100 of years. Microbial inactivation of digoxin, by decreasing its concentration in serum, has displayed a classical role of microbiome in pharmacology. Moreover, this provided the microbiota-dependent modulations of pharmaceutical or dietary interventions. The bacterium reduces the lactone ring and increases the concentration of reduced form in serum (Lindenbaum et al. 1981). Because of disturbed concentration range (appropriate range: 0.5–2.0 ng/ml) of active drug (Goodman 2011), it becomes ineffective. Moreover, reduction process decreases the target affinity by shifting the positioning within the binding pocket of the Na+/K+ ATPase (Farr et al. 2002). As per Haiser and coworkers, they tested in vitro the three *E. lenta* strains, including one type strain with digoxin reduction ability, which were not identifiable on the basis of common genetic markers. But reciprocal BLAST-P compared the three strains on proteome basis and found 90.5% and 79.4% similarity of type strain with other two strains. And, the cgr operon, the genetic loci responsible for drug inactivation, was only found in type strain. Hence, very fine cataloguing of microbiota, on the basis of abundance of cgr loci in gut microbiome of a population, is required to establish multivariate susceptibility groups. This would provide a better therapeutic regime according to respective gut microbiota and their metabolome. More numbers of active and follow-up studies are required, however, to establish a more clear

Table 7.3 Clinical examples of microbial role in efficacy and toxicity of drugs

Role of microbiome	Microbiota type (single species or mixed population)	Therapy used	Drug-microbiota interaction	Health effect (s)	References
Harmful	*Eggerthella lenta*	Digoxin; prescribed in heart failure and arrhythmias	Biotransformation of digoxin to dihydrodigoxin	↓ efficacy of digoxin	Haiser et al. (2013)
	Microbiota in dysbiotic state	Platinum chemotherapy and CpG-oligonucleotide immunotherapy	Lesser activation of immune response and reduced expression of immune mediators	↓ efficacy and ↑ tolerance toward therapy	Yu et al. (2014)
Beneficial	*Enterococcus hirae*, *Lactobacillus murinus*, *Lactobacillus johnsonii*	Cyclophosphamide as anticancer drug	Drug modulates and recruits the selected gram-positive species for translocation to specified organs	↑ efficacy of cyclophosphamide ↑ synergistic immunogenic effect of microbiota	Viaud et al. (2013)
	↑*Bacteroidetes* ↓*Firmicutes* ↓ *Firmicutes*/*Bacteroides* ratio	Vildagliptin for type 2 diabetes	The drug increased butyrate-producing bacteria; *Erysipelotrichaceae* and *Bacteroides*	The drug modulate the gut microbiota in a beneficial way	Zhang et al. (2017)
	Akkermansia muciniphila	Metformin; prescribed in type2 diabetes	Drug induced the abundance of bacterium	A synergistic mechanism of drug and bacterium has been proposed for drug efficacy	Lee and Ko (2014), Qin et al. (2012), and Forslund et al. (2015)

	Barnesiella intestinihominis and *Enterococcus hirae*	Cyclophosphamide; prescribed as anticancer immunomodulatory agent	Drug facilitated the microbes transfer to lymphoid organs with resultant increase in CD8/Treg ratio. Subsequently, microbes raised the infiltration of IFN-ɣ-producing gdT cells in cancer lesions in the colon	Drug efficacy improved synergistically in presence of these two species of gut microbiota	Daillère et al. (2016)
Beneficial or harmful	Mixed microbiota	Statin as drug reducing risk of cardiovascular disease	Microbially derived systemic bile acids and 2-hydroxyvaleric acid decide the efficacy of drug	Individuals can be differentiated from good "statin responders" to poor "statin responders"	Krauss et al. (2013) and Wishart (2016)

Note that action and efficacy of pharmaceutical drugs depend on the microbiota type present in hosts' gut
↓ decrease, ↑increase, *CD* cluster of differentiation, *IFN*-ɣ interferon-gamma, *Treg* regulatory T cells

relationship between host biomarkers, microbiome, and dosage regime. Some specified dietary interventions and/or supplements may revert back the drug inhibitory mechanisms (Haiser et al. 2013) or support the beneficiary actions of microbiota.

In another study (Yu et al. 2014), gut microbiota dysbiosis, using antibiotic cocktail, left the cancerous mice with less effective response to therapy in comparison to mice with intact microbiota. Genes responsible for immune activation, like phagocytosis, antigen presentation, and adaptive immunity, were found downregulated, whereas genes for cancer development, like metabolism, growth, and tissue development, showed upregulation in such mice. Moreover, microbiota-depleted mice also showed weak response to therapy of tumor-permeating myeloid-derived immune cells, resulting in poor production of reactive oxygen species and cytotoxicity after chemotherapy and minor cytokine production after CpG-oligonucleotide treatment. Hence, the intact gut microbiota can play a major role in successful implementation of any cancer therapy by modulating the immune system in tumor microenvironment (Iida et al. 2013; Yu et al. 2014).

An interesting study (Viaud et al. 2013) reported the microbiota-modulating and microbiota-migrating actions of drug, enabling the translocation of selective microbial species to secondary lymphoid organs and improved the action of drug. In germ-free or microbiota-dysbiotic mice, however, a resistance to antitumor drug cyclophosphamide was observed. This is a symbiotic-type action of drug and microbiota, inventing and recruiting the new subtypes of immune cells with immunogenic activity against tumors.

7.4.2 Short Chains Amino Acids as Microbial Metabolites for Clinical Outcomes

Short chain fatty acids are weak acids containing 2-5-carbon and present in colonic lumen in different concentrations, including acetate (C2; 60%), propionate (C3; 25%), butyrate (C4; 15%), and valerate (C5). These can be obtained in gut through microbial fermentation. The major substrates for such acids are the resistant starch and dietary fibers. They can be absorbed through lumen (Topping and Clifton 2001) and take part in host's metabolic processes as a precursor; acetate and propionate can promote lipogenesis and gluconeogenesis, respectively, in the liver (Wong et al. 2006). Besides these metabolic roles of fatty acids, tissue-specific (insulin action in peripheral tissues) and distal actions (metabolic regulation of integrated metabolic responses in the central nervous system) have also been reported in recent past and have been characterized on molecular level (Gao et al. 2009; Kimura et al. 2013; De Vadder et al. 2014). Besides other fatty acids, butyrate is the most important fuel source for colonocytes (Roediger 1980; Wong et al. 2006) and will be explained much in the present chapter.

Butyrate, a pluri-functional short chain fatty acid, is produced by colonic microbiota through fermentation of dietary fibers. Primarily, this metabolite provides energy to colonocytes and takes part in many cellular pathways (Leonel and Alvarez 2012). Lower gut microbiota has been well known to produce this fatty acid. A favorable acidic environment is required for butyrate production as acidity plays a growth-promoting role for gram-positive butyrate producers over gram-negative nonproducers such as *Bacteroides* spp. (Guilloteau et al. 2010). Lower pH values, pH 5.5, have been reported to be favorable for higher butyrate production in clinical trials as compared to high pH values, pH 6.5 (Walker et al. 2005). Although butyrate-producing capability is widely distributed among gram-positive anaerobic bacteria in the human colon, *Firmicutes*, majorly *Faecalibacterium prausnitzii* (clostridial cluster IV) and *Eubacterium rectale/Roseburia* spp. (clostridial cluster XIVa), are the main producers of this important metabolite (Louis and Flint 2009). Other major butyrate-producing genera have been reported in Table 7.4. At the intestinal niche, butyrate actively plays a regulatory (in oxidative stress, mucosal inflammation, visceral sensitivity and enteric motility) and inhibitory (in colorectal cancer) role to improve some pathological conditions (Canani et al. 2011).

In a published clinical report, higher short chain fatty acids are claimed to be produced in overweight individuals by a signature colonic microbiota in comparison to lean individuals with different intestinal microbiota (Rahat-Rozenbloom et al. 2014). In another report, gut microbiota-mediated butyrate production enables the better interaction with host receptors on leukocytes and intestinal endothelial cells and modulates Th1 and Th2 immunity in a beneficial way (den Besten et al. 2013). Moreover, microbiota-mediated butyrate production has also been reported, in a number of published reports, as a colon-cancer-preventing strategy due to

Table 7.4 List of major butyrate-producing microbial species

Bacterial species/group	Cluster
Butyrivibrio fibrisolvens	XIVa
Roseburia hominis	XIVa
Eubacterium hallii	XIVa
Eubacterium rectal	XIVa
Eubacterium ramulus	XIVa
Roseburia faecis	XIVa
Roseburia intestinalis	XIVa
Anaerostipes caccae	XIVa
Coprococcus eutactus L2-50	XIVa
Coprococcus catus GD/7	XIVa
Coprococcus comes A2-232	XIVa
Roseburia inulinivorans	XIVa
Eubacterium cylindroides	XVI
Anaerotruncus colihominis	IV
Subdoligranulum variabile	IV
Faecalibacterium prausnitzii	IV

histone deacetylase inhibitory and apoptosis-promoting activities of this molecule (Donohoe et al. 2014). In another interesting study, n-butyrate in the lumen was reported to inhibit histone deacetylase activity and resultant expression of pro-inflammatory cytokines in macrophages, thereby revealing the tolerance mechanism for most of the commensals gut microbes (Chang et al. 2014).

7.5 Probiotics as a Part of Gut Microbiota

One of the most influencing strategies for the maintenance and modulation of gut microbiota is the oral administration of selective microbes, which are known as probiotics with a definition of "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (Hill et al. 2014). The most extensively studied and commercially available probiotic organisms are majorly of two genera, *Lactobacillus* and *Bifidobacterium* (Douillard and de Vos 2014); however other microbes are also employed, including *Saccharomyces boulardii* (Feizizadeh et al. 2013) and *Escherichia coli* Nissle 1917 (Trebichavsky et al. 2010). Besides their probiotic nature, lactobacilli and bifidobacteria comprise a small but significant part of human microbiota (Leeber et al. 2018). These two genera are known for absence of any pathogenic species, and their abundance in human feces, along with other members of microbiota, is always considered beneficial (Harmsen et al. 2002).

7.5.1 Role of Probiotics in Disease Management

Probiotics are known to withstand the gastrointestinal barriers, to strengthen the gut integrity, to modulate the host immune system, to competitively exclude pathogens, to produce micronutrients (including vitamins), to complement host metabolism, and to support human health (Bhushan et al. 2017; Thursby and Juge 2017). Bifidobacteria and lactobacilli are considered as the two main health-promoting probiotic bacteria (Salminen et al. 1998). Decrease in the lactobacilli and bifidobacteria concentration in gut microbiota of elderly subjects have been reported with altered immunological and inflammatory responses (Hopkins and Macfarlane 2002; Kato et al. 2004). Hence, gut microbiota modulation through probiotic therapy has also been used for establishing a healthy gut microbiota with less inflammatory cytokine profile (Spaiser et al. 2015).

A myriad number of clinical studies, including humans and animals, have claimed for the disease-preventing and curing effects of probiotics (live and intact cells), their metabolites (Dorrestein et al. 2014), and surface molecules (Plovier et al. 2017). There has been an increase in evidences of health-promoting effects of effector molecules from both important probiotic organisms: lactobacilli and

bifidobacteria. Majority of probiotic-associated health claims have been summarized in the Table 7.5.

Besides abovementioned clinical health claims and specialized production of biomolecules, probiotics have also been known for biotransformation of micronutrient into more bioavailable forms (Saini et al. 2014, 2015) and also for bioremediation activities for removal of harmful heavy metals (Kumar et al. 2018) from in vivo systems.

7.6 Specialized Metabolites from Probiotics and Lactic Acid Bacteria (LAB)

7.6.1 Vitamins

Besides their complementary role in nutrient availability and metabolism of host (Neis et al. 2015), upper GI tract commensals have also been considered as a source of some vitamins, which are absorbed through the small intestine (Said and Mohammad 2006). The small intestine is known for its less diverse microbiome and majorly colonized by *Streptococcus*, *Lactobacillus*, *Veillonella*, and *Clostridium* clusters (Booijink et al. 2010; Wang et al. 2005). In a recently published report on B vitamin production capability of small intestinal bacteria, mRNA transcripts were identified including cobalt-precorrin-6A synthase from *Clostridium difficil*e, cobalt-precorrin-6x reductase from *Veillonella parvula*, and cobalt-precorrin-2 C20-methyltransferase from *Streptococcus sanguinis*. Moreover, transcriptomes for folate (B9), biotin (B7), pyridoxine (B6), riboflavin (B2), and thiamine (B1) biosynthetic enzymes were also detected primarily from *Pasteurellaceae* and *Streptococcaceae* species (Golomb et al. 2016). In a non-mammalian research, the gut microbiota-derived B vitamin complementation, particularly riboflavin, enables the flies to use low-nutrient or unbalanced diets (Wong et al. 2014).

Besides abovementioned genera, *Lactobacillus* has been considered as one of the most important ingested microbes. The diverse ecological habitation of lactobacilli (London 1976) has equipped these microbes with strain-specific metabolite (like vitamins)-producing capabilities (LeBlanc et al. 2011). Lactobacilli have also been recognized as a B vitamin producer, including production potential of B_1 (Saulnier et al. 2011), B_2 (de Valle et al. 2016), B_9 (Santos et al. 2008; Hugenschmidt et al. 2011), and B_{12} (Bhushan et al. 2016).

In one of the pioneer study, *L. reuteri* was reported as the first *Lactobacillus* species studied for vitamin B_{12} (B_{12} or cobalamin) production (Taranto et al. 2003). Furthermore, *L. plantarum* (Madhu et al. 2009; Masuda et al. 2012) and *L. rossiae* strains (de Angelis et al. 2014) were isolated from various fermented food products and reported for B_{12} production. It is one of the most important B-group vitamins and has been reported to play a significant role in hematological, cardiovascular, and neurological systems of human body.

Table 7.5 Clinical studies describing the roles of probiotics in disease prevention/management

Targeted disease/ syndrome	Probiotic strain (s) used	Study duration	Targeted subjects	Study design	Dose size and route	Pharmacological effect	References
Antibiotic-associated diarrhea	*L. rhamnosus* R0011 and *L. helveticus* R0052	10 weeks	Healthy individuals aged 18–50 years	A randomized, double-blind, placebo-controlled trial	8×10^9 CFU (oral route)	Duration of the diarrhea was significantly reduced in probiotic-treated adults	Evans et al. (2016)
	L. acidophilus, *B. lactis* and *LGG*	Duration of antibiotic treatment plus 1 week	70 children aged 1–12 years	Multisite, randomized, double-blind, placebo-controlled clinical trial	Ingestion of 100 g yogurt (twice a day) with final cell count of La-5 (8.3×10^9 CFU/day), Bb-12 (5.9×10^9 CFU/day) and LGG (5.2×10^9 CFU/day) Or A pasteurized yogurt, as placebo	A highly significant reduction in number of diarrhea and a higher compliance to therapy in yogurt-treated group	Fox et al. (2015)
Necrotizing enterocolitis	Multiple strains probiotics vs single strain probiotic	Initiated within the first 7 days and continued for at least 14 days	Preterm infants, aged ≤34 weeks' gestation and/or those of a birth weight ≤1500 g	An updated meta-analysis of randomized controlled trials	Enteral probiotic supplementation	Significant reduction in incidence of necrotizing enterocolitis and mortality with mixed probiotic use	Chang et al. (2017)
	A mixture of lactobacilli and bifidobacteria (Infloran) with/or breast milk	Routine supplementation from birth until discharge or 37 + 0 week GA	463 VLBW infants, <34/0 week gestation	–	Enteral feeding of 10^9 *B. infantis* and 10^9 *L. acidophilus*	Necrotizing enterocolitis was significantly reduced in probiotic fed group receiving mother's milk only	Repa et al. (2014)

	Infloran ; *L. acidophilus* and *B. infantis*, with/or breast milk	Routine supplementation from birth until discharged	367 very low birth weight (<1500 g) infants survived beyond the 7th day after birth	A prospective, masked, randomized control trial	Enteral feeding of 10^9 *B. infantis* and 10^9 *L. acidophilus*	Significant reduction (50%) in incidence and severity of necrotizing enterocolitis	Lin et al. (2005)
	B. bifidum and *L. acidophilus*	Twice daily for 6 weeks	444 very low birth weight preterm infants	Prospective, blinded, randomized, multicenter controlled trial	20 ml/kg per day per feeding An oral intake of 100 ml/kg per day was defined as complete enteral feeding	Reduction in necrotizing enterocolitis or the incidence of death. No adverse effects (such as sepsis, flatulence, or diarrhea) were seen	Lin et al. (2008)
Psychological (stress related) disorders	*S. thermophilus* SGSt01, *B. animalis* subsp. *lactis* SGB06, *S. thermophilus*, *B. bifidum* SGB02, *L. delbrueckii* spp. *bulgaricus* DSM 20081, *Lactococcus lactis* subsp. *lactis* SGLc01, *L. acidophilus* SGL11, *L. plantarum*, *L. reuteri* SGL01, maltodextrin, anticaking agent (silica), casein, lactose, and gluten	3-week	48 individuals were selected on the basis of body composition analysis, psychological profile, and eating behavior assessment by symptom checklist 90	A double-blind randomized clinical trial	1 bag of 3 g psychobiotics oral suspension containing cell numbers 1.5×10^{10} for all tested probiotic bacteria	A significant improvement in GI symptoms ($p < 0.05$), defecation frequency ($p < 0.05$), orocecal transit time and meteorism ($p < 0.05$)	Lorenzo et al. (2017)

(continued)

Table 7.5 (continued)

Targeted disease/ syndrome	Probiotic strain (s) used	Study duration	Targeted subjects	Study design	Dose size and route	Pharmacological effect	References
	L. helveticus R0052, *B. bifidum* R0071, or *B. infantis* R0033	6 weeks	581 college students stratified based on body mass index and randomized in semester exam days	A randomized, double-blind, placebo-controlled study	One study capsule per day with a meal. Or placebo (97% potato starch and 3% magnesium stearate) in hydroxypropyl methyl cellulose capsules	A significant reduction in stress-associated GI dysfunction and self-reported stress	Culpepper et al. (2016)
	L. helveticus R0052 and *B. longum* R0175	1 month	55 healthy Caucasian men and women	A double-blind, controlled, randomized, parallel study	During or just after breakfast, all volunteers took one stick of 1·5 g/day of PF (Probio' Sticks: batch no. 6533308; Institut Rosell-Lallemand, Blagnac, France)	Probiotic combination alleviated the psychological distress, cortisol level decreased after treatment	Messaoudi et al. (2011)
	3×10^9 cfu/stick				Or		
					Placebo (xylitol, maltodextrin, plum flavor, and malic acid)		

Acute diarrhea	Probiotic yeast *Saccharomyces boulardii;*	1 month	100 outpatients between 3 and 24 months old presenting with acute mild to moderate diarrhea of less than 7 days duration	A double-blind, prospective, randomized, placebo-controlled clinical trial	*Saccharomyces boulardii* was provided by the manufacturing company as capsules, to enable administration in liquid or food. The capsules contained 250 mg of *Saccharomyces boulardii* (or placebo); children under 1 year were given 1 capsule per day and those over 1 year, 2 capsules per day (every 12 h)	Reduction in the duration of diarrhea ($p < 0.05$), risk of prolonged diarrhea with accelerated recovery and more efficacy in case of early intake (within the first 48 h) of *Saccharomyces boulardii*	Villarruel et al. (2007)
	Saccharomyces boulardii at any dose and in any form; like capsule, sachet, yogurt	5–10 days	Children of age 0–18 years, male or female of any ethnic group with acute diarrhea (<14 days)	Meta-analysis based on randomized controlled trials	Most of the studies relied on daily dosage of 250–750 mg (10^9–10^{10} cfu) of *Saccharomyces boulardii*	Reduction in duration of diarrhea in the treatment group compared with the control group ($P < 0.001$)	Feizizadeh et al. (2013)
	LGG, 10×10^8 cfu	5 days	200 children (6 months–5 years of age) with acute watery diarrhea	An open-labeled, randomized controlled trial	In combination of standard World Health Organisation therapy, probiotic supplement or none was received by tested subjects	Both frequency and number of stool were found reduced only in probiotic-treated group ($p < 0.001$)	Aggarwal et al. (2014)

(continued)

Table 7.5 (continued)

Targeted disease/ syndrome	Probiotic strain (s) used	Study duration	Targeted subjects	Study design	Dose size and route	Pharmacological effect	References
	S. thermophilus, *L. rhamnosus*, *L. acidophilus*, *B. lactis*, *B. bifidum*, *and B. longum*	4 weeks	49 IBS patients	Randomized, double-blind, placebo-controlled trial	One capsule of probiotics/placebo twice a day to respective treatment group	Probiotic-treated group not only showed significant improvement ($p < 0.05$) in IBS symptoms by 4 weeks, but also the secondary symptoms (abdominal pain/discomfort and blotting) were alleviated	Yoon et al. (2014)
Prenatal therapy	*B. lactis*, Christian Hansen, Denmark, alone or in combination with LGG, ATCC 53013	14 days	43 women in late pregnancy and elective for cesarian section after 37 weeks of gestation	A randomized, double-blind placebo-controlled clinical trial	Supplementation of 10^9 cfu of probiotic or placebo (corn starch) in the form of water-dissolvable powder sachet for twice in a day, 14 days prior to the cesarian section	Expression of toll-like receptor genes both in the placenta and in the fetal gut was found to be significantly modulated using maternal probiotic supplementation, hence claimed for prenatal reprogramming of fetus to prevent postnatal long-term metabolic disorders	Rautava et al. (2012)

Sepsis	*L. plantarum* plus fructooligosaccharide	60 days	4556 healthy infants of minimum 35 weeks of gestation with no sign of sepsis	A randomized, double-blind, placebo-controlled trial	A capsule containing live cells of *L. plantarum* (~10^9) and fructooligosaccharide (150 mg) with maltodextrin (100 mg) as excipient	Significant reductions in sepsis were observed with lower respiratory tract infections in synbiotic-treated groups	Panigrahi et al. (2017)
							Jacobs et al. (2013)
For respiratory diseases	*L. paracasei* N1115, 3.6×10^7 CFU/mL	12 weeks	A total of 205 healthy volunteers (≥45 years) with similar living conditions	A randomized controlled open-labeled trial	300 ml/day of yogurt was orally taken as a supplement to the normal diet Or Normal diet without probiotic for control group	With the proposed mechanism of T-cell-mediated immunomodulation, the incidence of acute upper respiratory tract infection significantly decreased in the probiotic-supplemented group ($P = 0.038$)	Pu et al. (2017)
	LGG and BB-12w (10^9 cells) (Chr. Hansen A/S)	12 weeks	Framingham State University students living on campus	Prospective, randomized, double-blind, placebo-controlled trial	Administered in the form of probiotic containing flavored powder, 5 g using a stick on daily basis	The duration of respiratory tract infections was significantly reduced from 6 days (in placebo-treated group) to 2 days (in probiotic-supplemented group)	Smith et al. (2013)

(continued)

Table 7.5 (continued)

Targeted disease/ syndrome	Probiotic strain (s) used	Study duration	Targeted subjects	Study design	Dose size and route	Pharmacological effect	References
Rheumatoid arthritis	*L. acidophilus, L. casei* and *B. bifidum* (2×10^9 CFU/g of each)	8 weeks		Randomized, double-blind, placebo-controlled clinical study	Capsule containing probiotic combination	Metabolic status of rheumatoid arthritis patients got significantly improved. Disease activity score for 28 joints got improved; insulin level got decreased; and low density lipoprotein cholesterol level also significantly got improved	
	L. casei strain Shirota	4×10^{10} CFU	Oral consumption of fermented milk	6 weeks	Controlled single-blind clinical trial	Disease activity score for 28 joints was found to be observed in a favorable trend manner. Pro-inflammatory cytokine level got decreased, and anti-inflammatory cytokine level got increased in a positive manner with a limitation in statistical significance	

Anti-allergic	*B. longum* MM-2, *B. bifidum* G9-1, and *L. gasseri* KS-13 (1×10^8 cfu of each)	8 weeks	173 participants of age ~27 years with a history of seasonal allergies	Double-blind, placebo-controlled, randomized trial	Probiotic-/placebo-containing capsules (350 mg) were advised to be taken at the end of morning meal and another after having the evening food	The probiotic cocktail improved the ease of life of participants with mini rhinoconjunctivitis	Dennis-Wall et al. (2017)
	L. paracasei LP-33 (2×10^9 cfu)	5 weeks	Adults (18–60 years of age) oversensitized to grass pollens and showing persistent allergic rhinitis symptoms at least since last 2 years	A double-blind, randomized, placebo-controlled trial	A capsule containing dicalcium phosphate, magnesium stearate, and microcrystalline cellulose with probiotic (supplemented group) and without any beneficial microorganism (placebo) had to be consumed without meal	Quality of life was certainly enhanced by a significant and consistent improvement in ocular symptoms	Costa et al. (2014)
	L. paracasei 33 (5×10^9 cfu)	30 days	Ninety patients with perennial allergic rhinitis	A randomized, double-blind, placebo-controlled trial	2 capsules/day containing live or heat-killed probiotic or placebo were orally given to patients over a period of 30 days	Both frequency of AR and level of patient's botherness decreased with a highly significant manner ($p < 0.0001$ and $p = 0.004$, respectively). Overall significance ($p < 0.05$)	Peng and Hasu (2005)

(continued)

Table 7.5 (continued)

Targeted disease/ syndrome	Probiotic strain (s) used	Study duration	Targeted subjects	Study design	Dose size and route	Pharmacological effect	References
Anti-inflammatory	*B. longum* MM2, *B. bifidum* G9-1, and *L. gasseri* KS-13	13 week	118 older adults (age, 65–80 years)	A randomized, double-blind, placebo-controlled, crossover study	Probiotic/placebo twice daily	The probiotic-treated group showed healthy gut microbiota and less inflammatory cytokine profile as compared to placebo-treated group	Spaiser et al. (2015)
							Kato et al. (2004)
	L. salivarius CECT5713 (2×10^8 cfu)	4 weeks	40 healthy adults	A randomized, double-blinded, placebo-controlled human clinical trial	Probiotic/placebo capsule once a day	Probiotic treatment induced an increase in the proportion of natural killer cells and monocytes, in turn increase in innate immune responses ($p < 0.01$). Enhancement in regulatory cytokines, like IL-10 or TGF-b, is associated with immune system homeostasis	Sierra et al. (2010)

Oral health	*L. pentosus* strain b240 (4×10^9 cfu)	12 weeks	80 healthy elderly persons	Randomized placebo-controlled trial	Heat-killed probiotic in the form of water beverage (125 mL) or only the beverage in placebo group	Probiotic-treated group exhibited an increased (20%) level of salivary IgA antibodies, which was correlated with the better mucosal immunity and antimicrobial activity	Kotani et al. (2010)
Liver disease	*L. rhamnosus* DSMZ 21690 (2×10^9 cfu), *L. acidophilus* ATCC B3208 (3×109 cfu), *B. bifidum* ATCC SD6576 (2×10^9 cfu), and *B. lactis* DSMZ 32269 (6×10^9 cfu)	12 weeks	Participants of age between 10 and 18 years were chosen on the basis of body mass index (BMI ≥85) and sonographic conclusions of NAFLD	A randomized triple-blind placebo-controlled trial	One probiotic capsule or placebo with a frequency of once a day to respective group. Placebo and probiotic capsules were similar in appearance and dimensions	Probiotic group, in comparison to placebo group, showed significant reduction in the level of serum enzymes, alanine aminotransferase and aspartate aminotransferase, cholesterol, triglyceride, and low deficiency lipoproteins with normal liver sonography ($p < 0.001$)	Famouri et al. (2017)

(continued)

Table 7.5 (continued)

Targeted disease/ syndrome	Probiotic strain (s) used	Study duration	Targeted subjects	Study design	Dose size and route	Pharmacological effect	References
Irritable bowel syndrome (IBS)	*B. bifidum* MIMBb75 (1×10^9 cfu)	4 weeks	122 subjects of aged 18–68 years with mild to moderate IBS	A prospective, multicenter, randomized, double-blind, placebo-controlled, two-arm nutritional study	One probiotic capsule or placebo similar appearance and dimensions were given to respective groups daily	A highly significant reduction in the global assessment of IBS ($p < 0.0001$) with improved bloating/ distension and discomfort/pain	
	S. thermophilus KCTC 11870BP, *L. rhamnosus* KCTC 12202BP, *L. acidophilus* KCTC 11906BP, *B. longum* KCTC 12200BP , *B. lactis* KCTC 11904BP,and *B. bifidum* KCTC 12199BP. Total cfu per capsule were 5×10^9 with equal number of each probiotic species	4 weeks	49 IBS patients analyzed on the basis of Rome III criteria	A randomized, double-blind, placebo-controlled trial	One capsule/12 h containing lyophilized powder of probiotic mixture or placebo (maltodextrin) accounted for 13% of total weight (500 mg) of capsule	A significantly higher ($p < 0.05$) reduction in IBS symptoms in probiotic-treated group	Yoon et al. (2014)

L. Lactobacillus, *B. Bifidobacterium*, *S. Streptococcus*, *LGG Lactobacillus rhamnosus* strain GG, *CFU* colony-forming unit, *IBS* irritable bowel syndrome, *IBD* inflammatory bowel disease, *ATCC* American-type culture collection, *IgA* immunoglobulin A, *GI* gastrointestinal

In a recent report, two human-originated *L. plantarum* strains (BCF20 and BHM10) were reported for production of this vitamin (Bhushan et al. 2016). In a sequential work by the same research group, the authors claimed for the detection of three important B_{12} structural genes (cobT, cbiB, cbiA) on genomic DNA of *L. plantarum* species for the first time (Bhushan et al. 2017); however they were already known in other B_{12}-producing species. Moreover, the authors claimed for the highest production level (>100 μg/L), ever, from *L. plantarum* species. Concerning the daily B_{12} requirement of healthy human adult (2.4 μg/day) given by Institute of Medicine (1998), this much production level in a biofortified fermented food product, with a serving size of 200 g, could be complementary enough for attaining the recommended dietary allowance of this vitamin, if produced in bioavailable form. Few reports claim for the production of bioavailable form of this vitamin from *Lactobacillus* (Bhushan et al. 2017) and *Propionibacterium* species (Chamlagain et al. 2015; Deptula et al. 2015, 2017). However, all the studies were experimented in vitro, and some extra efforts are needed for replication of results in vivo.

In addition to the importance of *L. plantarum*, some *Lactobacillus* species (specifically *L. gasseri* and *L. reuteri*) are thought to be true GI commensals (Reuter 2001) and can be considered as vitamin suppliers to their host (LeBlanc et al. 2013), if retained and exhibited vitamin production capability in vivo.

Besides B_{12} production, lactobacilli have also been known for B_2 production. A recent report revealed the riboflavin production potential of *L. plantarum* in vitro (del Valle et al. 2014) and in vivo (del Valle et al. 2016). Interestingly, B_2 production has been suggested to enhance the probiotic action of *L. plantarum* (Arena et al. 2014). *Lactococcus* (LeBlanc et al. 2005) and *Propionibacterium* (LeBlanc et al. 2006) species have also been recognized as food-grade riboflavin-producing organisms. Moreover, the in vivo produced bacterial vitamin was found to correct the ariboflavinosis in mice model (del Valle et al. 2016). Humans get most of their B2 needs through milk and vegetables, cereals, yeast, and meats (Cooperman and Lopez 1991; Bacher et al. 2000). However, a certain amount has been suggested to be obtained from lower gut microbiota (Hill 1997). In that way, gut microbiota can play a role in compounding human's micronutrient supply (Burgess et al. 2004).

7.6.2 Bacteriocins

Bacteriocins are ribosomally synthesized, heat-stable antimicrobial peptides produced by various bacteria, including food-grade LAB. Besides their food-preserving effects (in vitro), these peptides have been known for their therapeutic potential owing to their killing nature against multidrug-resistant (MDR) pathogens (Perez et al. 2014). Recent high-throughput techniques (a combination of molecular mass analysis of obtained peptide and a statistical analysis of their antimicrobial spectra) enable us the better tracing and purification abilities to get novel

variants of bacteriocins (Perez et al. 2014). One of the major concerns of today's world is the lengthened list of multiple drug resistance pathogens; hence it is the need of hour to identify some novel antimicrobial peptides/bacteriocins from our microbiota. The research around clinical efficacy of bacteriocins has recently gaining its ground across the world due to their potent antimicrobial action against a number of gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) strain and vancomycin-resistant *Enterococcus faecalis* (VRE) strain (Okuda et al. 2013). Nisin, a food-grade bacteriocin, has been successfully tested for its efficacy in bovine model of mastitis and presently been suggested for further use (Cao et al. 2007). In a recent study, two potent bacteriocins (lacticin Q and nisin A) were reported to be more potent in killing of MRSA, in comparison to antibiotic vancomycin (Okuda et al. 2013). In a clinical setting, Oralpeace™ was reported to be efficacious in limiting the tooth cavities and gingivitis caused by *Streptococcus mutans* and *Porphyromonas gingivalis*, respectively (Yamakami et al. 2013; Perez et al. 2014).

7.6.3 Bioactive Peptides

Moreover, bioactive peptides have now been considered new-generation bioactive regulators that can prevent oxidation and microbial degradation in food system as well as used for the treatment of various medical conditions, thus increasing the quality of life (Lemes et al. 2016; Sánchez and Vázquez 2017). Bioactive peptides play important role in metabolic regulation and modulation by binding on target cell receptors and mediating specific functions same as hormones or drug. In contrast to synthetic substances, peptides are degraded into their basic amino acid units without leading to toxic metabolites (Singh et al. 2014). Similarly, food proteins have breakdown into di-, tri-, tetra-, or oligopeptides through the action of microbial enzymes during fermentation. These peptides have now been received excessive attention due to enhanced bioavailability and health benefits instead of latent protein. Therefore, these peptides can be called bioactive peptides and defined as "specific protein fragments that have a positive impact on body functions and may ultimately influence health." In addition to fermentation, bioactive peptides can be generated by enzymatic hydrolysis and food processing and during digestion of food proteins. These peptides have an array of activity and specific health effects, including antimicrobial, antioxidant, antihypertensive, antithrombotic, anti-adipogenic, dipeptidyl peptidase IV inhibition, opioid agonist and antagonist activities, immunomodulation, and mineral binding capacity (Singh et al. 2014, 2017; Singh and Vij 2017, 2018), and some are reviewed in Table 7.6.

Table 7.6 Overview of LAB-derived bioactive peptides

Peptide	Microorganism	Bioactivities	References
DKIHPFYQEPVL	*L. rhamnosus*	ACE inhibitory	Sánchez and Vázquez (2017)
VPP, IPP	*L. helveticus*	ACE inhibitory	
YPFPAVPYPQR, TTMPLW	*LGG*	Multifunctional	
YLLF	*Kluyveromyces marxianus*	ACE inhibitory	
ARHPHPHLSFM	*L. delbrueckii* subsp. *bulgaricus*	Antioxidative	
YPQRDMPIQ	*L. casei*	ACE inhibitory	Hajfathalian et al. (2017) and Marcone et al. (2017)
NVPVYEGY, ITALAPSTM, SLEAQAEKY, GTEDELDKY	*Bacillus subtilis* and *Bacillus amyloliquefaciens*	Antioxidative and ACE inhibitory	Jemil et al. (2014)
IKHQGLPQE, VLNENLLR, SDIPNPIGSENSEK	*L. acidophilus*	Antimicrobial	Hayes et al. (2006)
PYKLRP, DPYKLRP, GILRP, YKLRP	*Kluyveromyces marxianus*	ACE inhibitory	García-Tejedor et al. (2014)

L. Lactobacillus, *LGG Lactobacillus rhamnosus* strain GG, *ACE* angiotensin-converting enzyme

7.6.4 Conjugated Linoleic Acid (CLA)

Polyunsaturated fatty acids have been known for their impact on human physiology since decades (Renuka et al. 2016, 2018). Among them, CLA are of prime importance. They are the assorted group of positional and geometric isomers of linoleic acid which vary from each other based on the spatial arrangement of hydrogen bonds. Among various well-disclosed isomers of CLA, C9, t11 is known to have anti-inflammatory activity; t10, c12 for anti-obesity; and t9, t11 for anticancerous (Dahiya and Puniya 2015). Studies are available where LAB are able to synthesize CLA from external substrates such as linoleic acid, safflower, etc. under in vitro (Dahiya and Puniya 2017a) and in situ conditions (Dahiya and Puniya 2017b). Among them lactobacilli are preferred over other LAB because of their easy handing, minimal growth requirement, and safety status. In addition these bacteria can be directly utilized for CLA synthesis under in vivo conditions from dietary linoleic acid. Also, the dietary CLA is known to alter the gut microbiota composition, which plays a significant role in hosts' health and disease especially in obesity (Dahiya et al. 2017).

7.7 Microbial Effector Molecules for Host's Health and Disease

7.7.1 Effector Molecules from Host's Microbiota

A major advancement in culture-independent assays has now educated the host-microbiome research up in an advanced manner and provided the researchers a way to identify and characterize novel biologically active small molecules and metabolites derived from human microbiota. These include microbial products of primary metabolites (e.g., short chain fatty acids and vitamins) and a wide range of secondary metabolites, including both secreted and cell surface effectors (peptides or sugars) (Fischbach and Sonnenburg 2011; Lopez et al. 2014). These biomolecules have been well characterized as playing a therapeutic role, itself or as molecular target for drug discovery, in human physiology (Lemon et al. 2012). Effector molecules of microbiota have been described in Table 7.7.

Table 7.7 Overview of microbiota-derived effector molecules and their impact on human health

Microbe	Molecule/ metabolite	Health impact	Process involved	References
Bacteroides fragilis	Polysaccharide	Lymphoid organogenesis and regulating TH1/TH2 balance	Immunoregulation	Mazmanian et al. (2005)
Mixed microbiota	Pèptidoglycan	Sensitizing the immune system for T-cell-mediated systemic killing of pathogens *Streptococcus pneumoniae* and *Staphylococcus aureus*	Immunoregulation	Clarke et al. (2010)
Bacteroides fragilis	Polysaccharide	Protection of mice from *Helicobacter hepaticus* mediated experimental colitis by reducing pro-inflammatory cytokines production	Immunoregulation	Mazmanian et al. (2008)
Akkermansia muciniphila	Amuc_1100, an outer membrane protein	Protein administration elevated the expression of tight junction proteins in the gut, suggesting its role in alleviating the endotoxemia	Anti-inflammatory	Anhe and Marette (2017)

IL interleukin, *TH* helper T cells, *TNF-α* tissue necrosis factor-alpha, *MCP* monocyte chemotactic protein

7.7.2 *Effector Molecules from Probiotics*

As per the recent review meeting of the International Scientific Association for Probiotics and Prebiotics, probiotic health claims need to be validated with sound knowledge of effector molecules and molecular mechanism involved (Leeber et al. 2018). However, majority of probiotic claims lack the knowledge of underlying mechanisms; hence it generally becomes challenging to select the right probiotic strain for prevention/curing of a particular disease/syndrome. Hence, presently we are reviewing few of the well-documented effector-molecules-mediated health effects, especially for *Bifidobacterium* and *Lactobacillus* (Table 7.8).

Table 7.8 Cellular effector molecules of probiotics and their impact on human health

Probiotic organism	Effector molecule (s)	Mode of action	References
LGG	SpaCBA pili	Adherence to mucus and gut epithelial cells, cross talk with cells of innate immunity (monocytes and dendritic cells), and competitive exclusion	Lebeer et al. (2018)
	p40 and p75 (secreted proteins)	Prevention of cytokine-induced gut epithelial damage, prevention of colitis	Yan et al. (2013)
	Lipoteichoic acid	Negative modulation of colitis	Claes et al. (2010, 2012)
	CpG-rich DNA	Repression of IgE (in an allergen specific manner)	Iliev et al. (2008)
	Exopolysaccharides	Mitigation of adipogenesis	Zhang et al. (2016)
L. acidophilus NCFM	Surface layer proteins (SlpA, SlpB, and SlpC)	Adhesion to C-type lectin receptor, SIGNR3, thereby modulating intestinal epithelial barrier	Lightfoot et al. (2015)
L. plantarum WCFS1	StsP protein	NF-ƙB attenuation and modulation of cell signaling	Marco et al. (2010) and Lebeer et al. (2018)
L. salivarius UCC118	Bacteriocin Abp118	Inhibition of *Listeria monocytogenes*	Corr et al. (2007)
L. salivarius Ls33	M-tri-Lys (a muropeptide)	Enhanced expression of IL-10, a specific subset of dendritic cells and T-lymphocytes, thereby curing colitis	Fernandez et al. (2011)
L. reuteri 6475	Dgk (diacylglycerol kinase)	Anti-inflammatory mechanism by lowering down the histamine production	Ganesh et al. (2017)
B. breve UCC2003	Type IVb Tad (tight adherence) pili	Colonization at gut epithelium	Motherway et al. (2011)
B. longum 35624	Exopolysaccharides	Anti-inflammatory and anti-T_{H17} response	Schiavi et al. (2016)

L. Lactobacillus, *B. Bifidobacterium*, *NF-ƙB* nuclear factor kappa-B, *IgE* immunoglobulin E, *TH* helper T cells, *IL* interleukin, *SIGNR* specific ICAM-grabbing non-integrin related

7.8 New Perspective for the Role of Host's Microbiota in Pharmacology

In present times, microbiota and its interrelationship with host health have been shifted to a new horizon, and already deduced explanatory molecular mechanisms and compositional datasets have now been considered for designing a predictor model to assess individuals for disease proneness on the basis of their microbiota composition, thereby guiding the personified therapies.

On the same line, a concept of microbial global positioning system (mGPS) has been given by a group of active researchers in the field of microbiome research (Gilbert et al. 2016). After getting a comprehensive knowledge regarding human-associated microbes and their influences (positive and negatives) on human health, a need arises to move forward in developing a microbial global positioning system (mGPS) where the multivariate disease phenotypes would be placed in context of already established figurative maps of microbiome, and pharmacological interventions or diets would be decided in a decisive manner to achieve an end point (for example in Fig. 7.2) of totally healthy state (Gilbert et al. 2016). Directions to reach toward final point would be decided on the basis of previously standardized microbiome studies and present trackable position of diseased individual. After random positioning of diseased individuals, stratification would be done on the basis of

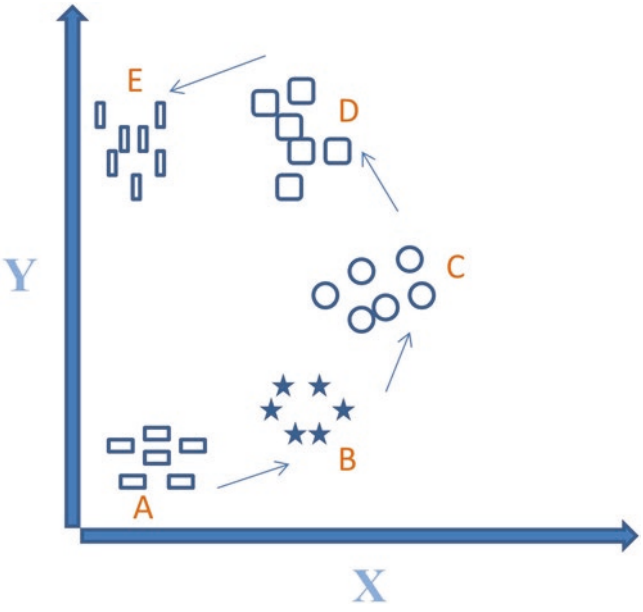

Fig. 7.2 Microbial global positioning system to stratify a population as per their microbiota in different physiological states of body and to guide their treatment toward healthy state (Gilbert et al. 2016). Denotations: *A to E* migration of patients physiology from highly diseased (*A*) to healthy state (*E*)

specific biomarkers (metabolites, genes, functions, or others), which would not only help in shifting participants from one position to another but also educate about treatment regime to move them from diseased to healthy state. This vision requires, although, a more advanced and cheaper way of analyzing microbiome than we have in present situations. Overall, this strategy will provide a highly powerful and relevant model for clinical therapeutics, once it has been assessed and implemented by regulatory authorities.

In a revolutionized transformation of pharmacology, using genome-wide association studies (GWAS) and microbiome-wide association studies (MWAS), we are moving forward from culture-based taxonomic inventory to genomic, transcriptomic, proteomic, and metabolomic world in search of development of personalized medicine. This approach is highly essential for getting a know-how of microbiome involvements in human health and diseases, along with the role of their metabolites for characterization of clinical biomarkers of various diseases. However, playing around a huge dataset, especially in multi-omics approach, in longitudinal studies costs too expensive and also exhibits a slow nature on clinically relevant timescales (Gilbert et al. 2016).

Thus, in order to really benefit with the knowledge from Human Microbiome Project, the Earth Microbiome Project, and American Gut for the development of mGPS, we have to develop higher-throughput and cost-effective techniques to process high number of samples generated during multi-omics studies, as well as need to improve advanced modeling techniques that obtain systems-level active factors from lower number of samples. In that way, it would be feasible for an individual to assess the past, present, and future (probable) picture of microbiome twist by twist, in a multivariate system, and they would be able to decide their future healthy path through their microbial companions.

7.9 Conclusion

Present book chapter reviewed the human associated microbes, especially bacteria, and their specialized metabolites and effector molecules in terms of their roles in human metabolism, physiology and pharmacology.

It should also be noted, however, that the host-microbiota-probiotic interaction is highly influenced by microbial mingle in the host's internal and external surfaces, host genetics, and multivariate environmental factors, including climate, food, and circadian rhythms. Moreover, majority of human-microbiota studies rely on fecal sampling and related microbial profiling for niche-specific phylogeny and gene expression. In this way, most of the generated data reveals the knowledge regarding colonic microbiota. Hence, more clinical data based on invasive sampling is required for exposing the microbiome of the human small intestine and its beneficial functionality.

In terms of challenging avenues, still a long scientific stride is required for actually analyzing the microbial metabolites specific health benefits of human

microbiota, until this revolution is culminated in to designing of clinical therapies. Besides the taxonomic dugout of various microbiome projects, roughly 50% of genes are still not characterized and demanding for proper characterization of novel biologically active microbiome-associated products. Hence, it is the demand of time to generate few achievable hypothesis and rational approaches in order to proficiently mine the pharmacological molecules from unexplored microbiome.

Interestingly, many successful probiotic claims have been generated, in the recent past, with animals and humans studies and opened a new path for more specified and cogent actions of probiotics in a huge number of clinical settings. In the present era of high antibiotic resistance, biotherapeutic approaches could be highly effective in prevention, as well as in curing of metabolic and infectious disorders. Probiotic modulation of gut microbiota and direct probiotic effects have been observed in a varied number of human altered physiological and pathological conditions, but still we are trying to find out the mechanistic insight in to the molecular mechanism underlying such health benefits.

Besides the positive health benefits (immunomodulation, improved metabolism, supply of vitamins) of healthy/diverse microbiota and probiotics, perturbed/unbalanced gut microbiota has profoundly been correlated with the altered metabolism of some important drugs consumed widely across the world for metabolic, cardiovascular, and other disorders. Hence, achieving a healthy and diverse microbiota has presently been recognized as an important factor for proper functioning of a drug in human system, and researchers have started working on the MWAS to explore the actual impact of microbiome on human physiology.

Another important point of attraction in the chapter was the role of microbial metabolites and surface effector molecules in human health and disease. Both gut microbiota and probiotics have been found as the important contributors of microbial specialized molecules and their positive and negative influence on physiological and immunological state of body.

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Chapter 8
Probiotics as a Dietary Intervention for Reducing the Risk of Nonalcoholic Fatty Liver Disease

Fouad M. F. Elshaghabee, Namita Rokana, Harsh Panwar, Knut J. Heller, and Jürgen Schrezenmeir

Contents

Abstract Nonalcoholic fatty liver disease (NAFLD) is characterized by an increase in triglyceride fat content of liver cells without excessive consumption of alcohol. It is the most predominant liver disease among different age groups including children and adults. Unhealthy foods such as high fructose and high trans-fatty acids in saturated fat seem to be associated with the pathogenesis of NAFLD. Different clusters of gut microbiota, e.g., *Firmicutes*, could regulate the energy balance and fat storage.

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Furthermore, different metabolites of gut microbiota, for example, endogenous short chain fatty acids and ethanol, are associated with increased levels of lipogenesis. Additionally, metabolism of endogenous ethanol leads to the formation of acetaldehyde resulting in increased oxidative stress and ultimately inducing liver injury. Lipopolysaccharides of the outer membrane of Gram-negative gut bacteria may also initiate some low grade inflammation in liver tissues. However, few reports from NAFLD patients showed ordinary serum endotoxin levels, excluding endotoxemia from the pathogenesis of NAFLD.

Beneficial gut microbiota, chiefly lactic acid bacteria and bifidobacteria, may induce positive effects through suppression of inflammatory cascades and exclusion of NAFLD promoting microbes, promoting gut barrier functions reducing levels of NAFLD pathogenesis. Therefore, selective probiotic strains with proven efficacy for NAFLD management and validated safety can be considered as a promising futuristic approach for NAFLD management. In this chapter, we review the normal gut microbiota, gut microbiota shifts in obesity, the role of some gut microbiota metabolites and dietary fructose in development of NAFLD, and the protective effect of different probiotics in reduction the risk of NAFLD.

8.1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is as of now the most widely recognized liver infection influencing both youthful and grown-ups. NAFLD is characterized as the fat buildup in the liver exceeding 5–10% of total liver weight, as decided from the level of fat-stacked hepatocytes. Lipid storage in liver cells (hepatic steatosis) and inflammation are often associated with nonalcoholic steatohepatitis, which is characterized by macrovesicular steatosis, lobular inflammation and hepatocellular ballooning in addition to steatosis (Neuschwander-Tetri and Caldwell 2003). In developing world, 20–30% of the population suffering from NAFLD is also suffering from other metabolic syndromes like obesity, type 2 diabetes, and dyslipidemia (Jimba et al. 2005; Byrne et al. 2009). Most patients with NAFLD suffer either from overweight or obesity (Kotronen and Yki-Jaervinen 2008).

The “two-hit hypothesis” is one of the many speculations proposed to comprehend the pathogenesis of NAFLD. In the “two-hit hypothesis,” the primary stage is steatosis, i.e., the aggregation of fat in liver cells (nonfat tissue). The second stage is oxidative stress, which is activated by various cytokines engaged with pro-inflammatory cascades, insulin resistance, and hepatocyte injury (Day and James 1998; Byrne et al. 2009). Gut microbiota and related metabolites have been linked to the advancement of either obesity or NAFLD (Tuohy et al. 2009; Baker et al. 2010). In vivo and human clinical trials indicate the role of gut microbiota in vitality homeostasis (Musso et al. 2010) and generation of noteworthy measures of different metabolites including

acetic acid derivation, which prompts lipogenesis framework (Daubioul et al. 2002), and generation of critical measures of ethanol, as observed in the breath of obese mice (Cope et al. 2000) and furthermore in human plasma (Volynets et al. 2012).

Additionally, gut microbiota serves as a source of acetaldehyde and lipopolysaccharides. Acetaldehyde is linked to ethanol metabolism, thus contributing to inflammation (Loguercio et al. 2002), and lipopolysaccharide endotoxin induces inflammation-related NASH (Miele et al. 2009). The composition of intestinal microbiota is dependent on several dietary factors. It can be altered by the intake of beneficial strains named probiotics or by consumption of un-digestible but fermentable polysaccharides, e.g., inulin or fructo-oligosaccharides. When linked with beneficial health effects, this reiterates the significance of pro-/pre- and synbiotics (Schrezenmeir and de Vrese 2001; Malaguarnera et al. 2012; Al-Muzafar and Amin 2017a, b). This chapter focuses on understanding the role of gut microbiota in both pathogenesis of NAFLD and also in management of the same through dietary intervention with different probiotic strains, reducing the risks associated with NAFLD.

8.2 Normal Gut Microbiota and Its Metabolites

Intestinal bacteria and fungi are represented by up to 2000 species. *Bacteroides*, *Firmicutes*, Eubacteria, peptostreptococci, bifidobacteria, and fusobacteria (*Faecalibacterium* sp.) are among the most predominant groups in the intestine of human adults in concentrations ranging from 10^{11} to 10^{12} cfu/L of large bowl contents. Intestinal microbiota produces several metabolites having an impact on human metabolomics (Salminen et al. 1998; Dethlefsen et al. 2006; Zihler 2010). Some of the metabolic end products by gut microbiota have been summarized in Table 8.1.

Lactic acid bacteria and bifidobacteria are considered as beneficial commensals of the gastrointestinal tract and are also generally recognized as safe (GRAS) (Thakur et al. 2016; Elshaghabee et al. 2017). They are classified as *Firmicutes* and *Actinobacteria* belonging to two major families *Lactobacillaceae* and *Bifidobacteriaceae*, respectively. The two major metabolites from sugar fermentation in the gut of these two groups of microorganisms are lactate and acetate. Both lactate and acetate play an important role in cross-feeding metabolic pathways of other bacterial species. This relationship is well recognized between lactate-producing *Bifidobacterium adolescentis* and lactate-consuming and butyrate-producing bacteria like *Anaerostipes caccae*/*Eubacterium hallii* (Belenguer et al., 2006) and for acetate-feeding from bifidobacteria to butyrate-producing bacteria like *A. caccae* and *Roseburia* sp. (Falony et al., 2006).

Table 8.1 Metabolic end products of major commensal bacterial groups in the human gastrointestinal tract

Bacteria/archaeon	Description	Range in feces log_{10}/g dry wt.	Metabolic niche	Fermentation end products
Bacteroides	Gram –ve rods	9.2–13.5	Different types of polysaccharides utilized	Acetate, propionate, succinate
Eubacteria	Gram +ve rods	5.0–13.3	Saccharolytic and proteolytic	Lactate, acetate, butyrate, ethanol
Bifidobacteria	Gram +ve rods	4.9–13.4	Polysaccharides and fructose utilization	Acetate, lactate, formate, ethanol
Clostridia	Gram +ve rods	3.3–13.1	Saccharolytic and proteolytic	Acetate, propionate, butyrate, lactate, ethanol
Lactobacilli	Gram +ve rods	3.6–12.5	Polysaccharides and fructose utilization	Lactate, acetate, ethanol, mannitol
Faecalibacterium F. prausnitzii	Gram –ve rods	5.1–11.0	Saccharolytic and proteolytic	Lactate, acetate, butyrate
Escherichia	Gram –ve rods	3.9–12.3	Saccharolytic	Mixed acid fermentation and ethanol
Ruminococcus	Gram +ve cocci	6.0–8.0	Saccharolytic	Acetate
Desulfovibrios	Gram –ve rods	5.2–10.9	Sulfate reduction, H_2 and lactate scavenging	Hydrogen sulfide
Methanobrevibacter	Gram +ve coccobacilli	7.0–10.5	H_2 scavenging	Methane

Adapted from Salminen et al. (1998), Finegold et al. (2002), Dethlefsen et al. (2006), Zihler (2010), and Elshaghabee et al. (2016)

8.3 Gut Microbiota Shift During Obesity-Related Nonalcoholic Fatty Liver Disease

The gut microbiota represents a key factor in pathogenesis of NAFLD (Abu-Shanab and Quigley 2010; Zhang et al. 2010). The number of studies dealing with the differences between intestinal microbiota in obese and lean subjects is increasing. Ley et al. (2006) reported that the proportion of *Bacteroides* increased in response to weight loss, while the proportion of *Firmicutes* decreased in obese subjects. More specifically, Nadal et al. (2009) reported that the counts of bacteria of the *Bacteroides*/*Prevotella* group correlated even better with the weight loss. The numbers of *Faecalibacterium prausnitzii* were significantly lower in lean than in obese children (Balamurugan et al. 2010). Analyses of clinical fluids of patients

suffering from nonalcoholic steatohepatitis showed high levels of *Weissella confusa* (Harlan et al., 2011).

Recently, analyses of gut microbiota from obese patients showed that the microbiota got depleted of *Methanobrevibacter smithii*. *Bifidobacterium animalis* was predominant in normal weight patients, while *L. reuteri* was associated with obese ones (Million et al. 2012). Overgrowth of bacteria in small intestine plays an important role in progression of NAFLD to NASH and increased intestinal permeability, as tested in humans and in animal models. Increase in the population of Gram-negative bacteria resulted in increased levels of endotoxin lipopolysaccharides and liver injury in a rat model (Pappo et al. 1992). Results reported by Miele et al. (2009) were first to point out that the NAFLD patients suffer from increased gut permeability (leaky gut) due to bacterial overgrowth in these patients. Using an animal model, Wu et al. (2008) showed that high fat diet resulted in alterations in aerobes (*E. coli*) but not in anaerobes (lactobacilli). An increase in counts of *E.coli* could be observed in the NASH group. In germ-free mice, dysbiosis refers to a condition in which microbial imbalance in the gut may trigger hepatic de novo lipogenesis by increasing the expression of lipogenic enzymes: acetyl Co-A carboxylase and fatty acid synthase (Backhed et al. 2004; Musso et al. 2009).

8.4 The Role of Gut Microbiota Components and Metabolites in the Pathogenesis of Nonalcoholic Fatty Liver Disease

Lipopolysaccharide layer of outer membrane of Gram-negative bacteria consists of a lipid and a polysaccharide moiety. Lipopolysaccharide acts as endotoxin resulting in high immune response in animals (McCarthy et al. 2003). The presence of lipopolysaccharide is recognized by human or animal cells via Toll-like receptor 4 (TLR4) which promotes secretion of pro-inflammatory cytokines and induces inflammatory cascades (Wang and Quinn 2010). Additionally, lipopolysaccharide plays an important role in the activation of kappa beta kinase (IKK), leading to phosphorylation and complete degradation of the kappa beta kinase beta (IKK-β) and NFκB inhibitor (Neal et al. 2006). In animal studies, induction of endotoxemia by lipopolysaccharide in lean mice resulted in increased weight gain of liver and adipose tissues (Cani et al. 2007a, b). Treatment with antibiotics like ampicillin and neomycin leads to reduced levels of endotoxemia in ob/ob mice or C57BL/6 J mice fed with high fat diet (Cani et al. 2008). In human studies, increased dietary intake of fat-carbohydrate diet leads to increased circulating lipopolysaccharide concentrations (Amr et al. 2008). Also, patients with NAFLD demonstrated increased gut permeability (Miele et al. 2009).

Ethanol (EtOH) and acetaldehyde are important metabolites of heterofermentative intestinal bacteria. EtOH can be metabolized to acetate and acetaldehyde. The latter might lead to formation of reactive oxygen species (ROS), thus increasing

oxidative stress inducing liver injury (Medina et al. 2004). Both EtOH and lipopolysaccharide promote the production of ROS in Kupffer cells due to increased expression of TLRs, particularly TLR4 (Gustot et al. 2006). Neomycin could reduce exhalation of ethanol in Lep ob/ob mice. This finding was believed to reflect suppression of gut microbiota and reduction of ethanol production. The latter is a factor considered to contribute to development of NAFLD (Cope et al. 2000). Additionally, detectable levels of breath ethanol were found in obese patients and upon intestinal yeast infection or bacterial overgrowth (Logan and Jones 2000; Nair et al. 2001). Baker et al. (2010) presented the first results indicating that alcohol produced by gut microbiota in NAFLD patients contributed to development of pathogenesis of NAFLD through increased alcohol in the circulation, induced host alcohol dehydrogenase and cytochrome P450 2E1 gene expression and activity, and elevated NADH levels. Recently, non-mannitol-producing intestinal heterofermentative lactobacilli (e.g., *Weissella confusa*) were producing high amounts of ethanol from fructose fermentation (Elshaghabee et al. 2016). All these factors are likely to favor fatty acids synthesis resulting in steatosis (Fig. 8.1).

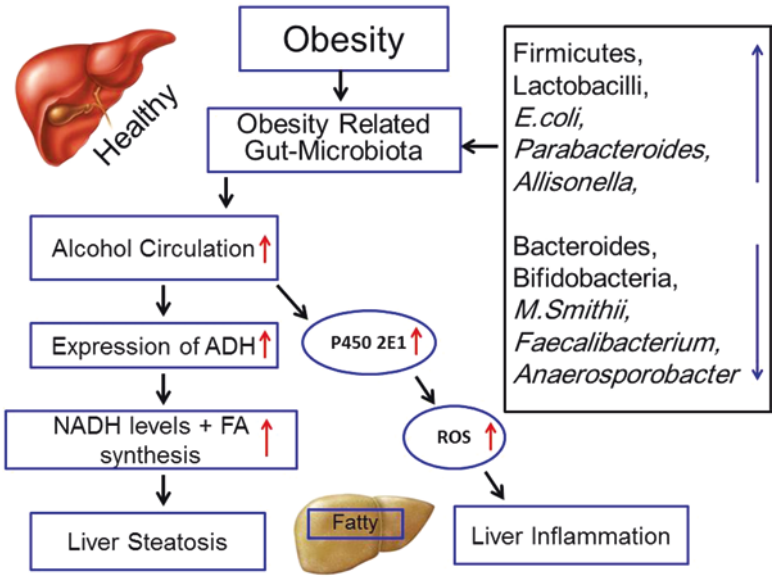

Fig. 8.1 Role of ethanol in development of non alcoholic fatty liver disease (NAFLD). (Adapted from Baker et al. 2010; Claire et al. 2010; Million et al. 2012; Wong et al. 2013). Obesity alters the gut microbiota composition, whereas levels of *Firmicutes*, lactobacilli, *Escherichia* (*E.*) *coli*, *Parabacteroides*, and *Allisonella* increased; however, levels of *Bacteroides*, bifidobacteria, *Methanobrevibacter* (*M.*) *smithii*, *Faecalibacterium*, and *Anaerosporobater* decreased. Furthermore, levels of alcohol circulation and inflammatory cascade were also increased resulting in fatty liver and afterward steatosis. *ADH* alcohol dehydrogenase activity, *FA* fatty acids, *P450 2E1* cytochrome is responsible for oxidizing alcohol, *ROS* reactive oxygen species

Gut microbiota produce different short chain fatty acids during fermentation of carbohydrates in the human intestine. Acetic and propionic acids are major sources of energy and stimulate salt and water absorption in the intestine. Butyric acid and its salts affect growth and differentiation of colonic epithelial layer. In addition, short chain fatty acids can modulate intestinal permeability in inflammatory bowel disease (Clausen et al. 1991; Salminen et al. 1998). On the other hand, increased amounts of short chain fatty acids produced by gut microbiota could act as precursors for fatty acids and cholesterol and elicitors of lipogenesis (de novo lipogenesis) and gluconeogenesis. In addition, binding of short chain fatty acids to specific receptors like G protein coupled receptor (GPR) increases peptide YY, which is responsible for decreasing the rate of intestinal transit, resulting in improved levels of nutrient absorption and leptin (Xiong et al. 2004; Delzenne and Cani 2011).

8.5 The Role of Fructose Metabolism in the Pathogenesis of Nonalcoholic Fatty Liver Disease

The main dietary sugar types are sucrose, glucose, and fructose. Sucrose is a disaccharide composed of fructose and glucose and is used in most sweetened beverages (Hanover and White 1993). In general, increased consumption of carbohydrates had been shown to be associated with many different metabolic syndromes including obesity, steatosis, or insulin resistance (Solga et al. 2004; Libuda et al. 2008). In contrast to glucose, fructose does not require insulin to enter epithelial cells; however, it requires phosphor-fructokinase to be metabolized. Absorption of fructose occurs via specific transporters: glucose transporter 5 (GLUT5) in the intestine and glucose transporter 2 (GLUT2) in the liver (Tran et al. 2009). Liver is a vital organ in mammals and plays an important role in fructose metabolism. The first step of fructose metabolism in the liver is phosphorylation of fructose to fructose-1-phospate (F-1-P) by fructokinase. Subsequently, F-1-P is cleaved into dihydroxyacetone phosphate and glyceraldehydes. The latter is phosphorylated into glyceraldehyde 3-phosphate which may either participate in de novo synthesis of fatty acids, gluconeogenesis, or inoxidation pathway (Tran et al. 2009; Iizuka 2017).

For better understanding the differences between glucose and fructose metabolism, mice were fed diets rich in glucose (30% glucose solution ad libitum for 8 weeks), which resulted in gaining weight without accumulation of fat in the liver. On the other hand, replacement of glucose with fructose at the same concentration caused accumulation of fat in the liver tissues (Bergheim et al. 2008). Chronic fructose consumption caused steatosis, increased storage of lipid in the liver tissues, decreased β-oxidation of fatty acids, and uncontrolled entry of fructose into glycolysis (Armutcu et al. 2005). Furthermore, results obtained by Lin et al. (2005) showed that fructose could activate several mechanisms related to insulin resistance, resulting in activation of peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1β and expression of different genes involved in lipogenesis and triglyceride secretion directly through co-activating sterol regulatory element-binding pro-

tein (SREBPs). Earlier, Spruss and Bergheim (2009) and Lim et al. (2010) described the influence of increased dietary fructose on development of NAFLD pathogenesis to be similar to chronic alcohol consumption. Increased dietary fructose has been connected to NAFLD through increased intestinal permeability, increased bacterial overgrowth, translocation of bacterial endotoxins into the portal plasma resulting in activation of Kupffer cells, increased formation of tumor necrosis factor alpha (TNF-α) as pro-inflammatory cascade, excess of mitochondrial acetyl-CoA being transformed into citrate, stimulation of de novo lipogenesis for fatty acids synthesis, and inhibition of hepatic lipid β-oxidation by excess of malonyl-CoA production.

8.6 Protective Potential of Probiotics Against Nonalcoholic Fatty Liver Disease

Probiotics are live microbial strains with health impact on host when they consumed daily with enough amounts (not less than Log 6 to Log 8 CFU/g) and incorporated into gut microbiome (Elshaghabee 2017). Probiotics have been proposed to have health benefits against several inflammatory and metabolic disorders including T2D (Aparna et al. 2013; Chauhan et al. 2014; Panwar et al. 2013, 2014, 2016a, b; Mallappa et al. 2012; Thakur et al. 2016). Probiotics in either free or microencapsulated form may have beneficial effects in NAFLD through modulation of gut microbiota via production of different antibacterial substances, simulation of immune stimulation and reduction levels of inflammation cascade (Fig. 8.2 and Table 8.2). However, the mechanisms of probiotics at molecular level are poorly understood (Iacono et al. 2011). Studies dealing with the therapeutic use of probiotics in modulating the gut microbiota under conditions of small intestinal bacterial overgrowths are still limited (Gabrielli et al. 2009). Administration of *B. lactis* could lower the bacterial translocation in rats by up to 80% (Eizaguirre et al. 2002). Also, *Lactobacillus johnsonii* La1 reduced bacterial translocation and attenuated endotoxemia in a rat model of cirrhosis (Chiva et al. 2002). Probiotic strains, e.g., lactobacilli, bifidobacteria, or enterococci, produce various antimicrobial substances like lactate, acetate, propionate, hydrogen peroxide, bacteriocins, and bacteriocin-like inhibitory substances (Tagg and Dierksen 2003), which inhibit growth of different pathogens like *Bacteroides vulgatus*, *Clostridium sp.*, *Enterobacter aerogenes*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica*, *Yersinia enterocolitica*, and enterotoxigenic or enteropathogenic *Escherichia coli*, regardless of the possibility that pathogens have adhered on intestinal cells before bio-therapeutics (Candela et al. 2005; Collado et al. 2007).

Additionally, probiotics also reduce gut microbiota metabolites like hydrogen sulfide and superoxide, which may be toxic to the intestinal epithelium cells (Attene-Ramos et al. 2007). *L. rhamnosus* GG, *L. plantarum* MB452, *B. infantis*, *B. lactis*, and *E. coli* Nissle 1917 were able to control either SIBO or bacterial translocation leading to decreased endotoxemia in animal models. This effect might be attributed to the abilities of probiotics to modulate tight junction proteins and mucins and to

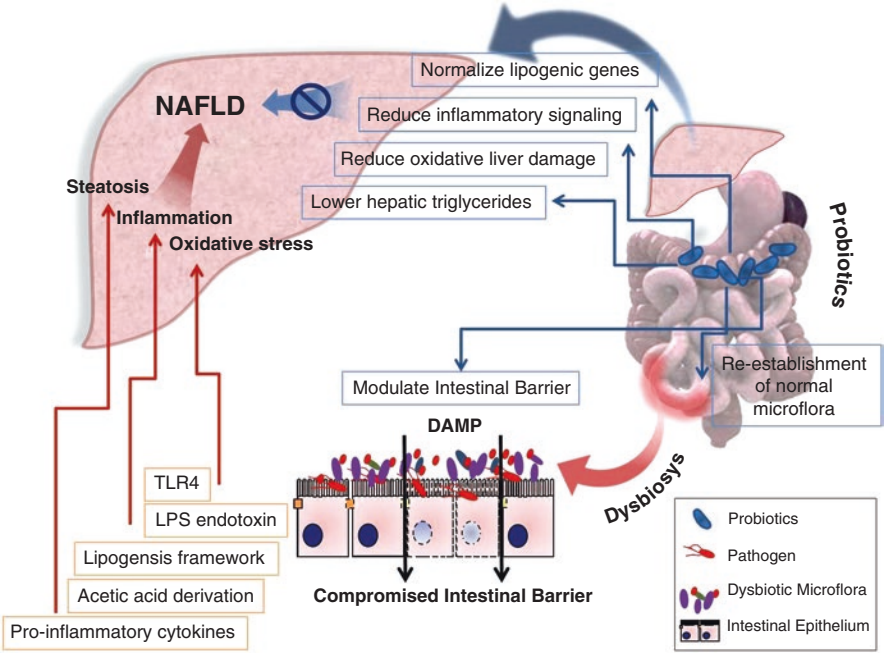

Fig. 8.2 Mechanism of action of probiotics for alleviation of non alcoholic fatty liver disease (NAFLD). The altered gut microbiota compromises intestinal barrier function which eventually contributes to development of NAFLD by elevating the level of blood endotoxin and proinflammatory cytokines. Probiotics resolve the adverse effects by re-establishing the normal gut microflora, reducing inflammatory signaling, and normalizing the lipogenic genes in the liver (*DAMP* damage-associated microbial pattern, *TLR4* Toll-like receptor 4, *LPS* lipopolysaccharide).

stimulate the nonspecific intestinal barrier defense mechanisms though protein kinase C and mitogen-activated protein kinase pathways, which could alter the levels of tight junction proteins occludin, ZO-1, and ZO-2 and regulate mucin 2 and mucin 3 genes (Johnson-Henry et al. 2008; Zyrek et al. 2007).

The lyophilized VSL#3 probiotic culture contains a mixture of viable bifidobacteria, lactobacilli, and *Streptococcus thermophiles* and could improve steatosis induced in either C57BL/6 mice or Sprague-Dawley (SD) rats by high fat diet. These effects might be due to increased hepatic natural killer (NK) T-cell numbers, reduced inflammatory signaling, and limited oxidative liver damage (Ma et al. 2008; Esposito et al. 2009). On the other hand, VSL#3 failed to ameliorate methionine-choline-deficient (MCD) diet-induced liver inflammation and steatosis in C57BL/6 mice (Velayudham et al. 2009). Different probiotic strains including *L. acidophilus* CGMCC 2106 or *B. longum* CGMCC 2107 attenuated amassing of fat in liver cells and reduced intestinal permeability in high fat-fed rats (Xu et al. 2012). *L. casei* Shirota could reduce the TLR4 signaling cascade in high fructose solution treated mice (Wagnerberger et al. 2013). Similarly, *L. reuteri* GMNL 263 resulted in decreased levels of IL-6, TNF-alpha, PPAR-gamma, and GLUT4 and in normaliza-

Table 8.2 Studies related to probiotic action for non alcoholic fatty liver disease (NAFLD)

Probiotic strains	Mode of action	References
Probiotic symbiter of 14 strains of *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Propionibacterium*	Reduced liver fat, aminotransferase activity, and the TNF-α and IL-6 levels in NAFLD patients	Kobyliak et al. (2018)
Lactobacillus acidophilus ATCC B3208, *Bifidobacterium lactis* DSMZ 32269, *Bifidobacterium bifidum* ATCC SD6576 and *Lactobacillus rhamnosus* DSMZ 21690	Improved pediatric NAFLD by decreasing mean cholesterol, low-density lipoprotein-C, and triglycerides level	Famouri et al. (2017a, b)
Probiotic mixture containing *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Bifidobacterium bifidum*, *Bacillus subtilis* fermentation extract, *Aspergillus oryzae* fermentation extract	Improved HFSD-induced steatosis through its effects on leptin, resistin, inflammatory biomarkers, and hepatic function markers	Al-Muzafar and Amin (2017a, b)
Bifidobacterium infantis and *Lactobacillus acidopilus* and *Bacillus cereus*	Delayed the progression of NAFLD via lipopolysaccharides/TLR4 signaling	Xue et al. (2017)
Lactobacillus paracasei Jlus66	Decreased body weight gain, serum triglyceride (TG), low-density lipoprotein (LDL) as well as aminotransferase (ALT) in rat models	Ye et al. (2017)
Multi strain probiotic mix containing *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus,* *Bifidobacterium breve*, *Bifidobacterium longum* and *Streptococcus thermophiles*	Reduced glycemic and inflammatory indices in patients with NAFLD	Sepideh et al. (2016)
Lactobacillus plantarum WCFS1, Lactobacillus rhamnosus GG, *and* anthraquinone	Upregulated CYP7A1, LDL-R, FXR, and PPAR-α protein produced in the process of fat metabolism while downregulated the expression of HMGCR, PPAR-γ, and SREBP-1c	Mei et al. (2015)
Lactobacillus acidophilus La5 and *Bifidobacterium lactis* Bb12	Improved hepatic enzymes, serum total cholesterol, and low-density lipoprotein cholesterol levels	Nabavi et al. (2015)

tion of the increased expression of lipogenic genes like Srebp-1c, FAS, and Elvol6 in high fructose (65%) diet-treated rats (Hsieh et al. 2013). Also, microencapsulated *L. fermentum* ATCC 11976 reduced hepatic triglycerides and decreased the expression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in hamsters fed with methionine/choline (Bhathena et al. 2013). Modulation of the composition of gut microbiota can be a useful strategy for treating different aspects of metabolic syndrome including obesity and NAFLD. *L. paracasei* CNCM I-4270, *L. rhamnosus* I-3690, and *B. animalis* subsp. *lactis* I-2494 could alter abundances of 49 of operational taxonomic units (OTUs), i.e., *Desulfovibrionaceae*, *Oscillibacter*, and *Clostridium* XIVa, in mice fed with high fat diet (Wang et al. 2015). Furthermore, probiotic *B. adolescentis* had protective effect of mice from NAFLD induced by

western diet through reduction of expression of 88 mRNA and portal endotoxin levels and activation of nuclear factor NF-κB and inhibits lipid peroxidation (Reicholda et al. 2014). Recently, the probiotic mixture containing *L. acidophilus*, *L. plantarum*, *B. bifidum*, and *B. subtilis* extract could reduce levels of IL-6 and improve leptin as well as lipid profiles (Al-Muzafar and Amin 2017a, b).

In clinical trials, patients administrated orally with a probiotic capsule twice daily for 28 weeks along with lifestyle change could lower liver biomarkers levels and fibrosis score (Vajro et al. 2011). Also, yoghurt cultures could decrease the BMI, cholesterol, triglycerides, and TNF-α in adult patients (Aller et al. 2011). *B. longum* mixed with fructo-oligosaccharides could attenuate NAFLD in adults after 24 weeks (Malaguarnera et al. 2012). VSL#3 (probiotic mixture culture) could also reduce BMI, levels of liver biomarkers, total cholesterol, glucose, and insulin in obese children after 16 weeks (Alisi et al. 2014). Recent findings of Famouri et al. (2017a, b) showed that administration with probiotic capsule (containing *L. acidophilus* ATCC B3208, *B. lactis* DSMZ 32269, *B. bifidum* ATCC SD6576, and *L. rhamnosus* DSMZ 21690 CFU) could reduce liver biomarkers, low-density lipoprotein-C, and triglycerides in obese children after 12 weeks.

8.7 Conclusion

Overgrowth of intestinal microorganisms and their metabolites, e.g., short chain fatty acids, endogenous ethanol, and lipopolysaccharides, are considered as a key player in the development of NAFLD. Available data suggest that food-based interventions could serve as an effective approach for controlling the NAFLD by exerting their beneficial effects on gut microbiota and intestinal health. Several types of probiotics in free or microencapsulated form may be easily applied in dairy or medical foods like VSL#3 and may reduce the risk of NAFLD through different mechanisms. However, the molecular mechanisms of potential health benefits of probiotics with regard to NAFLD are not yet completely explored. Further, more précised studies in this field will improve the understanding for utilization of these beneficial microbes as a natural and cost-effective intervention for the management of NAFLD.

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