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Contents

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An overview of three biocatalysts of pharmaceutical importancesynthesized by microbial culture

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A B S T R A C T

This article includes a general overview of the published research on a topic relevant to biomedical sciences research, pharma-industries and healthcare sector. We have presented a conciseinformation on three enzymes. These biomolecules have been investigated for their biocatalyticactivities beneficial in the detection of drugs and their metabolites present in micro-quantities insamples of blood, urine, and other body fluids, such as salicylate hydroxylase, and dihydrofolatereductase. Some enzymes are useful in biotransformation of compounds to convert them in anoptically active form, such as lipase. The information presented in this article has been collectedfrom the published studies on their catalytic function, and biosynthesis using selectedmicroorganisms. Several diagnostic assays are currently using enzymes as effective biocatalysts toperform the detection-test. For the marketing and consumer's convenience, pharmaceuticalcompanies have designed biosensors and diagnostic kits by incorporating specific enzymes for rapidtests required in pathology, as well as for the quantification of certain metabolites and chemicals inpathology samples in a shorter time. For such purpose use of enzymes synthesized by selectedspecific microorganisms is economical.

Keywords: Salicylate hydroxylase; Dihydrofolate reductase; Lipase; Aspirin; Methotrexate

1. Enzymes

Enzymes have been widely studied for their chemistry, reactivity and contribution in biochemistry and biological systems. Enzymes have been studied for their important activity asbiocatalysts to synthesize added-value metabolites, as well they are being used as important tool inthe diagnostics and therapeutics to improve human health. These important biocatalysts regulate therate of metabolic reactions that take place within a living cell. Enzymes contribute in a wide range ofvital functions in any living cell; therefore, these are important biomolecules for all biologicalsystems, whether in micro or macro organisms [1]. Enzymes have established their applications inmany sectors, including use of biocatalysts enzymes for bioresource utilization through bio remediation [2], medical & pharmaceuticals, textiles, paper and pulp, food and drink, etc. [3–6]. Inbiotechnology, enzymes have been manipulated to improve their quality in experiments to speed upbiochemical reactions [1,7].Enzymes are involved in all known metabolic pathways and are important tools for regulatingconditions in a homeostatic manner. These biocatalysts play significant roles in the metabolism ofmacromolecules for the survival of all living organisms. Inconsistent and weaker functioning ofenzymes might lead to the development of some metabolic disorders. Therefore, in recent years research has been focused on measuring the activity of relevant enzymes at various stages for earlydetection of health related disorders.

Currently in medical and healthcare sector, with increasing health problems and an ageing population in several geographical regions, the detection andtreatment of disease at an early stage has become very important. Pharmaceutical companies arecontinuously researching on new practical and affordable diagnostic systems and portable kits for thedetection of several diseases rapidly within a shorter duration [8,9]. This approach has advantages ofa rapid test and smaller volume of samples required in reaction. That could be a better and practicalalternative in many cases. The main disadvantages in the use of conventional analytical diagnosis methods are: first of allthe precautions should be taken during the collection of samples by patients at home or by staff atcare centers to maintain the sterility and avoid any contamination; secondly, sending requiredquantity of samples under proper storage conditions to pathology labs, without any spillage duringtransportation; thirdly the analytical methods of testing must be performed in a properly equippedlaboratory setting. That requires the availability of qualified skilled biomedical scientists andpathologists to perform these lab-based analytical testing using conventional methods. There areseveral important enzymes, which could be used for different assays, have been studied for theirbiosynthesis employing selective microorganisms [10–12].

1.1. Enzymes from microbial sources

The specific enzymes can be synthesized using purposely selected specific microbial strains in laboratory. They act as biocatalysts to perform reactions in bioprocesses in an economical and fasterway, compared to chemically-catalysed reactions. For specific applications, the differentfunctionality of biocatalysts are required, which includes tolerance to a varied range of pH, stabilityof enzyme activity over a range of temperature and other reaction conditions [1,3]. Such enzymeshave proven their utility in different situations and requirements. Therefore, several enzymes havebeen isolated, identified and characterized for a number of specific properties, and exploited forindustrial purposes [1–6].

Based on their applicability, certain microbial enzymes are categorized as thermophilic, acidophilic or alkalophilic. Each category of enzymes has a specific function but thermophilicenzymes have varied applications. Microorganisms producing thermostable enzymes that canperform reaction at higher temperatures would decrease the possibility of microbial contamination at industrial scale processing. In addition, the higher reaction temperature promotes the breakdown of raw materials. Furthermore, the thermotolerant nature of enzymes also helps in enhancing the masstransfer and reduction of the substrate viscosity during the progress of hydrolysis of substrates. Thermophilic xylanase, amylases and proteases are considered to be of commercial interest [4]. Microbial-synthesized enzymes, including protease, dihydrofolate reductase, lipase, esterase, salicylate hydroxylase and xylanase, have been investigated for their biosynthesis and application, but not all are produced by thermophilic microorganisms. In this article, we have included the published information on enzymes used in diagnosticassays and in biotransformation of drugs, which are of pharmaceutical importance, and those havebeen studied for their biosynthesis using specific microbial strains. These enzymes have theirapplication as an effective and prudent tool in biomedical and pharmaceutical sector, as discussed infollowing sections.

2. Application of enzymes in pharmaceuticals

The enzymatic diagnosis has become a reliable, simple and rapid diagnostic method in medical and health care. It is due to the unique characteristics of enzymes including, their specificity forsubstrate or chemical compounds, high-catalytic activity under mild conditions of tests.

Recent developments in prediction of an illness are the employment of biosensors as potential tools withbroader applicability. The enzymes that are well recognised as biological catalysts with specificityand selectivity have been studied for their use as the main component in fabrication of such devicesof biosensors [6,8]. The measurement of activity of specific enzymes in samples collected frompatients is used as the method of diagnosis. A particular enzyme is used as a marker for diagnosis ofa certain disease in specimens of blood, serum, urine and other body fluids.

Diagnosis by enzyme is a method by measurement of the concentration and changes of certainchemicals in the body to diagnose diseases, which is also done through the changes monitored inactivity of the original enzyme in the body fluids. Therefore, this approach of enzymatic diagnosticmethods is based on two parameters- firstly, by analysing the changes in the original enzyme activityin the samples taken from patients and secondly, by detecting the changes in some chemicals presentin body fluids, which are monitored in tests catalysed by enzym..

There are several enzymes used as biocatalysts in the diagnostic tests for the detection and also for the treatment of specific diseases [12–14], there are some examples presented as below:

1 Glucose oxidase enzyme is widely used to measure glucose level in the diagnosis of diabetes;
2 Cholesterol oxidase enzyme activity can be used to measure the amount of cholesterol present in the blood for diagnosis of condition of hyperlipidemia;
3 Urease enzyme is used to measure the level of urea in blood sample to diagnose liver and kidney lesions;
4 DNA polymerase has its application in testing the state of gene, for normal or any presence of oncogene in the body;
5 Glutaminase enzyme activity is required in the measurement of glutamine content in cerebrospinal fluid for the correct diagnosis of cirrhosis;
6 Trypsin enzyme can be used to dissolve blood clotting, to accelerate the process of wound healing, to remove necrotic tissue as well as in inhibiting the propagation of infecting microflora;
7 L-asparaginase can be used to treat cancer by depriving nutrients needed by the growth of cancer cells;
8 Protease enzyme easily available now in health shops is used to treat digestive disorders, and used by a person for easy digestion of protein-rich diets.

Several other enzymes including, esterase, thrombin, superoxide dismutase, L-asparaginase, lipase, natto-kinase, soybean meal plasmin, etc. have application for their catalytic activity in the medical and healthcare and for the treatment of certain diseases.

Biosensor, as the name states is a device, which uses biological molecules, especially enzymes or antibodies to detect the presence of chemicals in small amount of samples. This analytical devicemay contain an immobilized biological material (enzyme, antibody, nucleic acid, hormone, organelleor whole cell), which is designed specifically to interact with a specific chemical and producesphysical, chemical or electrical signals that can be measured [14,15]. A biosensor is a self-sufficientintegral tool designed which provides precise, quantitative and analytical information. Thisfabricated device uses a biochemical receptor that is in direct contact with.

The biosensor is mainly designed using three main parts: 1. A biological recognition element. 2. A transducer. 3. A signal processing system. Biosensors have become popular because of their accurate,

rapid, sensitive and selective detection strategies, which can be used routinely in daily life. Biosensors can be applied for rapid detection of different metabolites (pharmaceuticals) for the diagnosis of various diseases. Those enzymes could be of physiological importance, which arecapable of catalyzing a reaction with a chemical or a suspected metabolite under test conditions, these can be employed as a diagnostic entity within a biosensor [11–14]. Two diagnostic enzymesdiscussed in this review are salicylate hydroxylase [13,16], and dihydro folate reductase [17], whichare used for the estimation of specific drugs used in long-term treatment, such as aspirin and methotrexate.

2.1. Salicylate hydroxylase

Aspirin (Acetylsalicylic acid), which was first time introduced in medicine was more than a century ago in 1899. This synthetic compound is still one of the most commonly used component indrug formulations prepared for their therapeutic use, as analgesic, antipyretic, and in antiinflammatory drugs [18]. Aspirin has shown antiplatelet aggregation property, hence, therapeuticallythis compound acetylsalicylic acid is commonly prescribed to treat cardiovascular problems. Most ofthe absorbed aspirin enters in the blood circulation after its consumption by patients, in form ofsalicylate anions. These can be detected in the blood samples, at its highest concentration after twohours the patient has taken all those drug, which contain aspirin in their formulation. Some patients with certain illnesses are prescribed these drugs for a long-term treatment, therefore, in such cases salicylate levels in plasma samples of patients are measured regularly toavoid their toxic effects [19–21]. Though the short-term treatment of aspirin for analgesic/antipyreticpurposes are given in low doses, which actually produce considerably low salicylate concentrations, and hence these patients' blood samples do not require monitoring of aspirin levels. Aspirin, as an anti-inflammatory drug, is given to patients suffering with various forms ofarthritis. The blood samples of these patients, in cases of long-term treatment, are regularlymonitored to maintain salicylate level within the therapeutic range. Since there is a relatively small

difference between therapeutic and toxic dosages, a fast and accurate method is needed to assay the levels of salicylate in blood samples taken from the patients. Various methods for determining the level of salicylate have been reported in literature. These are avariety of analytical assays including potentiometry analysis using ion-selective electrodes (ISEs) andcolorimetric techniques as in the Trinder test [19]. The colorimetric method has severaldisadvantages and are not very specific [20]. High-pressure liquid chromatography and gas-liquidchromatography, fluorescence and ultraviolet spectrophotometry have also been used [21–23], butthese assays require time-consuming sample pre-treatment and therefore, such type of analysis arenot suitable for use in emergency situations, where a rapid test needs to be performed. Therefore, for amore specific and faster analysis, other assays such as an enzymatic method is used as a solution [24]. Salicylate hydroxylase enzyme (Salicylate 1-monooxigenase, E.C.1.14.13.1.) has been used todetermine the salicylate level in pharmaceutical samples and in blood serum [24]. The hydroxylationof the salicylate is catalysed by a biocatalyst, enzyme salicylate hydroxylase, and this biochemicalreaction can be used as an assay for the analytical purpose. Salicylate hydroxylase enzyme isrequired for the hydroxylation and simultaneous decarboxylation of salicylate to catechol. Following equation explains the enzyme reaction of salicylate with NADH and oxygen, producing catechol, NAD+, carbon di-oxide,

$$\text{Salicylate} + \text{NADH} + O_2 + 2H \rightarrow \text{Catechol} + \text{NAD} + H_2O + CO_2$$

In spectrophotometric measurement of enzyme activity, the absorbance decrease of NADH is monitored at 340 nm. The reaction is catalysed by enzyme salicylate hydroxylase, where NADH isused in reaction, as measured by the reduction in absorbance of NADH. The substrate salicylate isconverted to catechol, its formation in reaction is indicated by increase in absorption at 276 nm. Thestoichiometry between salicylate and NADH has been studied by Zhou et al using salicylatehydroxylase enzyme biosynthesised by *Pseudomonas putida* [13].

2.1.1. Microbial synthesis of salicylate hydroxylase

The interest in synthesis of salicylate hydroxygenase from microbial sources started due to its economical production and recovery of larger yield compared to its extraction from animal tissues. Various microorganisms have been studied for the biosynthesis of this enzyme, including*Trichosporon cutaneum* [25] and *Pseudomonas species* [26]. In our lab a research project was fundedby a pharmaceutical company to establish an economical microbial process for the biosynthesis ofsalicylate hydroxylase. Following a well-designed strategy of enrichment, isolation and screening, asalicylate-hydroxylase producing bacterial strain *Pseudomonas putida* was selected among severalisolates. This isolate, with an gradual acclimatisation to survive and grow at toxic concentration ofsalicylate added in cultivation medium up to 10 g per litre, was studied and selected for thebiosynthesis of enzyme [27]. Usually high salicylate concentration above 2 g per litre were foundtoxic to the growth of microorganisms and have inhibitory effects on salicylate utilising organisms, causing less bacterial cell mass, low biomass yield-coefficients, and overall affecting the enzymeproductivity by cells [28]. The biosynthesis process of salicylate enzyme in bacterial cultivationsystem was optimised for its batch production and then it was gradually scaled up from 500-ml flasksto fermenters with capacity of 5-litres to 80-litres [27].

Since for the recovery of this enzyme, which is not produced extracellularly in the fermentation medium, a large number of bacterial cells were required to release appreciable quantity of cell-boundenzyme for analytical assays. Therefore, to obtain larger volume of fermented medium, cultivation of *Pseudomonas putida* was optimised in a continuous bioreactor system, which yielded a large volumeoutput generating larger amount of bacterial biomass recovered in downstream processing [29]. Continuous cell culture has been reported beneficial to improve low cell densities, and low biomassyield coefficients, which otherwise will result in low enzyme productivity [29,30].

Pharmaceutically important enzyme salicylate hydroxylase was produced from an isolate Pseudomonas putida UUC-1 cultivated in an 80-litre fermenter in 16 h batch process. Afterdownstream processing and purification, each batch fermentation yielded ~9000 units per 60 litres ofworking volume in optimized process [27]. The enzyme produced by P. putida was purified, partiallycharacterised and used in the construction of a biosensor for the estimation of salicylate (aspirin) in bloodsamples. Zhou et al. [13] studied use of purified enzyme preparation from an optimised chemostatbiosynthesis process employing P. putida [16,29], for its application in the construction of biosensorsystems, e.g. carbon-paste electrodes, screen-printed carbon electrodes, and disposable carbonenzyme electrodes.

Although the enzymes are expensive and may increase the cost of the analysis, but the immobilization of enzyme for its repeated use can significantly reduce the cost. The immobilizationcan be done on solid supports, which are then packed in bioreactors, or closely to an electrode surface. Biosensor for aspirin/salicylate is based on the generation of catechol by the passag of samples with salicylate and

nicotinamide adenine dinucleotide (NADH) through an electrode device containing salicylate hydroxylase, which was immobilized in controlled porosity glass beads. Thelinear range of the disposable carbon enzyme electrode in response to salicylate was achieved to 1.8 mmol/ml [13].

2.2. dihydro folate reductase

Methotrexate (MTX), trimethoprim, and pyrimethamine are well known antifolate drugs, and are clinically used as anticancer, antibacterial and antimalarial drugs, respectively, which inhibit dihydrofolate reductase activity. MTX belongs to group of medications termed as antimetabolites. As the MTX treatment slows down the growth of cancer cells and this medication reduces the growth of skin cells to stop forming of scales in condition of psoriasis. Methotrexate is also used in decreasing the activity of the immune system in patients of rheumatoid arthritis [31,32]. The mechanisms of action MTX in inflammatory arthritis has been recently reviewed in detail [33]. This has been reviewed that despite the use of several agents for the treatment of forms of inflammatory arthritis, therapy with MTX given in low doses is most effective. Therefore, for the treatment of psoriatic arthritis and other forms of inflammatory arthritis MTX is a widely used primary drug.

Dihydrofolate reductase (DHFR) enzyme activity is inhibited by MTX, which prevents the reduction of dihydrobiopterin to tetrahydrobiopterin. That causes aggravation in sensitivity of T cells to apoptosis, as a result immune responses are weakened [34]. MTX can be analysed in serum and plasma samples of patients on long-term treatment taking this drug, DHFR enzyme-inhibition assay is based on principle of MTX inhibiting DHFR by binding to it. The mechanism used in the detection of its concentration, lies in the fact, that the biocatalytic activity of DHFR is inversely proportional to the MTX level in samples. DHFR activity is measured spectrophotometrically at 340 nm as decrease in absorbance of NADPH, which is affected by the presence of MTX in samples.

2.2.1. Microbial synthesis of dihydrofolate reductase

DHFR enzyme (EC1.5.1.3) was initially prepared from animal tissues [35,36], which was complicated process producing a very low-yield at high cost. Therefore, the biosynthesis of such important biocatalyst was studied using microorganisms particularly from bacterial strains [37,38]. For higher yield synthesis of DHFR, its production was investigated by six MTX-resistant bacterial strains (PFR-1 to 6) isolated from soil samples. Isolates were subjected to develop resistance for MTX by cultivating them in MTX supplemented medium with the gradual increase in concentration of MTX [17]. Two strains PFR1 and 3 were selected for biosynthesis of DHFR in fermentation process, for their resistance at higher concentration of MTX. High yields up to 4950 Units of enzyme per ml were synthesised by bacterial isolate PFR3 in flask experiments. The production of enzyme was further optimised to obtain an increased yield of 5737 units from 4950 units per litre in scale-up cultivation of bacteria from flasks to 5-litre fermenters [17].

A methotrexate-resistant strain of *Escherichia coli* Type 1 was optimised to produce exceptionally high levels of DHFR enzyme (EC1.5.1.3) within six hours of its fermentation in shake flask-batch process [39]. Further this strain was employed in a chemostat continuous process to obtain a larger yield of enzyme units. A high rate of enzyme biosynthesis was achieved with 10,360 Units per litre in 5-litre fermenters under chemostat culture conditions using a fast growing bacterial strain of *E.coli* [39].

Table 1 summarises published work on two diagnostic enzymes salicylate hydroxylase and dihydrofolate reductase, prepared and characterised using purposely isolated and standard strains of microorganisms, including bacteria and yeasts.

2.3. Lipase

The lipase (EC 3.1.1.3) is well-thought-out as a natural catalyst, in humans and animals it is present in the stomach and pancreatic juice as a naturally produced enzyme. The catalytic activity of lipase is required for the digestion of lipids and fats. An active lipolytic system is necessary in helping to maintain function of a healthy gallbladder, in proper digestion of a diet composed of fatty or oily ingredients, regardless from plant or animal source. Lipases belong to the enzyme family of hydrolases, they act on carboxylic ester bonds. They are involved in catalysing the hydrolysis of triglycerides into low density lipoprotein molecules [1,40].

Table 1. Application of microbial Enzymes in diagnostic assays.

Microbial Source	Catalytic Functions	Useful Enzymes	Ref
Pseudomonas putida UUC-1	Construction of a biosensor for Aspirin levels	Salicylate hydroxylase	13
Batch culture of *Pseudomonas putida*	Detection of salicylate in body fluids	Salicylate hydroxylase	16
methotrexate-resistant bacterial soil isolates	Antifolate drugs, Methotrexate level in blood samples	Dihydrofolate reductase	17
Trichosporon cutaneum	Determining salicylate level in body fluids	hydroxyquinol 1,2-dioxygenase, and Salicylate hydroxylase	25
Pseudomonas putida ATCC 29351	electron-transfer properties in reduction of salicylate to Catechol	Salicylate hydroxylase	26
Bacterial Soil isolates	Measurement of salicylate	Salicylate hydroxylase	27
Continuous chemostat culture *P. putida UUC-1*	reduction of salicylate to Catechol	Salicylate hydroxylase	29
Pseudomanas cepacia ATCC 29351	Measurement of salicylate in aspirin	Salicylate hydroxylase	30
methotrexate-resistant murine LY5178 cells	Methotrexate level	Dihydrofolate reductase	36
Amethopterin-resistant strain of *Streptococcus faecium*	Level of antifolate drugs	Dihydrofolate reductase	37
Amethopterin-resistant mutant of *Escherichia coli*	Methotrexate level	Dihydrofolate reductase	38
Continuous culture of a methotrexate-resistant *Escherichia coli*	level of antifolate drugs	Dihydrofolate reductase	39

Lipase is the one such widely used and versatile enzyme for the reason that the catalytic mode of lipases could be very different and unique in different reaction medium. For example, at the water-lipid interface lipase have the ability to hydrolyse fats into fatty acids and glycerols, but this reaction could be reversed if lipase working in a non-aqueous medium. This catalytic ability of lipase can be easily exploited for a specific purpose, in catalytic reaction medium with water interface or in absence of water. This possibility of multi-faceted activity of lipase makes it most widely used enzyme for various industrial as well as pharmaceutical applications [41]. The high potential for uses of lipases in health care and medicine are evolving rapidly and currently they are being widely used in treatment. Researchers have explored in intensive studies, lipases can be employed as an important tool in substitution therapy [42]. In some patients lipase enzyme deficiency occurs during illness and poor health conditions, then the required doses of lipase are administered externally, to compensate the low lipase level in patients. Under normal metabolic condition occurring in healthy human beings, lipase are playing their role in breaking down fats present in diet. As a result fat content is digested and absorbed in the intestine, but in the compromised health condition the deficiency of lipases leads to malabsorption of fats and also other fat-soluble nutrient and vitamins [41,42].

Lipases therapy can be used in the treatment of Alzheimer's disease, atherosclerosis cystic fibrosis, and for prevention and therapy for cancer. They have potential in their application as a diagnostic tool for the detection of their presence or varied levels in blood, which can indicate the detection of certain disease or infection [42]. Obesity is a serious health issue of concern around the world, the state of obesity causes metabolic disorders. The practical pharmacological approach considered in the treatment of obesity is by inhibiting the activity of digestive lipase, which will reduce fat absorption due to its non-assimilation in body of patient or a person with condition of obesity [43]. Lipase enzymes can be applied as biocatalysts to perform the conversion or transformations of highly regio- and stereo-selectivity under milder conditions, compared to chemical reactions. For the wider utility in several industries, lipases from microbial sources have been studied, as they are used as important tool for their catalytic activity of biotransformation [40,44]. The Lipase is significantly important in pharmaceutical and agro-chemical industry therefore, stereo-selectivity of lipase has been exploited for the preparation of single enantiomers from racemic mixtures, for the desired activity of biotransformation. The work has been published related to enzymatic bio-transformation of chiral compounds for application in pharmaceutical industry [45,46]. Lipase enzyme preparation from selected yeast strains has been used in the biotransformation studies by Muralidhar et al, for the racemic resolution of RS-Baclofen [47], and for the resolution of (RS)-proglumide [48].

2.3.1. Microbial synthesis of lipase

The activity of these enzymes are adequately stable and the supply of enzyme can be obtained from *in vivo* synthesis sources including, animals and plants. But to get better and higher yields of lipase, this can be *in vitro* synthesized in laboratory employing selected microorganisms. Therefore, for large-scale requirement of lipase in industries for commercial use, lipases significantly synthesized by microbial strains are being used [1,40]. Extensive work has been published on various substrates and methods studied for the biosynthesis of lipase employing microbial strains [1,40,44]. Lipase has been synthesized using different microbial strains (Table 2).

The synthesis of lipase by microorganisms mainly depends on culture conditions used in the fermentation [49]. It is important to select a suitable process for biosynthesis depending on type of

carbon sources being used [50]. Researchers have studied different states of fermentation process, including batch, fed-batch to increase the production rate of enzyme [51]. For the synthesis of higher yield of lipase enzyme, a yeast strain of Candida cylindraceae NRRL Y-17506 was employed using two types of carbon sources olive oil, glucose and combinations of glucose/olive oil in submerged fermentation. A response surface approach was adopted for the comparison of lipase production, with various medium composition using glucose/olive oil, malt extract, yeast extract, peptone and tween 80 [52]. An effective assay for lipase enzyme activity has been recommended by Horiuti et al, which is a titrimetric method by substrate emulsification using olive oil as a substrate in reaction mixture and Tris-HCl buffer. One lipase unit is calculated as the release of one micro-equivalent of fatty acid per minute under standard assay condition of 27 °C at pH 8.0 [53].

A novel solvent stable lipase enzyme has been synthesised by a bacterial culture Pseudomonas and characterised for its stability in solvent medium [54]. A purified preparation of lipase produced by fungal strain Aspergillus fumigatus has been characterised for its suitability in selected applications [55]. Lipases have established their use in the construction of biosensors [56], due to their wide substrate specificity in the conversion of triglycerides and other esterified substrates. Moreover, now lipases are commercially available for several applications, including medical diagnosis and bioassays [57–59].

Table 2. Application of microbial enzymes in biotransformation.

Catalytic Functions	Useful Enzymes	Microbial Source	Ref
Industrial uses for lipolytic activity	Lipase	Several microbial strains	40
racemic resolutions	Lipase	Several yeast strains	44
Captopril synthesis	Esterase	Bacterial strains	45
Stereo-selectivity resolution	Lipase	Several microbial strains	46
Racemic resolution of RS-Baclofen	Lipase	*Candida cylindraceae*	47
Resolution of (RS)-Proglumide	Lipase	*Candida cylindraceae*	48
Resolution of antibiotics	Acidic Lipase	*Rhizopus arrhizus*	49
Industrial uses	Lipase	*Candida rugosa*	50
Industrial uses	*Pseudomonas fluorescence*-lipase	Recombinant *Escherichia coli* in Fed-batch cultivation	51
Stereo-selectivity resolution	Lipase	*Candida cylindracea*	52
Industrial uses	novel solvent stable lipase	*Pseudomonas reinekei*	54
Industrial uses	Purified lipase	*Aspergillus fumigatus*	55

3. Conclusion and future perspectives

With a change in lifestyle and an increasing ageing population in many countries, diseases and health-disorders are also increasing. Therefore, accurate test methods results are needed to deal with the increasing number of health issues, and for the timely treatment of a particular diagnosed condition. Different enzymes, diagnostic kits and biosensors are being evaluated for monitoring and diagnosis of

the types of suspected ailments. Diagnostic tests have been widely used to detect and quantify different biomarkers to attain the critical information about health care, which is an important step in making accurate medical decisions. The major challenges with use of enzymes for diagnostic purposes are their yield, shelf life and the stability, if these enzymes were extracted from non-microbiological sources, such as animal tissues. Several diagnostic methods used in pathology labs require costly reagents, equipment, and time for analysis of samples, as well as the availability of trained pathologists and technical staff to operate the process. Hence, there is a focus on research and development of tools that are cost effective, stable and simple-to-operate. The enzyme-based detection of various health disorders have been investigated and in some cases it is fully established; but high costs are involved in enzyme production, purification, their shelf life and storage conditions. For an economical production of enzymes by a humane technique (avoiding their extraction from animal tissues), the use of microbial fermentation technology is a preferred option. Enzymes synthesized by selected microbial strains can be purified for high specific activities and characterized for their stability. Such bioactive enzyme preparations, either in a free-state or in an immobilized-state, can be utilised for diagnostic purposes with specificity and accuracy.

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Conflict of interest

The authors declare there are no conflicts of interest in this review article.

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Social Networking of Quasi-Species Consortia drive Virolution via Persistence

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A B S T R A C T

The emergence of cooperative quasi-species consortia (QS-C) thinking from the more accepted quasispecies equations of Manfred Eigen, provides a conceptual foundation from which concerted action of RNA agents can now be understood. As group membership becomes a basic criteria for the emergence of living systems, we also start to understand why the history and context of social RNA networks become crucial for survival and function. History and context of social RNA networks also lead to the emergence of a natural genetic code. Indeed, this QS-C thinking can also provide us with a transition point between the chemical world of RNA replicators and the living world of RNA agents that actively differentiate self from non-self and generate group identity with membership roles. Importantly the social force of a consortia to solve complex, multilevel problems also depend on using opposing and minority functions. The consortial action of social networks of RNA stem-loops subsequently lead to the evolution of cellular organisms representing a tree of life.

Keywords: virolution; quasispecies consortia; virus persistence; membership roles

1. Introduction

A crucial question in the evolutionary tree of life concepts remains how variations, especially genetic variations occur that are object of biological selection. The neo-darwinistic narrative of the last century always assumed that the variation process is the result of random replication errors (mutations). It remained a curious explanation to reconstruct the emergence of organismic complexity by selection of accumulated beneficial errors. In contrast to this we assume both the evolutionary relevant genetic variations are the result of natural genome editing by viruses and related genetic parasites and the transfer and integration of virus derived genetic content into cell based organisms by infection events. Such events were coopted and exapted by the host and integrated remaining viral defectives such as mobile genetic elements and non-coding RNAs served to reprogramm gene expression and epigenetic markings. This we term virolution, virus driven evolution. The application of virolution overall to issues regarding the origin and evolution of life can provide us with a very distinct and new perspective on how living organisms emerge.

2. Virus-like genetic parasites can build cooperative networks

The involvement of a virus-like process in the origin of life seems illogical. How can a molecular genetic parasite of the host system (virus) be involved in the origin of the host, even if the host is a more simplified RNA based living system? Would this not require the pre-existence of the host? But the term

'virus-like' includes parasites of RNA (stem-loop) replicators, even those simple RNAs that must have been ancestral to any RNA based life forms [1]. Due to high rates of generating diversity, parasitic replicators emerge at the very origins of RNA quasi species [2,3]. However, parasites have previously been treated as presenting a major problem for origin of life scenarios [4,5]. Recently, we have come to realize that virus evolution can often involve complex cooperative as well as antagonistic group behaviors [6,7]. By embracing a fundamental role for diversity and parasites in the origin of life, we can understand how cooperation of both kinds of RNA replicators-very similar ones and even distinct ones-can emerge with more complex function and why this fundamentally generates networks (RNA societies), not fittest type individuals [8–10].

3. Virolution drives complex adaptations

Virolution means virus as mediators of evolution and as competent agents to edit host code [11,12]. Virolution in an RNA virus employs consortial, quasispecies swarms that retain swarm (network) identity [13]. By then following the 'virolution' (cooperative consortia) of such stem-loop RNA communities, we can better understand the origin of virus, tRNA and the ribosomes [14–17]. Virolution also allows us to understand more complex adaptations that affect group identity, immunity and multiple identities such as various cell types [18]. Such complex adaptations can create and modify existing networks [19]. The social force of networks of stem-loop RNAs to solve complex problems did not terminate with the emergence of RNA based life [20].

4. Virus mediated evolution: virolution

A 'virus first' perspective for understanding human evolution will likely seem counter intuitive or even preposterous to many readers [21,22]. Surely these most selfish and destructive of agents cannot be proposed to have contributed substantially to the many complex features that make us human. Viruses are genetic parasites, often capable of transmission and dependent on their host for replication and/or maintenance [23]. They are thus fundamentally able to both, interact with and contribute to host genetic and epigenetic content [24]. Both capacities allow viruses to be editors of host genetic content [25–27]. We know viruses to be agents of disease, often serious and even fatal. In what way can these capacities relate to the complexity needed to generate capabilities of organisms in all domains of life [28]? Besides the direct interaction on host genomes viruses are also capable of colonizing and persisting in host genomes and becoming one with them with more far reaching consequences [29,30]. In this case viruses may introduce instruction sets to host cells [31]. Such new and diffuse instruction sets can promote new regulatory networks with new capacities [32,33]. This process has been called 'virolution'; virus mediated evolution [34]. And it is the persisting viruses that are highly host specific which, usually sexually transmitted also have the ability to differentially affect host survival. The relationship of persisting viruses to its host population has been proposed to contribute significantly to host survival and affect the whole tree of life [35–37]. Such a process is fundamentally symbiogenic [38].

5. Consortial, cooperative, multifunctional and transmissive RNA

Why would viruses promote novelty via the formation of complex networks able to contribute to host phenotypes [39]? The currently accepted view is that viruses are simply providing an extended source of errors (diversity) that can occasionally become 'exapted' by their host for host purposes [40]. An infected individual host variant will survive and somehow adapt virus information for its own survival

[18]. Networks are then created from this information in step wise series of selection events. The real answer, however, lies much deeper then is likely to be appreciated. Indeed, it relates directly to the earliest events in the evolution of life reaching all the way back into the RNA world [41,42]. This world is characterized by consortial, cooperative, multifunctional and transmissive RNA agents that operate in groups that can identify network membership and preclude non-members which clearly are the forerunners of cell-based immune systems [8]. More recently it was detected that RNAs use signaling molecules which drive RNA communication networks to coordinate cooperative interactions [43]. We have long focused on the modest genetic adaptations associated with neo-Darwinian variation and selection. But whenever a host genome becomes colonized by non-ancestral endogenos retroviruses and related elements that replicate via RNA, a quasi-species consortia mediated process again applies to modify existing RNA networks that provided identity (being necessary for control and immunity) superimposing new and often multiple uses of stem-loop RNAs that are now engaged in and generate new identity networks [18,44]. This means such a colonizing event may activate posttranscriptional active RNAs out of former conserved functions into a new context of genomic plasticity. This is a much more creative and punctuated process, able to promote complex regulatory shifts, but one that still essentially uses invasive stem-loop RNA agents.

6. Definition problems

The term virus has a broad and almost instinctive meaning to many people with respect to disease. It is however, worthwhile exploring a current definition of this term in order to employ it with greater consistency and precision [45–47]. Since it is well known that many viruses can infect and exist within their host with no disease, clearly disease cannot be a defining characteristic [48]. Nor is uncontrolled replication a defining characteristic since many viruses have highly regulated replication cycles. Some do encode proteins involved in membrane synthesis. Some do not even encode their own capsid or membrane proteins so this too cannot be a defining characteristic. But so far, no virus has been observed to code for a complete ribosome [17]. Nor do they appear to encode many of the most fundamental metabolic proteins. Thus viruses are fundamentally molecular entities that are parasitic to living systems with ribosomes and energy production. But some viruses are parasitic to other viruses with all their persistent viruses and defectives (thus parasitic to living systems plus virus) and most viruses can generate defective versions of themselves which are parasitic to the host system plus self full virus which represents a never ending modularity [29,49,50]. These situations can be very important for some specific viral life styles. Thus our definition must be inclusive of all of these situations. We therefore propose the following characteristics for defining virus:

- A virus is a molecular genetic parasite.
- A virus must be competent in the instruction system of its host system
- A virus must superimpose (edit) new instructions onto the host system (extending the code, bringing novelty, promoting symbiosis)
- Viral instructions must promote maintenance of the virus (i.e. self identity compatible) which includes directed replication needed for either maintenance and/or transmission.
- Virus instructions can also simply include compelling the host to ‘maintain’ the viral instruction set (persistence) and replicate it along with the host.
- Viral instructions must oppose (i.e. damage) competitive instruction sets (i.e., host immunity and/or virus competition).

• These viral instructions may subvert (colonize) and manipulate opposing or competing instruction sets so as to maintain a coherent viral instruction system.
• The simplicity of RNA virus instructions requires that they be a coherent consortia of diverse RNA instructions (QS-C).

In addition to these defining characteristics present in quasi-species consortia we would propose that the original 'viral' instruction system were simple stem-loop RNA replicators, as proposed for the RNA world [51]. These parasitic replicators were able to transfer and occupy (ligate) their other RNA stem-loops, including their own quasispecies. Such self invasion promotes the emergence of more complex functions such as ribozymes and a consortial ribosome for example [52,53]. RNA viruses still depend on these stem-loop instruction agents for basic identity and replication. The host (DNA) has become a habitat for these RNA societies [54].

7. Social interactions are not a mathematical expertise

There are other important problems involving definitions that should be mentioned. These include the terms networks and systems. The real problem, relates to attempts to mathematically define these terms so that calculation based approaches can be applied to them [39]. For example, a network stems from the concept of a net, with knots (nodes) connected to each other and originally used in fishing techniques. In network theory this was applied to mathematical modelling. Similarly, formal complex systems posits a mathematical foundation for defining systems [55]. However, in the context of diverse but coherent RNA agents (QS-C), it is not possible to mathematically set either the potential interactions or the nature of these interactions for a single RNA stem-loop as it will have conditional, context and history dependent activities (uses) within the population [56,57]. Although statistical modelling may generate results to find quantitative traits that are based on unequivocal data, contextual and history dependent properties of quasispecies consortia can be defined unequivocally only in rare cases [58].

However, it does compel us to use the terms networks and systems in a less mathematical (but popular) way. The concept of network in particular will be important for our discussions as it will relate directly to vital group identity which will require the specification of network membership characteristics [59]. For an RNA agent, being a member of a network relates directly to its identity markers (often stem-loops). We will often consider the issue of group identity and group behavior as these are the proposed to provide the foundations of social interactions. Therefore we seek to define a network from the perspective of a consortia of RNA agents and apply the strategies of these diffuse transmissive agents to explain the creation of new networks and redirecting existing ones [39]. However, it will be very difficult to think about and communicate these consortial or social issues. This is not because they are so inherently complex, but more because they are fundamentally interactive social phenomena that resist a formalizable (mathematical) explanation [60]. A social system will have individual agents such as RNA stem-loops that will fundamentally have multiple (often opposing) activities and uses [61]. This is most apparent in the study of viral QS-C presented below. Social behaviors can be best investigated by analysing action motifs resulting in coordinated group behavior and communication (as documented by e.g., the cells of the immune system) [62]. Especially the social force of communicative interactions cannot be quantified by formalizable procedures, because emerging properties of social (not individual) interactions such as cooperative group behavior depending on contextual an historical circumstances can be better analyzed by social science terminology than by mathematical equations [63].

8. Addiction modules: how persistence drives complexity

Another main question is the evolution of complexity. Let us remind the fact, that cellular life represent rare islands in a sea of viruses. As a consequence every cell based life is under constant infection pressure from its beginning until death [64,65]. Genomic habitats of cellular lifes are a rare resource for genome invading genetic parasites such as persistent viruses, their defectives and an abundance of infection derived mobile genetic elements and non-coding RNAs all characterized by repeat sequence formations, such as transposons, retrotransposons, long terminal repeats (LTRs), non-long terminal repeats (non-LTRs), long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), Alu's, group I introns, group II introns, plasmids, long noncoding RNAs, miRNAs and many others [66–70]. Addiction modules represent at least two competing genetic parasite consortia (viral clouds), which try to invade host genomes. The competition targets not solely each other, but host immune system. This may result both the competing genetic parasites and the host immune system, can together install persistence and remain as a counterbalancing module [71–73]. The most interesting aspect here is, that if these persistent agents are conserved into the genomic identity of the host, the former identity changes dramatically. New features are part of the host genomes which did not exist before. In such addiction module integration events, up until 100 new genes can be transferred into the host genome in a single event [18].

This means a rather complex genetic set may be transfered into a host genetic identity via a single infection event. For example, in the case of a restriction/modification module, this means 52 restriction enzymes are counterbalanced by 52 modification enzymes. This indicates how complex addiction modules may be constructed [74,75]. One of the best known addiction modules in bacterial life is the restriction/modification (R/M) system, which is a common feature with immune functions. One part consists of an antitoxic modification enzyme, which represents an unstable protective agent. The counterpart consists of a toxic restriction enzyme, which is a stable but destructive. [71,76]. Most importantly, this new information representing genetic novelty by a persistent integration of counterbalanced coded genetic elements is not the result of error replication, but a result of module-like linked genetic contents. This fundamental difference to error replication narratives proposes new nucleic acid sequence constructions by integration of larger content arrangements into a coherent syntax without destroying the already existing sequence content [77]. The explanatory model of the last century in which error prone replication of quasi-species dominated, now can be revisted into quasi species consortia with an inherent never ending capability of creative productivity. 'Error-treshold'in this perspective is overproduction that cannot be conserved or serve as beneficial genetic content.

This is an unique social force, because several RNA stem loop consortia cooperate by competition together with the host immune systeme and change host genetic identity by complementing into a counterbalancing module. The new capabilities that derive from integrated addiction modules are absent in the same host species which are not object to such infection events. Both, the competing viral clouds and the host genetic identity reach a cooperative upgrade of genetic complexity and addictive dependency on such infection events. Contextual circumstances (stress, environmental change, etc.) may destabilize such addiction modules and counterbalance may get weak or even out of control, the destructive toxin element may become active again and harm or even kill host.

9. Networks of non-related infectious agents constitute information in host genomes

The development of neo-Darwinian thinking in 1930's stems directly from the foundation that natural selection acting on variation (mostly from replication errors) in individuals selects for the survival of fittest type variant. Thus the variation in offspring originates from the direct ancestor to the selected individual [78]. However, when 'non-ancestor' virus derived genes are seen to occur in host genomes, it is typically reasoned that such genes simply represent another form of variation (errors) that were also somehow associated with individual survival. The surviving host individual was then able to adapt (exapt) these genes for its own purpose and survival [79]. This explanation still invokes a central role for individuals. What then results are various scenarios, such as kin selection, or arms race ideas involving a serial one-upmanship and linear process of selection. Any networks that emerge will then need to stem form this same serial process. The process is not prone to punctuated bursts in evolution nor is it particularly prone to rapid emergence of complexity or novelty. Also, any associative or group behavior that emerges, such as altruistic behavior, will similarly stem indirectly from individual survival, as described by various kin selection or game theory models. This view has been well accepted for numerous decades and many current evolutionary biologist no longer question its basic tenants. Some even like to think of this as laws of evolution. But this is a view that emerged well before we understood the broad and ancient prevalence of virus [27]. In the last several decades analysis of comparative genomics and metagenomic sequencing of numerous habitats has shown us that virus derived sequences dominate in all habitats so far evaluated [80].

The term virosphere has been introduced to describe this vast cloud of genetic information [81,82]. And within the genomes of organisms, virus derived information is almost always the most dynamic component of host DNA for all domains of life [83,84]. Much of this virus derived host DNA has long been seen to play no useful role, it was 'junk' DNA that was the product of selfish replicators [85,86]. Yet much of this 'junk' was clearly viral derived and often its expression was associated with reproductive tissue. More recently, such 'junk' has seemed much more important for the functioning of the organism [87,88]. But it is still basically seen as 'exapted' stuff, put to some inadvertent good host use following individual selection. But the existence of a vast virosphere should compel us to think differently about virus derived information in host genomes. All domains of life must survive in this ancient, unrelenting and extremely adaptable virosphere.

10. Cooperative RNAs

How does virolution affect host evolution? Can it be providing some core, essential function needed for life? And more fundamentally, does virolution operate with additional principles, such as consortial group identities (QS-C) that can colonize and transform host, which fundamentally promote networks and complexity? If so, can these principles help us better understand the origin of life. Such virolution is what promotes the creation of new 'systems', not serial selection from errors [89,90]. But this looks like errors since most of the instructions are subviral [91]. Viruses, the ultimate and nearly invisible selfish agents have finally taught us about the social force of consortia. It is a big lesson and it applies to all levels and eras of life. But why would a consortia of viral agents act to promote complexity? It is for the sake of superimposing group identity and group survival? The QS-C has to incorporate a new viral derived identity onto the host [92]. This colonization will also clearly affect host survival in its extant virosphere via updated immune functions [93]. The virosphere matters for the success of all life. Such a colonizing event must promote the survival of this information and new viral identity/ecology that results. This is a very different perspective then that of selfish individual type selection. And although virolution supports various forms of multilevel selection, it does not conflict with traditional individual type selection which emerged with DNA based cells and virus. But whenever infectious sets of RNA

based replicating agents successfully colonize a host, they will again bring to bare the creative, cooperative and distributed power of QS-C selection to their host [94]. This is a most ancient process that still operates on DNA, using DNA as a stable habitat. The RNAs have multiple regulatory capacity which leads to a better understanding RNA cascades and networks, which are the products of or promoted by serial colonization of virus (and often provide antiviral activity). These regulatory stem-loop RNAs will mostly occupy introns, 3' UTR and some 5' promoter regions. We will aso see that older identity/regulatory systems become subjected to manipulation (repurposed) or elimination following successful colonization.

11. Fittest individual type reconsidered

RNA viruses have long been recognized as distinct agents from their host cells in that they were the sole survivors of the RNA world that still used RNA as a genetic molecule [95]. That they could replicate so readily and be characterized in the laboratory made them ideal systems to study variation in RNA replication [96]. The variation was considered to result mostly from copy errors of a low fidelity polymerase. And since viruses could be 'cloned' they apparently adhered to the concept of individual fittest type selection. Since it was realized early on that RNA replications at the dawn of life in the RNA would also replicate with high error rates, this seemed to present a problem for the origin of life and the origin of the genetic code [97]. It was from this perspective in the 1970's that Manfred Eigen developed the quasispecies equations to explain the quantitative behavior of RNA populations that were generated via errors of the master fittest individual type template [79]. The basic assumptions were then that there was a master fittest type RNA template that would generate a cloud of RNA progeny due to copy errors, but that this cloud would have certain overall behaviors (such as error threshold). Many more theoretical papers followed this early publication by Eigen, by his colleagues representing the exploitation of the formal mathematical analogy of quasispecies dynamics and statistical mechanics [98]. This should finally lead to a theory of evolution based on biochemical kinetics [78]. And in the following decades, a large number of laboratory studies by RNA virologist sought to evaluate and measure various aspects of quasispecies theory [99]. It became very clear that the quasispecies behavior of RNA viruses was very important for understanding clinical outcomes of human infections. And indeed, some of the insights of quasispecies theory were observed, such as error threshold. The concepts of variation of the master fittest type became entrenched during this period as there seemed to be no conflict with more traditional neodarwinian selection.

12. The example of retrotransposon activity in human brain evolution

Current knowledge about the evolutionary origin of placenta organ in mammals clearly indicates natural genome editing by persistent retroviruses [100]. Another intrugiung example is human brain evolution. For many years, molecular biologist assumed that the complex RNA expression patterns observed by various techniques (such as hybridization kinetics) in the mammalian brain was due to the expression of many genes, which was expected for such a complex organ [101]. However, comparative genomics has made clear that gene transcription differs little between human and great apes [102]. Indeed total gene numbers differ remarkably little between the simplest animals (C. elegans) and humans. But by far the biggest differences between human and chimpanzee genomes were due to indels (insertion and deletions) [103–105]. The great majority of these indels are the result of retrotransposon activity of various types such as ERVs, LTRs, LINES and alus being most numerous [106]. Of these, the alu elements and transcripts are particularly active and affecting RNA editing and intron splicing in the

human genome [107]. In addition, they are frequently involved in epigenetic control and can emerge or expand rapidly in genomes [108]. Such a large scale retroposon colonization would seem to pose a highly genotoxic situation for the human genome, an idea which seems supported by genomic analysis [109,110]. And yet this noncoding DNA is species specific [111], evolving quickly in humans [112], but also appears to be under very strong selective constraints [113,114]. This seems problematic in several ways: this is an inherently destructive event that should seldom result in novel or complex phenotype, plus it is both rapidly changing between species yet sometimes highly conserved. Indeed, this high rate of change was previously used to argue for the idea that it must be junk DNA. Yet, these are the changes that must be addressed and included to explain the emergence of the large and social human brain [115]. How then can we understand the origin of the most complex organ known (human brain) in the context of such massive introduction of errors? Clearly we cannot. But perhaps the concept of 'errors' is itself in error as implied above [116].

Indeed a major correction in our thinking has emerged from the ENCODE project. This project is a consortia of researchers that has sought to characterize all the RNA transcribed from the human genome, including RNA that is not cytoplasmic polyadenylated mRNA but is non-coding RNA [117,118]. It is now quite clear that most of this 'junk' is transcribed and that 95% of the transcripts are from repeated sequences that were retrotransposed [119]. These transcripts include a previously poorly studied class of long non-coding RNA [120,121]. Furthermore, these non-coding transcripts appear particularly relevant to human brain and cognitive development and evolution [122,123]. Additionally, long term memory also seems to use non-coding RNA [124]. These observation have led John Mattick to propose that genetic programming in higher organisms (including human) has been misunderstood for 50 years [117]. Regulatory RNA derived from retrotransposons is key to eukaryotic complexity, compelling us to abandon the concept of selfish junk DNA. But in this realization we also come to realize this regulatory RNA is operating mostly as stem-loop RNAs that have complex, multilevel and even opposing functions. It is clearly operating and evolving as a network. But networks of stem-loop RNAs are also thought to have been crucial for the origin of RNA based life [125,126]. Could it be that the creative social force of networks of stem-loop RNAs involved in the origin of life are still at work during recent human evolution? If we look at the synaptic plasticity in humans, arc-a key protein in memory storage-derived from a retroviral infection event [127,128].

13. To make a network from a collective: quasi-species-consortia (QS-C)

In the ensuing several decades, many laboratory observations were made that indicated more complex collective behaviors for viral quasispecies then were predicted by the quasispecies equations [99]. The most recent compilation of these studies outlines many of the collective behaviors that have been made with quasispecies [40]. The culmination study that most clearly reported that quasispecies have more complex collective behaviors was the study from the Andino group of poliovirus pathogenesis in a mouse model in which diversity and cooperation were key to viral fitness [129,130]. The quasispecies collectives have distinct and measurable fitness.

- They can compete with and exclude related populations.
- They have minority populations that are crucial for overall fitness [131,132].
- They can display heterogeneity important for fitness that is not observed in the consensus type [133].
- They can suppress their own replication through lethal defection [134].
- They can be composed of members that can complement and interfere with replication of the collective

and many of these features can be observed in clinical infections such as humans with hepatitis C virus [135].

Thus quasi species are collectives that have positive and negative interacting members that are bound together for a combined fitness that depends on diversity [136–138]. It is thus ironic in that it is from the viruses, the most selfish of all genetic entities, we experimentally observe the characteristics of cooperative, collective behavior. And it was the 'fittest type' assumptions of Eigen that generated quasispecies equations and theory which stimulated the development of this modern collective quasispecies view. But we are left with a conceptual contradiction. Modern quasispecies observations do not depend on the master fittest type and the consensus sequence may not predict to the fitness of the diverse collective. Diversity itself seems crucial.

Such dynamic diversity allows a population of otherwise rather simple agents (such as HIV-1) to defeat a highly complex and evolved system of adaptive and innate immunity in their human host [139]. If such infections were limited to the fittest type individuals, they would fail to overcome such a complex system. Not only can the social force of quasispecies defeat our human immune system, it has also largely defeated our combined human technology by frustrating the development of effective vaccines for 30 years. All this impressive biological competence from a small and 'simple' virus. The term QS-C will indicate a 'collective' of 'cooperative' character to the population. That way the original term, quasispecies (QS), can still apply to fittest type models. With this clarification, it should become apparent that all RNA replicators (especially simple ones) will have high rates of diversity generation (not error). In contrast to the error replication narrative such high rates of diversity generation can be termed also as high levels of non-directed creative productivity reminding us that living agents in populations does not replicate mechanistically in a machine-like manner but may search also for innovative solutions for unexpected context [89]. In addition, all genetic entities that replicate via RNA will also be prone to QS-C social (collective) behaviors [140].

Importantly these behaviors will include both cooperative and competitive interactions, even within the same individual molecule. RNA, however, is not simply providing a syntax for genetic information. It is more than code. It can also provide structure (stem loop), identity (stem-loops, 5', 3' ends) and functional (ribozyme) activity. And it can be dynamic (e.g. pseudoknots) and responsive to the environment (riboswitches) and even frameshifting. A ribosomal frameshift is a natural technique to process alternative translation of an mRNA sequence by changing the open reading frame [141–143]. Because of this much extended capacity relative to DNA, RNA can be considered as more active agents, with group behaviors that make it able to function as an agent-based population to affect its own activity and survival [11,26]. It was from this perspective that we proposed that DNA should be considered as a habitat for these active RNA agents [54]. But this discussion of simple RNA replicators suggest that the concept of QS-C should also apply to the ideas and experiments concerning the 'RNA world' hypothesis. Yet curiously, very little 'RNA world' research has addressed any issues regarding quasispecies, see [144,145], let along the more modern QS-C idea. As many are starting to think that life originated in a cooperating situation [146].

14. Virolution drives the origin of life

To evaluate the QS-C and infectious perspective on the RNA world hypothesis, we will apply and explore the RNA-agent concept introduced above to the role of stem-loop ribozymes in the origin of life.

The main objective is to incorporate the historically absent QS-C and parasitic perspective (with its inherent feature for group fitness) into the process that creates social RNA networks out of prebiotic elements. We will not explore early chemical evolution that might have led to the emergence of RNA molecules but will instead assume RNA has come into existence and follow its features from this perspective. One immediate consequence of this perspective is that we will be focused on collective features of RNA populations and will thus evaluate the chemical consequence of ribozyme QS societies, not individual replicators. This foundation immediately creates a situation in which collectives of molecules with multiple behaviors will have the primary role in promoting the origin of life. It will also be important early on to consider how these systems maintain coherence (group identity), as this is an essential feature. Indeed, a basic and continuing theme will be that a core function of stem-loop RNAs is to provide molecular identity through all of evolution, including recent human evolution [147]. The idea is then that individual members of stem-loop RNA populations were collectively able to invade (ligate into) each other to form a more stable and capable (ribozyme active) consortia with emergent, transformative and unpredictable abilities. These collective would lead to the origin of various ribosome and other RNA groups within cellular organisms still linked to its stem-loop tRNA origin [148–150]. Such a scenario also introduces the basic role of cooperation in the origin of life and thus the communication of RNA stem loops [43]. It does not, however, eliminate competition, preclusion or extinction which are also inherent features of QS-C behaviors. Competition is not disolved, but preliminary counterbalanced by a sophisticated creation of flexible hierarchies. Furthermore, the identity and transmissive role for stem-loop RNAs sets the early (precellular) foundation for the origin of viruses whose emergence will further drive host evolution via persistent colonization. The cooperative and parasitic features of QS-C will also promote the early participation of peptides in the identity and evolution of the abundant groups of ribonucleoproteins [151].

The maintenance of these RNA societies as a coherent collective will generally be mediated by addiction modules, which underlie group identity and immunity in all living systems. With this foundation, the emergence of genes, DNA, cells and individual fittest type selection can all be coherently described although alternative concepts contradicting the RNA-world first hypothesis are still discussed [152]. But the emergence of DNA and cells and Darwinian evolution does not terminate the central role for transmissive RNA societies in the evolution of life. DNA becomes a habitat for these stem-loop 'identity' RNAs. One issue should already be clear. This scenario posits that collective and cooperative behaviors were and remain essential for the emergence of living complexity [41].

15. How RNA hair-pins generate identity networks

On the origin of the RNA world, short RNA oligomers formed by chemical processes needed to become longer RNAs able to perform template based catalysis. It has been proposed that the initial chemical formation of hairpin-like RNAs (stem loops) could provide ribozyme activity following a ligation based modular evolution that would yield ribozyme auto catalysis [28,153,154]. But according to the parameters of QS-C evolution, for a consortium of RNA stem-loop replicators to survive, they must form a coherent population. They must share their identity and survival [19]. The identification of the stem-loop sequence itself by catalytic agents could provide such common identity. Alternatively, chemical markers or initiators of catalysis could also mark the common population for priming or replication. Thus it is very interesting that the smallest ribozyme so farreported consists of just 5 nucleotides able to catalyze aminoacylation of the 3' end [155,156]. The addition of an amino acid to an RNA molecule has many interesting chemical implications. A ribozyme has rather limited chemical potential compared to

proteins. This is mostly due to proton dissociation constant of various amino acid moieties which are not close to pH neutrality. Thus amino acids are much more capable as chemical catalyst for this reason. Without the participation of amino acids, ribozymes must attain complex folds, often with some dynamic character (pseudoknots) to be effective catalyst allowing them to cleave and ligate RNA. Given this chemical advantage, we might expect that RNA evolution was greatly facilitated (but not coded) by peptides that contribute catalytically, as stabilizers or selectors for specific RNA sequences [157]. In addition, such a modified RNA would likely also provide a chemical marker that could distinguish this RNA population. Indeed this molecular identity idea is developed below as a way to better understand the origin of tRNA and its role in initiating replication of so many RNA viruses, as well as how this chemical marker could promote the natural genetic code.

16. Emergence of RNA group identities

A good starting point for the accumulation of complexity seems to be hairpin ribozymes whose activity can be controlled by external effectors [158]. Structural variation in these ribozymes allows progeny RNA to have different functions from their parental RNAs. The objective is to replicate RNA with RNA which hairpin ribozymes can perform via a sequence of ligation reactions that produce a longer ribozyme [159]. Along these lines, two short hairpin RNAs can catalyze their own ligation to form larger RNA constructs [160]. Thus we see interactions that promote more complex progeny. However, for a fully active ribozyme, complex RNA folding is needed. And such folding is cooperative [161]. Folded ribozymes can also interact with other small molecules promoting their function as riboswitches [162,163]. This includes amino acids which could promote either catalytic control or group identity marking [164].

And the ribozyme folds can also be dynamic and context sensitive as seen in pseudoknots [165]. But ribozymes can also be invasive, including self invasive [166]. Thus stem-loop RNA have many behaviors that would allow them to function as a mixture of agents involved in their own identification and synthesis [167,168]. Of particular interest is their ability to self ligate as this could promote the emergence of social RNA networks with group identity [169,170]. We can also think of tRNA as stem-loop RNA with various functions and histories. Indeed, it appears that tRNAs evolved from two separate hairpins [171], in which each of the stem loops interacts with a different ribosomal RNA subunit. This is a very interesting observation from an social RNA network perspective. The invasive nature of intron ribozymes (endonuclease) also applies to tRNA from archaea, but here four distinct specificities are known [172–174]. This very much resembles an identity system in which introns are marking central cellular (self) agents (such as tRNAs) for group identity but should destroy similar tRNAs lacking the intron marking. It is thus also interesting that tRNA with various linked amino acids themselves have been proposed to have originated before the translation system as genomic 3' tags needed for RNA ribozyme replication [175–178]. This early function can also be explained as having served as a tag for group identity and could better explain the polyphyletic nature of the origin of tRNA [179].

17. Network membership–basal need to belong

The perspective social interacting networks of quasi-species consortia allows us to consider the role of stem-loop RNAs in the origin of the RNA world in which the action of individual agents can cooperate and be combined into a more capable collective action of a population. Thus the origin of spontaneous cooperating networks of stem-loop RNA replicators can be understood from this perspective [41].

However, we will use the term network to include some distinct features specifically network membership. To designate this situation we apply the term social network to distinguish networks that have no social membership criteria.

Basically, for a network to be coherent and able to act collectively, it must limit membership to promote coordination by communication [43]. Otherwise it is simply a abiotic collection of uncoordinated entities and there will be no selection for maintaining the network existence. If we are examining a network composed of stem-loop RNAs, it will be necessary for the individual RNAs to have some behaviors that maintains membership such as replication and identification of self and non-self members. As mentioned by Nelson and Breaker this requires signal mediated interaction. If only one type of RNA is supported (e.g. high fidelity replication), there can be no complementation and complex function (i.e. ribozyme) for the collective. A diversity of behavior and type will be essential. Recall however, that these RNAs act as agents in which various (multiple) behaviors will be possible even for the same sequence. This means there is diversity of interaction as well as diversity of type. Thus overall interaction of an RNA agent with the collective must promote coherence and continued existence. What then are the features that promote continued existence (selection) for a social network?

This does not require that only positive (e.g. replication) interactions be supported. Negative interactions, including interference will also be needed. For example, highly efficient run-away replicons would overtake a QS collective and yield only one RNA type. Thus the QS would lose complementing functionality and would also consume all substrates if they were not regulated. This situation presents a problem in those habitats with limited substrates, likely a very common state. Therefore, some level of self regulation (negation) in the collective would promote the survival of the collective, especially if these RNAs could interact with the substrate in a regulatory (e.g., riboswitch) manner. That efficient replicators become susceptible to parasitic replicators would provide an inherently spontaneous process of self regulation. Yet the collective will still need to promote replication when it is favored. Accordingly, it becomes important for members of the collective to be subjected to both positive and negative self regulation via RNA-RNA interactions. However, here too there must be some limits to self regulation as the collective cannot tolerate overly active self regulating members that will extinguish the collective.

Thus we see that being a successful member of a collective has many (and multiple) behaviors associated with it. On top of that, as a QS-C replicates, these features will drift with time in a dynamic manner. In this context we can see that a random RNA stem-loop or a stem-loop RNA from a different QS collective would likely not be coherent with the other members of a particular QS. A social QS network is generally rather specific for its members. Group selection has already started. Indeed, many experiments with RNA viruses infecting humans and animals have shown a particular QS will exclude other QS of the same virus which results into immune function. Additionally such society membership is also time dependent in that the serial passage of the same viral QS will usually result in subsequent QS that preclude prior individual members of the QS. This behavior has often been called a 'Red Queen' behavior, but such a classical neo-Darwinian view does not incorporate or acknowledge the issue of group membership [180]. The membership view, on the other hand, allows us to understand the maintenance of minority types in the collective since these members can provide a needed but complementing catalytic control. Thus a social QS consortia is a network that will naturally promote the emergence of membership. And as noted, defective interfering agents can also contribute to membership control.

18. To be or not to be part of group identity

As previously proposed group membership can also be promoted by the combined action of toxic agents linked to antitoxic agents [72]. A common version of a toxic agent is an endonuclease that will cleave sequences that are identified as foreign. The antitoxin in this case prevents the action of the endonuclease e.g., via a bound protein or methylated base, dsRNA with another molecule, altered RNA fold etc. In this light, the endonuclease and ligation activities of stem-loop ribozymes are particularly interesting [181]. A stem-loop ligase could identify non-member stem-loop RNAs and destroy them by ligation. Recall, however, that serial ligation can also be used to copy a stemloop RNA. But such a situation has several very interesting implications. One of the problems with a group identity of stem-loop RNAs is that to attain their combined function, they need precise physical molecular placement relative to one another. This would normally require a high concentration dependence to counteract diffusion. By ligation, however, we could build a group identity of stem loop RNAs that have covalently placed the various stem-loops in the correct functional (or dynamic/regulatory) context and have lost their concentration dependence. It seems likely that such a process would involve invasive self colonizing stem-loop RNAs that results in one molecular entity with a common identity function. This would generate one entity that evolved from the ligation of a mixed set of stem-loop agents that now have a highly enhanced (collective) functional capacity. This collective would also have a highly enhanced capacity for persistence as it need not continually replicate individual stem-loop RNA agents to maintain its membership. The collective, however, would still need to oppose non-members or other parasite participation. Additionally, a collective might attain a conditional (regulated) replication capacity if it incorporates stem-loop RNA riboswitches. It is by such a process that we can now consider the origin of the ribosome [182,183]. Membership is thus crucial for living networks to emerge. In examining the literature relevant to QS, the RNA world and RNA network formation, we can indeed find some experimental evidence that supports QS and the spontaneous emergence of RNA networks [41,160]. But almost completely lacking from such experiments is any evaluation of the membership issue. For example, quasispecies-like behavior has been observed with in vitro RNA replicator studies [184]. Nonenzymatic template (peptide) directed autocatalytic systems can show network behavior [185,186]. And communities of RNA ribozyme replicator sets can also show lateral evolution [187].

Also rule based computing simulation have been applied to similar systems in an effort to understand the emergence of parasites and antiparasites [10,188]. Along these lines, the hypercycle kinetic model was proposed to be a system of cross catalyzing RNA replicators which depend on cooperation for growth [189]. But hypercycles as proposed are not able to tolerate networks of non-related parasites, let along depend on them for development. Yet the biggest problem of all such studies is that there are few assumptions regarding the basic importance of social network membership [190]. Without a social network membership concept and its attending strategies, authentic collective action does not emerge. The dynamic nature of network membership and collective action poses many unsolved problems for existing theory. For example, how is the multi-potential of an individual RNA to be evaluated within the QS-C if we cannot specify all the other interactions and how they change with time? We cannot apply our current ideas of fitness to this individual RNA as the historical and population context is key [191,192]. Increasing research on history and context of such QS-C are detecting the pathways how RNA stem loops with different ancestral history contribute to newly arising consortia [193,194].

Network membership needs to be prominently considered if we are to understand the origin of the ribosome and the genetic code. The emergence of the genetic code is not solely a molecular biological

invention but the result of social interacting RNA consortia which needed many code using agents [195,196]. For in contrast to neo-Darwinian evolution, network members will generally have distinct ancestral histories. These members will mostly originate from separate parasitic lineages that were able to penetrate defenses and join the network, sometimes in mixtures. They don't need to descend from one individual or even be from the same type of agents such as virus, transposon, intron, inteines and others [197–199]. From this perspective we can understand why the two halves of tRNA have distinct evolutionary histories, yet tRNA is a core agent for evolution of life [200,201]. Thus neither the amino acid based (peptide) ancestors or the RNA based ancestors need a common origin to participate in a symbiogenic network. [202,203].

19. Conclusions

The perspective on social networking of quasi-species consortia (qs-c) provides us with the opportunity to coherently explain how RNA stem loop groups mediate evolutionary novelty and genetic variations. The presented contribution basically traces social stem-loop RNAs from providing replicators, marking group identity, to the invention of ribosomes and translation. The half a century outlasting quasi species theory with its individual fittest type selection narrative is now revisited into social networking of quasi species consortia that provide us with a model how communicative interacting RNA groups generate genetic novelty, transfer even complex genetic content to cellular host organisms via infectious genetic parasites and evolutionarily change their genetic identity by persistence. The error-replication narrative of the last century can be replaced now by current knowledge about creative productivity of quasi-species consortia. The crucial roles of quasi species consortia membership which includes also minority types elucidates how such RNA populations may compete and cooperate in parallel in an indefinite and unpredictible way. As main drivers of genetic novelty they represent virolution being the essential pathway of the evolution of the tree of life.

Author contributions

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DNA-based detection of Mycobacterium avium subsp. paratuberculosis in domestic and municipal water from Porto (Portugal), an area of high IBD prevalence

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ABSTRACT

Mycobacterium avium subsp. paratuberculosis (MAP) may play a role in the pathology of human inflammatory bowel disease (IBD). Previously, we found a high frequency (98% in patients with active disease) of MAP DNA detection in the blood of Portuguese Crohns Disease patients, suggesting this cohort has high exposure to MAP organisms. Water is an important route for MAP dissemination, in this study we therefore aimed to assess MAP contamination within water sources in Porto area (the residential area of our IBD study cohort). Water and biofilms were collected in a wide variety of locations within the Porto area, including taps connected to domestic water sources and from municipal water distribution systems. Baseline samples were collected in early autumn plus further domestic water samples in early winter, to assess the effect of winter rainfall. DNA was extracted from all 131 samples and IS900-based nested PCR used to assess the frequency of MAP presence. Our results show high MAP positivity in municipal water sources (20.7% of water samples and 41.4% of biofilm samples) and even higher amongst domestic sources (30.8% of water samples and 50% of biofilm samples). MAP positivity in biofilms correlated with positivity in water samples from the same sources. A significantly higher frequency of MAP-positivity was observed during winter rains as compared with samples collected in autumn prior to the winter rainfall period (61.9% versus 30.8%). We conclude that domestic and municipal water sources of Porto region have a high burden of MAP contamination and this prevalence increases with rainfall. We hypothesize that human exposure to MAP from local water supplies is commonplace and represents a major route for MAP transmission and challenge which, if positively linked to disease pathology, may contribute to the observed high prevalence of IBD in Porto district.

Keywords: Mycobacterium avium subsp. paratuberculosis; inflammatory bowel disease; MAP detection; water contamination

1. Introduction

Mycobacterium avium subsp. paratuberculosis (MAP), a member of the *Mycobacterium avium* complex, is the aetiological agent of Johnes Disease (JD), an enteritis particularly affecting ruminant species (bovine, ovine and caprine). MAP has a wide-ranging host spectrum, causing enteritis in many wild animal species, including deer, rabbits and macaques [1,2]. Europe and North America consistently report moderate/high incidence of JD (resulting in important economic losses) that persist because of limited availability in efficient pathogen eradication policies that are disadvantaged mostly by the lack of a commercial vaccine able to fully prevent non-clinical faecal shedding [3–5]. Common pathological traits between JD and Crohns disease (CD) in humans, along with consistent reports of MAP detection in CD patients [6,7] have suggested a possible role of MAP in the pathology of this type of inflammatory bowel disease (IBD). However, MAP has not been detected in every CD patient and can be commonly found in the gut and peripheral blood of many apparently normal human controls [8] . The aetiology of IBD (including CD) is thus likely to be multifactorial including the presence of host genetic (and epigenetic) susceptibility traits [9,10] , composition changes in gut microbiota (dysbiosis) and long term colonization/ chronic exposure to particular pathobionts that include MAP and other species such as adherent-invasive Escherichia coli (EC) [7,11–14]. These indications are supported from studies showing significant influencing factors in disease progression include diet and associated exposure to particular water and soil environments, many of which contain MAP and EC, and the long-term remission observed in a number of patients receiving either faecal transplantation and/or anti-MAP therapy [15] . The current evidence thus suggests that MAP may represent only one possible aetiological agent and that a variety of etiological factors contribute singular aspects to the triggering of this syndrome which in themselves exert diverse degrees of influence on individual hosts depending on existing susceptibilities .

Humans can become infected by MAP through consumption of meat or dairy products from contaminated animals [16] and the environment. Water is an important vehicle for MAP dissemination and source of human exposure [17]. Indeed, water is used in multiple ways (consumption, oral hygiene, vegetable growth) and is diet-independent, thus, not restricted to any one particular diet regimen. Inhalation of exposure from contaminated water aerosols may also be a route for MAP entry [18,19]. Several studies have demonstrated that MAP remains viable for long periods (years) in soils and water, bound to solid particles in suspension and also inside amoebae [20–22]. A pharmaco-epidemiological study conducted between 2003–2007 and reported by Azevedo et al. [23] showed a trend to increasing inflammatory bowel disease (IBD) incidence in Portugal, in particular in the Lisbon and Porto areas. In previous work [24] we have shown high prevalence of MAP and EC DNA in the peripheral blood of Portuguese patients from the Porto area with CD and ulcerative colitis. We found a high frequency of CD patients positive for both MAP and EC DNA in blood, regardless of disease activity, suggesting that both microbial agents may play a part in CD progression. Interestingly, about 38% of the subjects included in our healthy control group were also positive for MAP DNA in blood, suggesting high exposure to MAP in the Porto area. MAP has been shown to highly and persistently contaminate soils and water downstream of areas where animal farms were located [21,25,26]. In many of the suburban zones of Porto, cattle and dairy farming remains commonplace. High apparent MAP infection prevalence rates have been reported throughout Northern Portugal including cattle, sheep and wild animals such as boar and deer [27].

This suggests a consistent falloff of MAP from these sources into the abundant river systems from which all Porto potable water is reservoired and sourced. Thus, in the present work we aimed at assessing if MAPcontaminated water is present in the human water supply chain of the Porto area, constituting a potential environmental risk for IBD that could help explain the high incidence we have previously found in this region.

2. Material and methods

2.1. Sample collection

Water and biofilm samples were collected by volunteers from Universidade Fernando Pessoa in different municipalities of Porto Metropolitan Area (PMA) and/or Porto District (PD) (Figure 1). Samples were collected from taps connected to either domestic water sources (including household dug wells or pumped groundwater sources) or the municipal water distribution system. Porto municipal water is collected in the Crestuma-Lever catchment located in Douro river watershed, upstream the city of Porto, in the municipality of Vila Nova de Gaia.

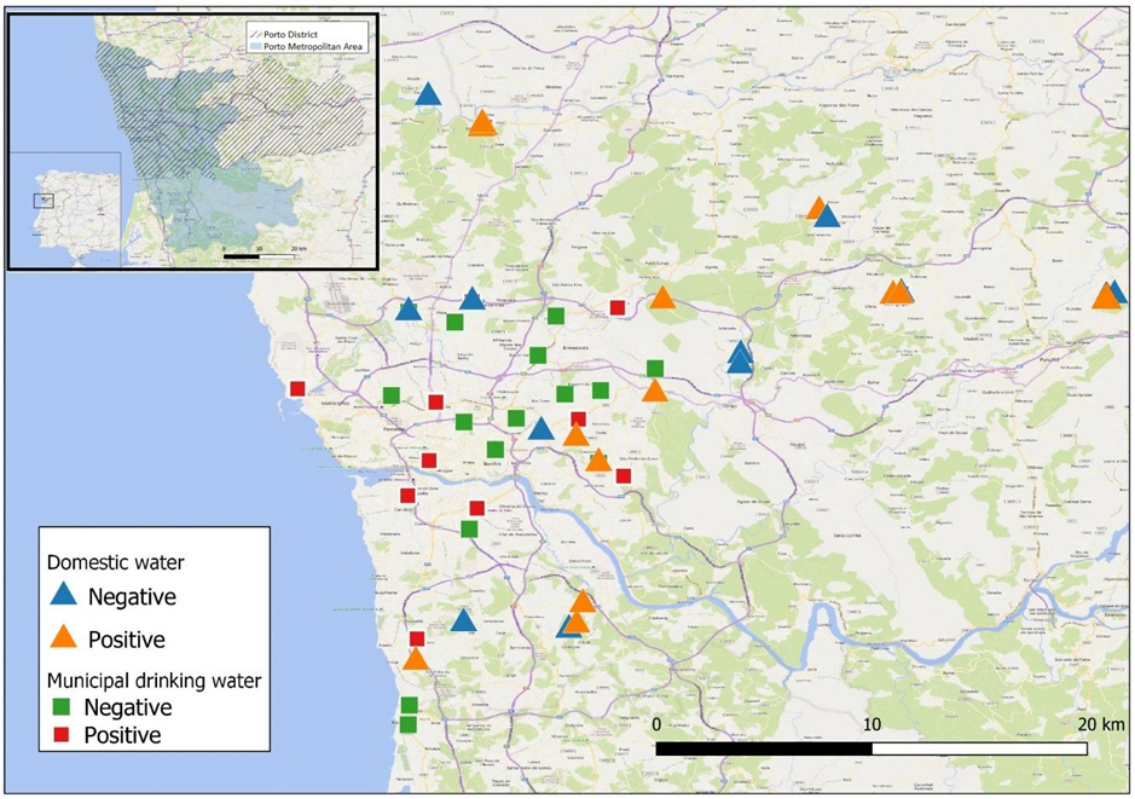

Figure 1. Geographical representation of Porto District/Porto Metropolitan Zone and distribution of domestic (triangles) and municipal (squares) collection sites. Red/orange symbols represent positive sites for MAP DNA and green/blue symbols represent negative sites for MAP DNA. MAP DNA detection was performed as described in Material and Methods. Dashed area, Porto District; shaded area, Porto Metropolitan Zone.

Domestic sources comprised 26 collection sites from 9 municipalities (Amarante, Gondomar, Paços de Ferreira, Paredes, Maia, Trofa, Valongo, Vila do Conde and Vila Nova de Gaia). Municipal sources comprised 29 collection sites from 8 municipalities (Gondomar, Paredes, Porto, Maia, Matosinhos, Trofa, Valongo and Vila Nova de Gaia). Samples were collected initially in September/October 2018 (Collection 1) with additional 21 domestic water samples collected from 7 municipalities (Gondomar, Paços de Ferreira, Paredes, Maia, Trofa, Vila do Conde and Vila Nova de Gaia) in December/January 2019 (Collection 2) to evaluate possible effects of dilution caused by winter rains. Samples included also biofilm collections from taps accounting for a total of 131 samples of which 73 were domestic samples (26 water samples + 26 biofilm samples collected in autumn + 21 water samples collected in winter) and 58 municipal samples (29 water + 29 biofilm samples collected in autumn).

2.2. Water sample collection and filtration

Municipal (treated public water supply) and domestic (dug wells or groundwater) water samples (1 L) were collected using sterile water collection flasks (bacteriology grade) (VWR). Each sample was filtered under vacuum using a sterile filter funnel with a 0.45 μm membrane (MicroFunnel™, Pall Laboratories). Filter membranes were removed with sterile forceps, placed in a 5 mL PowerWater DNA bead tube (Qiagen) and frozen at −20 °C until processing for DNA extraction using the DNeasy PowerWater kit (Qiagen), according to manufacturers instructions. At the end of the procedure, extracted DNA was eluted with solution EB (supplied with the kit), to a final volume of 100 μL.

2.3. Biofilm collection and preparation

Biofilms were collected in households from taps connected to the municipal drinking water system or to household water sources (dug wells or groundwater) [28] . Biofilm samples were collected using a cotton swab immediately immersed and shaken in 1 mL sterile water (molecular biology grade) (Sigma-Aldrich) to allow biofilm detachment. The swab was then discarded and the water containing biofilm residues centrifuged at 13,000 xg for 1 minute. Supernatant was removed and the pellet frozen at −20 °C until processed for DNA extraction using the DNeasy PowerBiofilm kit (Qiagen), according to manufacturers instructions. At the end of the procedure, extracted DNA was eluted with solution EB (supplied with the kit), to a final volume of 100 μL.

2.4. MAP DNA detection by PCR

Confirmation of MAP DNA was carried out by real-time nested PCR (for increased sensitivity), using the real-time PCR LightCyclerTM 1.5 Carousel-based system (Roche Applied Science). For the first round of PCR, primers L1 (CTTTCTTGAAGGGTGTTCGG) and L2 (ACGTGACCTCGCCTCCAT) were used [29] . These primers amplify a region in the insertion sequence IS900, resulting in a 398 bp amplification product. The PCR reaction was performed on LightCycler™ capillaries (20 μL) (Roche Applied Science) in a final volume of 20 μL, using the following reaction conditions: 10 μL SSO Advanced Universal inhibitor-tolerant SYBR Green Supermix, 4 μL of molecular biology grade water, 0.5 μL of each primer L1 and L2 (0.5 μM final concentration) and 5 μL of template DNA. Cycling conditions were as follows: Initial denaturation/enzyme activation at 95 °C for 3 min, followed by 25 amplification cycles consisting of 15 sec at 95 °C, 15 sec at 58 °C, 30 sec at 72 °C and fluorescence acquisition after 5 sec at 85 °C. Melting point analysis was then performed using 1 cycle at 95 °C for 15 sec, 47 °C for 15 sec and then continuous

fluorescence monitoring while ramping to 96 ℃ at 0.2 ℃/sec. Carrousel was then cooled to 30 ℃ for 30 sec. For the nested PCR, primers AV1 (ATGTGGTTGCTGTGTTGGATGG) and AV2 (CCGCCGCAATCAACTCCAG) were used [29]. The PCR reaction was performed in a final volume of 20 µL, using the following reaction conditions: 10 µL SSO Advanced Universal inhibitor-tolerant SYBR Green Supermix (Bio-Rad), 8 µL of molecular biology grade water, 0.5 µL of each primer AV1 and AV2 (0.125 µM final concentration) and 1 µL of template DNA. Cycling conditions were as follows: initial denaturation/enzyme activation at 95 ℃ for 3 min followed by 40 cycles of amplification consisting of 95 ℃ for 15 sec, 62 ℃ for 15 sec and 72 ℃ for 20 sec (fluorescence acquisition at end). Melting point analysis was then performed using 95 ℃ for 15 sec, 47 ℃for 15 sec and then continuous fluorescence monitoring while ramping to 96 ℃ at 0.2 ℃/sec. Carousel was then cooled to 30 ℃ for 30 sec. To minimize the risk for amplicon contamination, the PCR mixes were prepared in a clean isolated room and DNA samples used in the first and second round PCR were added in different laminar flow hood biosafety cabinets located in different rooms. As an additional safety measure these rooms were equipped with ceiling UV lamps. Both the biosafety cabinets and the rooms where they are located were UV-treated for at least 30 minutes before and after DNA addition. Reaction mixes were prepared in a separate room that was kept sample free at all times. Molecular biology grade water was used for the negative controls of both PCR rounds. This was added to the corresponding PCR capillary at the end of sample addition, keeping the capillary open during the entire process. Positive control DNA was prepared from a CFU quantified MAP culture aliquot. The positive control used was close to the sensitivity limit. Sensitivity of the assay was determined by 10 fold serial dilutions of CFU quantified MAP culture dilutions and found to be of 20 CFU per reaction (data not shown). Filter tips and molecular biology grade reagents and disposables were used throughout the procedure.

2.5. Statistical analysis

Comparison of MAP DNA prevalence between domestic and municipal water and/or biofilm samples, as well as between domestic water collection 1 and 2, was made using Fishers exact test. In all tests, a significance of $P < 0.05$ was considered. The program Prism 9 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and graphical construction.

2.6. Design of geographical maps

Geographical maps were constructed using the QGIS 3.16 software for windows (https://qgis.org/en/site/).

3. Results and discussion

3.1. Geographical distribution of collection sites

Water/biofilm collection sites are depicted in Figure 1. Since water and biofilms from domestic sources were collected from taps connected to household dug wells or to pumped groundwater sources, collection sites were more likely to be located in peripheral municipalities of Porto metropolitan area or in the Porto district, in less urbanized areas. Contrastingly, the majority of municipal drinking water and biofilm samples were obtained more centrally and associated with more urbanized areas.

3.2. MAP contamination of domestic and municipal water sources

In this study, MAP DNA was more frequently detected in domestic samples (domestic water 8/26 or 30.8%; municipal water 6/29 or 20.7%; domestic biofilm 13/26 or 50.0%; municipal biofilm 12/29 or 41.4%). Although differences between domestic and municipal water sources were statistically not significant (Figures 2 and 3) our results are in line with the findings reported by Pickup et al. [21].

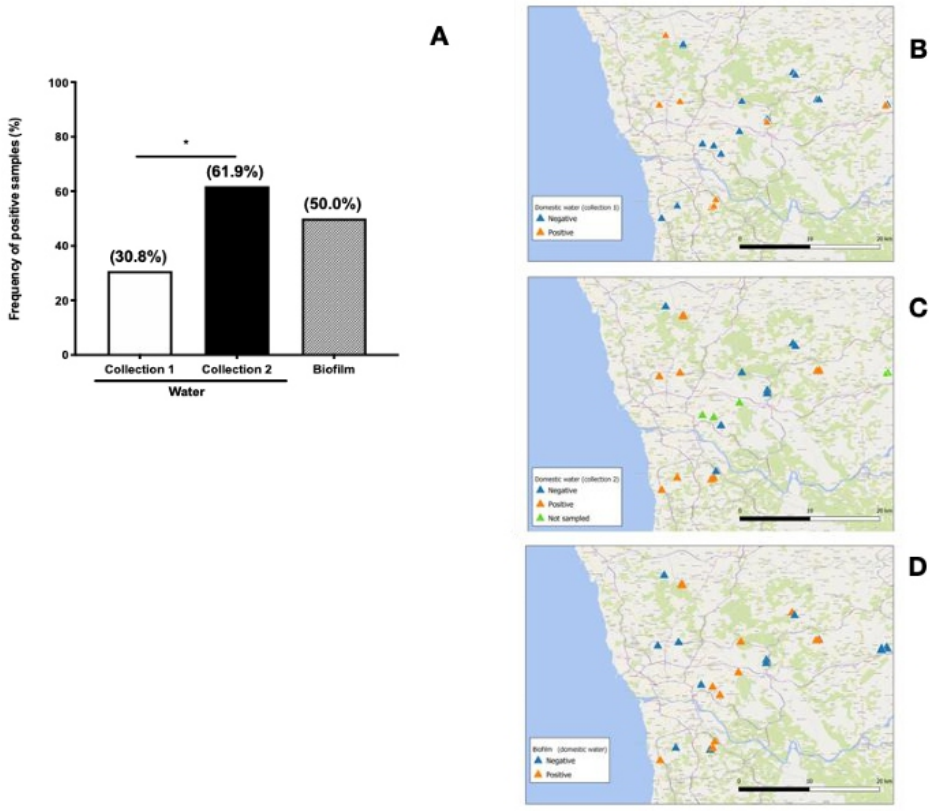

Figure 2. MAP contamination of domestic water sources. (A) Frequency of samples from domestic water sources positive for MAP DNA. Water samples were collected prior (Collection 1) and during (Collection 2) the winter rainfall period. Biofilm samples were collected at Collection 1 time point, only. MAP DNA was extracted and analysed as described in Materials and Methods. (B),(C),(D) Geographical distribution of domestic water samples positive and negative for MAP DNA. (B) Water sample results at Collection 1 time point, (C) Water sample results at Collection 2 time point and (D) biofilm sample results. *p < 0,050.

It has been reported that soil and rivers near cattle farms show heavy MAP contamination [16,21,25,26] , which leads to contamination of groundwater that is sourced for domestic water collection. Water treatment can substantially reduce MAP contamination, as described in a number of studies where MAP was more frequently detected upstream than downstream of water treatment works [21,30]. This might be explained mostly by removal of suspended organic solids, since MAP associates with these particles but is resistant to chlorination [31] . Nonetheless, water treatment processing does not totally eliminate MAP organisms and some may escape to form biofilms on water pipes, building up over time and leading to persistent contamination of drinking water systems [20,32].

Indeed, we found a higher trend for MAP positivity in biofilm samples collected from both domestic and municipal sources as compared to their corresponding water samples (Figures 2 and 3), although these differences were not statistically significant. The frequency of treated municipal water samples positive for MAP DNA in our survey (6/29 or 20.7%) was considerably higher than previously reported in South Wales (2%) [21], Italy (3%) [33] and a national US survey (0%) [28] , but lower than reported in Northern Ireland (45%, by IS900 PCR) [30] and a US midwest region (80%) [28]. These differences are not attributable to the type of detection as all these studies used PCR. However, at least partially methodological issues may still play an important role as in some cases small samples (50 mL) were used [21] and this may severely impact the sensitivity of the detection [21]. In one of the cited studies, culture assays, were also used in parallel but found to render negative results even in cases where as high as 50% of samples turn PCR positive [30], a finding that may be explained by the difficulty of culturing MAP. Together these findings led us to choose to test MAP presence exclusively by the Nested qPCR and to use 1 L sample volume for the present study to minimize false negatives. Our findings suggest that both domestic water and municipal drinking water may be an important source of MAP contamination and transmission in the Porto region.

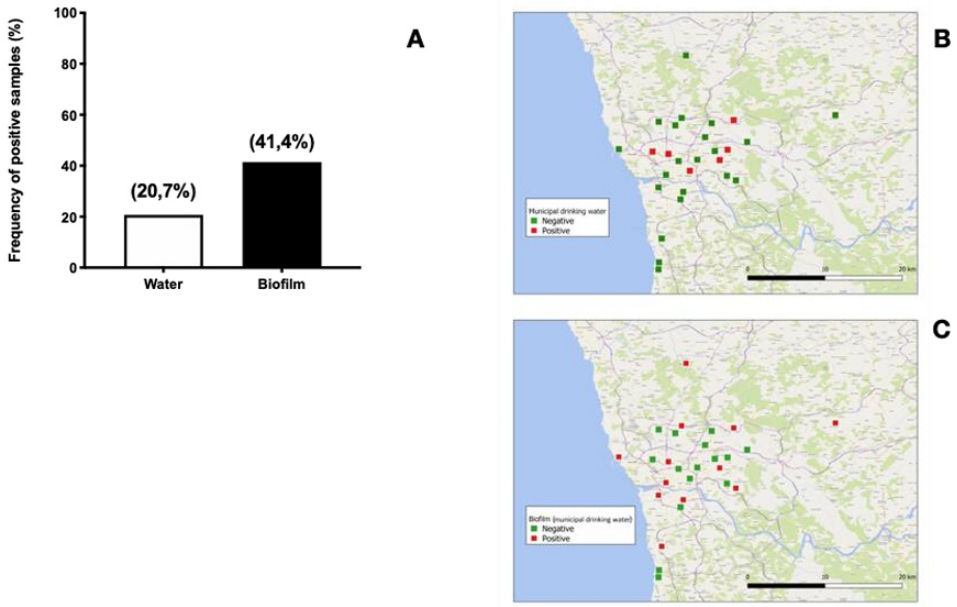

Figure 3. MAP contamination of municipal drinking water sources. (A) Frequency of samples from municipal water sources positive for MAP DNA. Water samples were collected at Collection 1 time point (prior to winter rains). MAP DNA was extracted and analysed as described in Material and Methods. (B) and (C), Geographical distribution of positive and negative samples for MAP DNA in municipal water sources. (B) water sample results, (**C**) biofilm sample results.

3.3. Effects of rainfall on MAP contamination

A significantly higher frequency of MAP-positivity was observed among domestic water samples collected during winter rains (Collection 2–13/21 or 61.9%), as compared to samples collected before winter rains, at the beginning of autumn (Collection 1–8/26 or 30.8%) (Figure 2). Increased river height and flow rate was associated with higher MAP detection on rivers Taff and Tywi, at South Wales, UK

[21,25]. Higher MAP contamination was also found on river Tywi when heavy rainfall was observed in the preceding days of sample collection [21] . In a comprehensive geographical survey on soil samples conducted in Great Britain, Rhodes and collaborators found a strong association between MAP contamination and the presence of cattle farms [26], although soil contamination was widespread and not confined to cattle areas. They also found that MAP contamination was decreased in soils located in areas of higher rainfall, suggesting that soil runoff can be a major contribution for a rise in river and aquifer MAP contamination. Although not included in the present study, effects of winter rains in the catchment water would also be important to evaluate, since a rise in MAP contamination during winter can also impact municipal treated water. Future work can address this hypothesis. Publicly available data on existing animal farms (2019 agricultural census from the Portuguese National Statistical Institute–INE) does not have sufficient geographical resolution to allow comparison with our data. Nevertheless, the north of Portugal (including the Porto region) was an area of high cattle breeding until the late 1970s, for milk or meat consumption, with some cattle and sheep farms still remaining nowadays, including in the peripheral municipalities of Porto area as reported for the broad agricultural Northern Region of Portugal in the 2019 agricultural census from the Portuguese National Statistical Institute–INE. Although information on paratuberculosis prevalence in northern Portugal is limited, a serological study using ELISA for detection of antiMAP antibodies on 3900 sheep belonging to 150 flocks in northeast Portugal showed that 46.7% of flocks had positive animals. According to the ELISA test sensitivity and specificity, the true paratuberculosis prevalence was 6.7% [34] . In another study, MAP DNA (IS900) was detected in faeces from 22 out of 24 asymptomatic bovines belonging to 4 farms in diverse locations of northern Portugal [35]. Positive MAP culture was also obtained from 12 faecal samples among the 22 MAP DNA positive ones [35]. These reports point to high paratuberculosis prevalence in animal farms of northern Portugal, which may lead to soil contamination by animal shedding and subsequently to water contamination by soil runoff.

4. Conclusions

Weconclude that domestic and municipal water sources of Porto region show evidence of MAP contamination, making it a potential environmental IBD risk to take into consideration. Differences in the presence of MAP before and after rainfall suggests that it can be associated with soil runoff to rivers and groundwater from animal sources leading to the formation of MAP biofilms on water pipes. We hypothesize that this represents a major route for human exposure and colonization of MAP which, if able to trigger disease states in susceptible hosts, could contribute to the observed high prevalence of IBD in Porto district.

Acknowledgments

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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Exploring endophytes for in vitro synthesis of bioactive compounds similar to metabolites produced in vivo by host plants

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ABSTRACT

Endophytes represent microorganisms residing within plant tissues without typically causing any adverse effect to the plants for considerable part of their life cycle and are primarily known for their beneficial role to their host-plant. These microorganisms can in vitro synthesize secondary metabolites similar to metabolites produced in vivo by their host plants. If microorganisms are isolated from certain plants, there is undoubtedly a strong possibility of obtaining beneficial endophytes strains producing host-specific secondary metabolites for their potential applications in sustainable agriculture, pharmaceuticals and other industrial sectors. Few products derived from endophytes are being used for cultivating resilient crops and developing non-toxic feeds for livestock. Our better understanding of the complex relationship between endophytes and their host will immensely improve the possibility to explore their unlimited functionalities. Successful production of host-secondary metabolites by endophytes at commercial scale might progressively eliminate our direct dependence on high-valued vulnerable plants, thus paving a viable way for utilizing plant resources in a sustainable way.

Keywords: Endophytes; biomolecules; secondary-metabolites

1. Importance of medicinal plants

Different types of microbial species as symbionts of a plant, living most of their lifetime within the tissues showing no symptoms, are recognized as endophytes [1]. Normally plants have always been a primary source of food and medicine since time immemorial. Medicinal plants have always remained a primary source for treating common ailments and diseases in some parts of the world lacking basic healthcare facilities. Several allopathic drugs are either transformed or derived directlyfrom plant parts thus putting pressure on already depleting plant resources. Alternative source of some of the metabolites commonly derived from plants would eventually reduce our dependence on plant-based bio-resources. The herbal medicines derived from plants have been well documented since ancient civilizations of India, Egypt, China, Central Asia, Greece, etc. These civilizations, over several centuries, have played a

considerable role in exploring and reporting beneficial properties of diverse group of plant species [2–4]. Medicinal plants and their derivatives remain a major source of medicine for regular ailments in developing countries as they are reasonably priced and easily accessible [5]. Last few decades have again received a considerable interest towards the search for unique metabolites from natural sources [6]. Several components of drugs are still derived directly from plant parts while few others are transformed from the molecules obtained from various plants. Even after exploring for natural compounds all these years, plants continue to hold treasure house of unknown metabolites [7]. Demand for Ayurvedic and Chinese herbal medicines are very high due to inadequate facilities for allopathic treatment and poor healthcare system in these regions [8]. About 70% of people across the globe continue to rely on herbal medicines as remedies and for treating numerous diseases [9]. There is a considerable growth in consumption of medicines derived from plants even in Western and European countries [10]. Herbal products occupy fair share in overall drug market across the globe which will continue to grow steadily [11,12]. Medicinal plants continue to hold a significant place in various therapeutics and health care systems leading to massive demand for plant-based bio-resources [13].

2. Characteristics of endophytes

The microorganisms such as fungi, bacteria including actinomycetes and viruses that reside within plant tissues are known as endophytes [14]. The endophytes have been classified as true endophytes or transient endophytes depending upon their diversity, biological nature, classification and method of transmissions [15]. Endophytes were further classified by Rodriguez et. al. into clavicipitaceous (class 1) and non-clavicipitaceous (classes 2, 3 & 4) based upon the narrow or broad range of hosts, types of tissues colonized, pattern of colonization in plants that is either extensive or limited, in planta bio-diversity that could be high, low or unknown, vertical or horizontal types of transmission through different generations and habitat or non-habitat adapted fitness benefits. Tolerance to drought conditions and enhancement of growth are common non-habitat adapted benefits, irrespective of origin of habitat, whereas benefits of habitat adapted are specific to the habitat with selective pressures that include salinity, pH and temperatures [16]. Considerable attention in the extensive investigation of beneficial microorganisms from the plant tissues fully demonstrate their unique abilities to produce secondary metabolites of the host plant and collection of functionalities (Figure 1) with their possible applications in agriculture, pharmaceutical and industrial sectors [17–20]. Importance of endophytes came into light only after the demonstration of toxic syndrome in cattle caused by endophytes of pasture grasses [21,22].

Endophytes are abundant in nature and have been found in all those plant species that have been studied so far. These microorganisms share an obligate or facultative relationship with the plant while causing no harm to their host [23]. Endophytes have characteristic of producing bioactive compounds, as they have been isolated from the tissues of roots, leaves and stems of their host plant, which produce similar metabolites [24,25]. Identification of fungal endophytes has been carried out by studying the morphological characteristics after sporulation. However, classification of non-sporulating fungi is problematic and it is carried out through phylogenetic analyses of rDNA-ITS sequences after the amplification of DNA extracted from the fungal mycelia [26,27]. Similarly, phylogenetic analyses of the 16s sequences obtained after the amplification of rDNA would help to identify the bacterial endophytes [28].

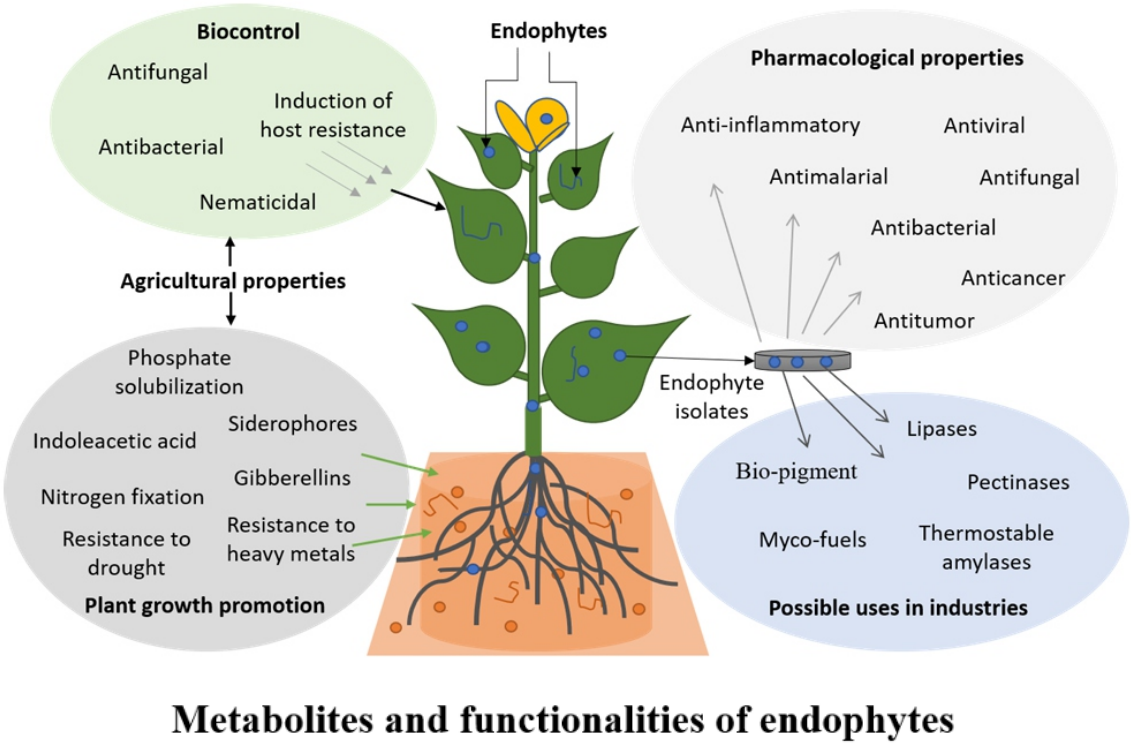

Figure 1. Possible applications of metabolites and functionalities derived from endophytes in different sectors.

3. Metabolites and activities of endophytes

Microbial endophytes are well-known for their ability to produce a wide range of pharmacologically important compounds with enormous therapeutic potentials; which have been identified as antiviral, antifungal, antibacterial, antitumor and anticancer agents. A number of endophytes are prospective source of plant growth promoting factors, and plant hormones. They can synthesize compounds of applications in the field of agriculture, iron chelating agents, compounds with nematocidal, insecticidal activities and abiotic stress tolerant properties. Some endophytes have shown their ability to secrete wide range of extracellular enzymes, such as phosphatase enzyme to convert insoluble phosphates to soluble form for its easy assimilation by plants. Endophytes produce molecules suitable for the production of bio-fuels and degrade complex organic and inorganic substances with suitable use in industrial sectors. The useful properties of endophytes are listed below with their potential significance in respective sectors.

3.1. Potential significance of endophytes with respect to agriculture

Published work state that endophytes are definitely an excellent source of metabolites and desired functions that could prove to be beneficial in organic farming system. Some of the endophytes could be used as bio-pesticides against phytopathogens due to their antimicrobial, nematocidal and insecticidal qualities.

3.1.1. Pesticidal properties of endophytes

The extracts from a perennial grass native to most of Europe Phleum pratense, demonstrated myco-toxic properties, which were secreted by a systemic grass symbiont fungal endophyte Epichloe typhina. The antifungal properties of the extracts were detected against Cladosporium herbarum [29]. A strain of fungus, L1930, obtained from Larix laricina displayed insecticidal property against larvae of spruce budworm. Chitinase, known to degrade chitin polymers that are essential part of a fungal cell wall, was produced by bacteria, an endophyte of Sinapis arvensis. The bacterial endophyte was identified as Bacillus cereus strain [30] and was known to play a defensive role against a phytopathogen Rhizoctonia solani [31]. Strain of Neotyphodium sp. (AR601) producing large quantities of alkaloids such as loline and ovaline inoculated into a cultivar 'Jackal' of turf tall fescue have shown birds deterring ability [32]. Several endophytes have consistently shown to induce effective resistance in plants against common phytopathogens, by producing proteins related to pathogenesis. Fungal endophytes found from the leaves of trees typically growing in Indian states of Western Ghats, and Tamil Nadu, were able to secrete chitinase and chitosanase, which could increase defenses in host plant against phytopathogens, by initiating host defenses and increasing resistance [33,34].

3.1.2. Plant growth promotion by endophytes

Endophytes have been identified to solubilize phosphates, produce siderophores, secrete plant growth promoting factors and increase soil nutrition by degrading complex organic molecules. It was observed that ericoid plants were able to thrive in extreme conditions due to the presence of an endophyte, Hymenoscyphus ericae, that produced several enzymes along with phosphate solubilization properties [35]. Lu et al isolated Colletotrichum sp. B501 from the healthy stems of Artemisia annua L, secreted IAA and 3β-hydroxy-ergosta-5-ene, these compounds that showed properties for plant growth [36]. The production of a range of factors and plant hormones have been reported from both fungal and bacterial endophytes [37–41]. Some endophytes have been found to increase tolerance of plants in soils contaminated with heavy metals [39,42,43]. In vitro investigation of endophyte-plant interaction in Echinacea purpurea demonstrated that colonization potential of bacterial strains belonging to Pseudomonas and Arthrobacter genus were tissue specific in host plants from which they were originally obtained but did not show similar specificity in non-host plants [44]. Further, plant growth promotion (PGP) was observed in inoculated plants due to the secretion of Indoleacetic acid by endophytic bacteria. Physiology of plants were influenced by compounds secreted by endophytes and plant metabolites, in turn, they regulated the growth of endophytes. Similarly, endophytic strains of Bacillus sp. isolated from Thymus vulgaris demonstrated plant growth promoting traits in Solanum lycopersicum L under salt stress along with showing antagonistic activity against Fusarium oxysporum and reduced the antioxidant stress on plants [45]. Antagonistic properties against human pathogens were observed in cultivable bacteria obtained from different segments viz. roots, stem, leaf and flower of Origanum vulgare L [46]. Pseudomonas and Bacillus were the most represented genera of endophytes in Lavandula dentata that demonstrated multiple PGP traits [47]. All these aspects make these endophytes a potential source of bio-fertilizer, bio-pesticide, plant growth promoter and maintain overall growth and development of the plants.

Some of the bacterial and fungal endophytes and their potential applications in agriculture are listed in Table 1 (1.1 and 1.2).

3.2. Potential significance of endophytes with respect to pharmaceuticals

Products derived from natural sources are a major area of research for discovering the range of their functions, that could be used in pharmaceutical industries [66,67]. Microorganism from different biotypes have repeatedly proven to be a constant source of secondary metabolites with novel and unique properties, which have found a major place in medical sector [68]. Since the discovery of endophytes and their ability to produce plant secondary metabolites and other bioactive compounds, several reports are available on mining of novel secondary metabolites [69,70]. Different saponins showing antagonism were extracted from Fusarium sp. PN8 isolated from Panax notogensing [71].

3.2.1. Antimicrobial properties of endophytes

Some species of endophytes are known to produce antimicrobial compounds. Phomopsichalasin (**11**) an antimicrobial agent was extracted from Phomopsis sp., isolate no. MF6031 obtained from the twigs of Salix gracilostyla var. melanostachys. The compound **11** exhibited antibacterial activity against Bacillus subtilis, Salmonella gallinarium and Staphylococcus aureus with some amount of antagonism towards Candida tropicalis [72]. Findlay et al isolated an endophytic fungus from the needles of Larix laricina (Du Roi) K. Koch [53]. which produced 6-oxo-2-propenyl-3, 6-dihydro2H-pyran-3-yl ester (**12**) showing antibacterial activity against Vibrio salmonicida, S. aureus and Pseudomonas aeruginosa.

In another study, a Colletotrichum sp. isolated from internal stem tissues of Artemisia annua L. showed antifungal, antibacterial and fungistatic properties. Its metabolites 6-isoprenylindole-3 carboxylic acid (**19**), 3β,5α-dihydroxy-6β-phenylacetyloxy-ergosta-7,22-diene (**21**), 3β-hydroxyergosta-5-ene (**15**), 3-oxo-ergosta-4,6,8(14),22-tetraene (**16**), nd 3β,5α-dihydroxy-6β-acetoxyergosta-7,22-diene (**20**) showed antibacterial activity against Gram-positive and Gram-negative bacteria. Compounds **15**,**19** and **21** had antifungal properties, compound **15**,**19** and **20** demonstrated fungistatic property [36]. Several other researchers have also studied endophytes possessing antimicrobial properties [71,73–77].

Table 1.1 Bacterial endophytes with potential significance in agriculture sector.

Sl. No.	Functionalities	Endophytes	Properties	Host plant	Ref
1.	Chitinase	*Bacillus cereus* strain 65	Antifungal	*Sinapis arvensis* L.	[31]
2.	Jasmonates, Abscisic acid and phosphate solubilization	*Bacillus* sp., *Achromobacter* sp., *Alcaligenes* sp.	Plant growth and development	*Helianthus annuus* L.	[38]
3.	Leu-surfactin (**8**)	*Bacillus mojavensis* RRC 101	Biocontrol of *Fusarium verticillioides*	*Bacopa monnieri* L.	[48]
4.	Nitrogen fixation	*Rhizobium leguminosarum*	Biofertilization, increase rice yield.	*Oryza sativa* L.	[40]
5.	Phosphatases, Siderophore, Nitrogen fixation	*Rahnella* sp. and *Pseudomonas* sp.	Bio-fertilization	*Musa* L.	[49]
6.	Siderophore	*Streptomyces* sp. GMKU 3100	Promote plant growth	*Oryza sativa* L	[50]
7.	Plant growth promoting factors	*Enterobacter* sp. FD17	Enhancement of maize yield	*Zea mays* L.	[51]
8.	IAA, Siderophore, Phosphate solubilization	*Serratia* sp., *Enterobacter* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Stenotrophomonas* sp., *Agrobacterium* sp., *Ochrobactrum* sp., *Bacillus* sp. and *Tetrathiobacter* sp.	Plant growth promotion in *Zea mays*.	*Zingiber officinale* Roscoe	[41]

Table 1.2. Fungal endophytes with potential significance in agriculture sector.

Sl. No.	Functionalities	Endophytes	Properties	Host plant	Ref
Clavicipitaceous					
1.	Ethyl trans-9.10-epoxy-ll-oxoundecanoate (**1**), Ethyl 9-oxononanoate (**2**), Ethyl azelate (**3**), Hydroxydihydrobovolide (**4**).	*Epichloe typhina*	Antifungal	*Phleum pratense* L.	[52]
2.	8,1',5'-trihydroxy-3',4' dihydro-1'H-[2,4']binapthalenyl-1,4,2'-trione (**5**)	Fungus L1930 (unidentified)	Insecticide	*Larix laricina* (Du Roi) K. Koch	[53]
3.	Phosphatase, Protease, Cellulase, Hemicellulases, Pectinolytic enzymes, Ligninase	*Hymenoscyphus ericae*	Phosphate solubilization, Protein breakdown, Cell wall lysis.	Ericoid plants	[35]
4.	Indole-3-acetic acid (IAA) and 3β-hydroxy-ergosta-5-ene (**6**)	*Colletotrichum* sp. B501	Plant growth hormone	*Artemisia annua* L.	[36]
5.	Phosphate solubilization	*Penicillium* sp.	Bio-fertilization	*Triticum aestivum* L.	[54]
6.	3- Hydroxypropionic acid (**7**)	*Phomopsis phaseoli and Melanconium betulinum strains*	Nematicidal	*Broad leaved tree of tropical rainforest, Betula pendula Roth. And Betula pubescens Ehrh.*	[55]
7.	Volatile organic compounds	*Muscodor albus*	Mycofumigation	*Cinnamomum zeylanicum* Blume	[56]
8.	Protease amylase, lipase, laccase, cellulase and pectinase.	Various fungal species	Enhance resistance of grasses to multiple stresses.	*Catharanthus roseus* L. (G. Don.), *Calophyllum inophyllum* L., *Bixa orellana* L., and *Alpinia calcarata.* Roscoe	[57]
9.	Gibberellins	*Penicillium* sp. M5.A and *Aspergillus* sp. M1.5	Promote plant growth and development.	*Monochoria vaginalis* (Burm.f.) C. Presl ex Kunth	[37]

Continued on next page

Sl. No.	Functionalities	Endophytes	Properties	Host plant	Ref
Clavicipitaceous					
10.	Siderophore	*Phaeotheca* sp. *Fusarium sp.*, *Penicillium* sp. and *Arthrinium sp.*	Antibacterial	*Pinus sylvestris* L. and Rhododendron *tomentosum* Harmaja	[58]
11.	1,8-cineole (monoterpene) (**9**)	*Hypoxylon* sp.	Antimicrobial	*Persea indica* (L.) Spreng.	[59]
12.	Chitosanase, chitinase.	*Xylariaceae* sp., *Aureobasidium pullulans*, *Colletotrichum* sp., *Lasiodiplodia theobromae*, *Phomopsis* sp. and *Fusarium* sp., Botrytis sp., *Trichoderma* sp., *Alternaria* sp., *Nodulisporium gregarium*, Nigrospora *oryzae*, *Drechslera* sp., *Pithomyces* sp. *Sordaria* sp. and *Pestalotiopsis* sp.	Pathogenesis related proteins, phytoalexins and proteinase inhibitors in plants. Acts against phytophagous nematodes and plant pathogenic fungi.	Leaves of different tree species of Western Ghats.	[33]
13.	Phosphate solubilization	*Penicillium* sp.	Bio-fertilization	*Camellia sinensis* (L.) Kuntze	[60]
14.	Gibberellins and Indole acetic Acid	*Penicillium* sp. LWL3 and *Phoma glomerata* LWL2	Promote plant growth	*Cucumis sativus* L.	[61]
15.	Plant growth promoting factors	*Phoma* sp.	Bio-fertilizatzion	*Tinospora cordifolia* (Thunb.) Miers and *Calotropis procera* (Aiton) W.T. Aiton	[62]
16.	Trichodemin	*Trichoderma brevicompactum*	Antifungal against phytopathogens	*Allium sativum* L.	[63]

Continued on next page

Sl. No.	Functionalities	Endophytes	Properties	Host plant	Ref
Clavicipitaceous					
17.	Indole acetic acid, Gibberellins and Reactive oxygen species.	*Galactomyces geotrichum* WLL1	Promote growth of plants in heavy metal contaminated soil.	*Trapa japonica* Flerov	[42]
18.	Not identified (ethyl acetate extract)	*Aspergillus* sp. and *Emericella* sp.	Insecticidal properties	*Rhizophora mucronata* Lam.	[64]
19.	Plant Growth promotion and Resistance to heavy metals	*Phialocephala fortinii*, *Rhizodermea veluwensis*, and *Rhizoscyphus* sp.	Growth enhancement, Nutrient uptake, Decrease Heavy metal concentration	*Clethra barbinervis* Sieb. Et Zucc.	[43]
20.	Not identified (ethyl acetate extract)	Several fungal isolates belonging to Ascomycota and few Zygomycota.	Antifungal properties against root rot pathogens.	*Panax notoginseng* (Burkill) F. H. Chen ex C. Y. Wu & K. M. Feng	[34]
Non clavicipitaceous					
21.	Indole Acetic Acid (IAA)	*Rhodotorula* sp. and *Rhodosporidium* sp.	Plant growth	*Populus* L.	[65]
22.	Plant growth promoting factors and reduce cadmium toxicity	*Piriformospora indica*	Enhance plant growth in cadmium toxic soil.	*Triticum aestivum* L.	[39]

3.2.2. Other medicinal properties of endophytes

Few endophytes show the medicinal properties as anticancer and antitumor in their metabolites. An endophyte isolated from Taxus brevifolia Nutt., Taxomyces andreanae, was able to produce Taxol, the host secondary metabolite, in a broth culture medium [78]. Similarly, different metabolites with anticancer properties were obtained from the microbial species isolated from different plant species [79,80]. A metabolite, Hypericin, with anti-viral, antimicrobial and anti- inflammatory properties was produced from a microbial strain isolated from Hypericum perforatum L. [81]. Lovastatin was produced in significant amount by an endophyte Phomopsis vexans isolated from Solanum virginianum L. [82]. The secondary metabolites and other functions from endophytes could have potential applications in therapeutics without causing damage to the respective plant species. The bacterial and fungal endophytes suitable for therapeutic purposes are listed in Table 2 (2.1 and 2.2).

Table 2.1. Bacterial endophytes with potential significance in therapeutic sector.

Sl. No.	Functionalities/ Metabolites/Compounds	Endophytes	Properties	Host plant	Ref
1.	Xiamycin (**62**), methyl ester of Xiamycin (**63**)	*Streptomyce* sp. GT2002/1503	Antiviral	*Bruguiera gymnorrhiza* (L.) Savigny	[83]
2	Agarwood	*Bacillus pumilus.*	Antimicrobial, Laxative, sedative, digestive, etc.	*Aquilaria* species	[84]

Table 2.2. Fungal endophytes with potential significance in therapeutic sector.

Sl. No.	Functionalities/Metabolites/Compounds	Endophytes	Properties	Host plant	Ref
Clavicipitaceous					
1.	Taxol (**10**)	*Taxomyces andreanae*	Antitumor	*Taxus brevifolia* Nutt.	[78]
2.	Phomopsichalasin (**11**)	*Phomopsis* sp. isolate no. MF6031	Antimicrobial	*Salix gracilostyla* var. melanostachys	[72]
3.	Cryptocandin (**13**)	*Cryptosporiopsis quercina*	Antimycotic	*Tvipterigeum wilfordii* Hook. f.	[85]
4.	3β,5α,6β-trihydroxyergosta-7,22-diene (**14**), 3β-hydroxy-ergosta-5-ene (**15**), 3-oxo-ergosta-4,6,8(14),22-tetraene (**16**), 3β-hydroxy-5α,8α-epidioxy-ergosta-6,22-diene (**17**), 3β-hydroxy-5α,8α-epidioxy-ergosta-6,9(11),22-triene, 3-oxo-ergosta-4-ene (**18**), 6-isoprenylindole-3-carboxylic acid (**19**), 3β,5α-dihydroxy-6β-acetoxy-ergosta-7,22-diene (**20**) and 3β,5α-dihydroxy-6β-phenylacetyloxy-ergosta-7,22-diene (**21**).	*Colletotrichum* sp.	Antibacterial, antifungal and fungistatic.	*Artemisia annua* L.	[36]
5.	7-butyl-6,8-dihydroxy- 3(R)-pent-11-enylisochroman-1-one (**22**), 7-but-15-enyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one (**23**), 7-butyl-6,8-dihydroxy-3(R)-pentylisochroman-1-one (**24**)	*Geotrichum* sp. Ccre7	Antifungal, antituberculous and antimalarial	*Crassocephalum crepidioides* (Benth.) S. Moore	[74]
6.	Asperfumoid (**25**), Asperfumin (**26**), Monomethylsulochrin (**27**), Fumigaclavine C (**28**), Fumitremorgin C (**29**), Physcion (**30**), Helvolic acid (**31**), 5α,8α-epidioxy-ergosta-6,22-diene-3β-ol (**32**), Ergosta-4,22-diene-3β-ol (**33**), Ergosterol (**34**), *Cyclo* (Ala-Leu) (**35**) and *Cyclo* (Ala-Ile) (**36**).	*Aspergillus fumigates* CY018	Antimycotic	*Cynodon dactylon* (L.) Pers.	[73]

Sl. No.	Functionalities/Metabolites/Compounds	Endophytes	Properties	Host plant	Ref
Clavicipitaceous					
7.	Brefeldin A (**37**)	*Cladosporium* sp.	Antimicrobial	*Quercus variabilis* Blume	[75]
8.	Ampelopyrone (**38**), macrosporin (**39**), 3-O-methylalaternin (**40**), methyltriacetic lactone (**41**), citreoisocoumarin, macrosporin (**42**), 3-O-methylalaternin (**43**), desmethyldiaportino (**44**), desmethyldichlorodiaportin (**45**), ampelanol (**46**), altersolanol A (**47**), alterporriols D (**48**), alterporriols E (**49**) and altersolanol J (**50**).	*Ampelomyces* sp.	Cytotoxic and antimicrobial	*Urospermum picroides* (L.) Scop. ex F.W. Schmidt	[86]
9.	Paclitaxel (**51**)	*Fusarium solani*	Anticancer	*Taxus celebica* (Warb.) H. L. Li	[79]
10.	Usnic acid (**52**), Cercosporamide (**53**), Phomodione (**54**).	*Phoma* sp. isolate No. 2323	Antibacterial	*Saurauia scaberrinae* Hemsley	[77]
11.	Phomopsin A (**55**), Phomopsin B (**56**), Phomopsin C (**57**), Cytosporone B (**58**), Cytosporone C (**59**)	*Phomopsis* sp. ZSU-H76	Antifungal	*Excoecaria agallocha* L.	[87]
12.	Not identified	*Fusarium* sp. DF2	Antimicrobial	*Taxus wallichiana* Zucc.	[88]
13.	Deoxypodophyllotoxin (**61**)	*Aspergillus fumigatus* Fresenius	Anticancer	*Juniperus communis* L. Horstmann	[80]
14.	Benquinol (**64**), Benquoine (**65**)	*Phomopsis* sp. CMU-LMA	Antibacterial and cytotoxic	*Alpinia malaccensis* (Burm. f.) Roscoe	[89]
15.	Terpene (**66**)	*Phomopsis* sp.	Antibacterial	*Allamanda cathartica* L.	[90]
16.	8-octadecanone (**67**), 1-tetradecene (**68**), 8-pentadecanone (**69**), octylcyclohexane (**70**) and 10-nonadecanone (**71**).	*Fusarium solani*	Antimicrobial	*Taxus baccata* L.	[91]
17.	Emerimidine A (**72**), Emerimidine B (**73**), Emeriphenolicins A (**74**), Emeriphenolicins D (**75**), Aspernidine A (**76**), Aspernidine B (**77**), Austin (**78**), Austinol (**79**), Dehydroaustin (**80**), and Acetoxydehydroaustin (**81**)	*Emericella* sp. (HK-ZJ)	Antiviral	*Aegiceras corniculatum* (L.) Blanco	[92]

Continued on next page

Sl. No.	Functionalities/Metabolites/Compounds	Endophytes	Properties	Host plant	Ref
Clavicipitaceous					
18.	Guignardin A (**82**), Guignardin B (**83**), Guignardin C (**84**), Guignardin D (**85**), Guignardin E (**86**), Guignardin F (**87**), Palmarumycin C1 (**88**), BG1 (**89**) and JC1(**90**).	*Guignardia* sp. KcF8	Antimicrobial, Cytotoxic, Protein inhibitor	*Kandelia candel* (L.) Druce	[93]
19.	Lovastatin (**91**)	*Phomopsis vexans*	Lower blood cholesterol	*Solanum xanthocarpum*	[82]
20.	Unknown	*Luteibacter* sp. NORREL-Li2	Bio convert major ginsenosides into minor ginsenoside	*Platycodong randiflorum* (Jacq.) A. DC.	[94]
21	Saponins	*Fusarium* sp. PN8 and *Aspergillus* sp. PN17	Antimicrobial	*Panax notoginseng* (Burkill) F. H. Chen ex C. Y. Wu & K. M. Feng	[71]
Not identified					
22.	Protocatechuic acid (**92**) and acropyrone (**93**).	Fungal endophyte	Antibacterial	*Citrus jambhiri* Lush.	[76]
23.	Hypericin (**60**)	INFU/Hp/KF/34B	Antibiotic, antiviral, anti-inflammatory, seasonal effective disorder, relief from sinusitis	*Hypericum perforatum* L.	[81]
24.	6-oxo-2-propenyl-3,6-dihydro-2H-pyran-3-yl ester (**12**)	L1930 (unidentified)	Antimicrobial	*Larix laricina* (Du Roi) K. Koch	[53]

Table 3.1. Bacterial endophytes with potential significance in industrial sectors.

Sl. No.	Functionalities	Endophyte	Properties	Host plant	Ref
1.	Pectinase	*Paenibacillus amylolyticus*	Pectin lyase	*Coffea Arabica* L.	[95]
2.	Thermostable α-amylase	*Nocardiopsis* sp.	Starch degradation	*Pachyrhizus erosus* L.	[96]
3.	Thermostable glucoamylase	*Streptosporangium* sp.	Starch degradation	*Zea mays L.*	[97]
4.	Protease	*Bacillus halotolerans* strain CT2	Alkaline protease	*Solanum tuberosum* L.	[98]

Table 3.2 Fungal endophytes with potential significance in industrial sectors.

Sl. No.	Functionalities	Endophyte	Properties	Host plant	Ref
Clavicipitaceous					
1.	Amylase, cellulase, xylanase and ligninase.	*Fusarium* sp., *Phomopsis* sp. *Phoma* sp., *Colletotrichum* sp.,	Wood degradation	*Brucea javanica* (L.) Merr.	[99]
2.	Microbial oil and cellulase	*Phomopsis, Cephalosporium, Microsphaeropsis, and Nigrospora.*	Production of bio-fuel	*Taxus chinensis* var. mairei Mast, *Cupressus torulosa* D. Don, *Keteleeria davidiana* varchienpeii, *Sabina chinensis* cv. Kaizuca and *Keteleeria evelyniana* Mast.	[100]
3.	Myco-diesel	*Gliocladium roseum* (NRRL 50072)	Energy production and utilization	*Eucryphia cordifolia* Cav.	[101]
4.	1,4-Cyclohexadi-ene (**94**)	*Hypoxylon* sp.	Oxidizes to benzene (component of crude oil)	*Persea indica* (L.) Spreng.	[59]
5.	Polyurethanases	*Pestalotiopsis microspora* E2712A	Degrade polyester polyurethane	Ecuadorian Amazonian plant	[102]
6.	Lipase	*Candida guillermondi*	Synthesis of methyl oleate	*Ricinus communis* L.	[103]

Continued on next page

Sl. No.	Functionalities	Endophyte	Properties	Host plant	Ref
Clavicipitaceous					
7.	Amylase	*Alternaria* sp., Phoma sp., *Nigrospora* sp.	Starch hydrolysis at alkaline pH and low temperature	*Eremophilia longifolia* (R.Br.) F. Muell.	[104]
8.	Bio-pigment	*Phoma* sp.	Bio-pigment production	*Clerodendrum viscosum* L.	[105]
9.	Xylanases	*Trichoderma harzianum*	Xylan degrading enzyme	*Sargassum wightii*	[106]
10.	Laccase	*Hormonema* sp. and *Pringsheimia smilacis*	Degrade Lignin	*Eucalyptus globules* Labill.	[107]
11.	Cellulase and Xylanase	*Acremonium* sp. *Aspergillus* sp.	Degrade cellulose and Xylan	*Memecylon excelsum* Blume, *Glochidion borneese* Mull. Arg.) Boerl.	[108]

Non clavicipitaceous					
12.	Lignocellulolytic enzymes	*Bjerkandera sp.*	Wood degradation	*Drimys winteri* J. R. Forst. & G. Forst. and *Prumnopitys andina* (Poepp. ex Endl.) de Laub.	[109]
13.	Microbial oil and cellulase	*Sclerocystis*	Production of bio-fuel	*Taxus chinensis* var. mairei Mast, *Cupressus torulosa* D. Don, *Keteleeria davidiana* varchienpeii, *Sabina chinensis* cv. Kaizuca and *Keteleeria evelyniana* Mast.	[100]

3.3. Potential significance of endophytes in industries

Microorganisms and their derivatives play a significant role in processing of substrate into several products for use in industrial sectors. There are many reports of enzymes being produced by endophytes isolated from different plant species. Enzymes like amylase, pectinase and lipases obtained from different endophytes have been known to hydrolyze starch, pectin and oils, respectively. Other enzymes include cellulases, xylanases, amylase, laccase, and proteases, which have application in various industrial sectors [98,104,106,107]. An endophyte, Nocardiopsis sp.,isolated from Pachyrhizus erosus L., was found to secrete a thermostable α-amylase, which is useful for starch degradation [96]. Similarly, Candida guillermondii from Ricinus communis L. produce lipase and helps in the synthesis of methyl oleate [103].

Some endophytes are also known to produce bio-fuels, as alternate source of conventional fuels. A fungal isolate, Hypoxylon sp. from Persea indica (L.) Spreng. was found to secrete 1,4Cyclohexadi-ene (**94**). The compound **94** readily oxidizes to benzene, which is a main component of crude oil [59]. In another work, an endophyte Gliocladium roseum (NRRL 50072) isolated from Eucryphia cordifolia Cav. produced a bio-fuel known as myco-diesel [101]. While some of the endophytes are known to degrade polyurethane which are of great value to the industrial sector [102], others are known to produce pigment suitable for use in food industry [105]. Bacterial and fungal endophytes with their ability to produce bioactive compounds with their potential applications in industries are listed in Table 3 (3.1 and 3.2).

3.4. Understating the potential of endophytes through genome mining

Some microorganisms are known to synthesize only a limited number of secondary metabolites (SMs) as compared to the ones estimated through genome mining [110]. SMs are synthesized through pathways that utilize multiple enzymes. Biosynthetic gene cluster (BGC) comprises set of genes that encode for proteins required during a pathway. Diverse methods could be employed to activate the cluster of genes that remain silent under in vitro conditions. The genome mining approach could reduce the time taken to identify the putative genes required for the synthesis of secondary metabolites [110].

The sequencing of genes have helped in the identification of genes related to SMs and enhanced the characterization process [111]. Nielsen and Nielsen have suggested three approaches for understanding the unknown BGCs that include targeted approach: where the similar BGCs are compared to form a probable BGCs, untargeted approach involves the use of different databases to mine for information and lastly through the use of metabolomics techniques [112]. Wang et al. [113] developed bacteriophage recombinases to quickly identify and stimulate BGCs that are cryptic in strains of Burkholderia species. Poplar trees augmented with a modified strain of endophyte, Pseudomonas putida W619-TCE, showed increased reduction (90%) of trichloroethylene evapotranspiration under field tests [114].

4. Progress and developments

In an effort to meet the increasing demand of food and feeds, chemical fertilizers and pesticides have been commonly used in agricultural system for improving soil fertility and controlling pests, respectively. The adverse effect of use of toxic chemicals in agriculture has resulted in increasing interest in sustainable farming practices [115,116]. Biofertilizers and Biopesticides derived from microorganisms have been effective in dealing with phytopathogens as well as Biofertilization of the soil. Bacterial and fungal endophytes have shown positive effects in plant growth promotion, pest management and improving soil health [117,118]. Numerous endophytes have shown their ability to promote plant growth and antagonism against phytopathogens under in vitro conditions. Some of the strains have found their place in modern agricultural practices, such as perennial ryegrass (PRG) due to its endophyte, Neotyphodium lolii, was able to protect the host plant from Argentine stem weevil infection without producing any toxic compounds harmful to livestock. A product of Rye grass, Ar1, infected with the endophyte has been beneficial for livestock production in places with lesser number of black beetles. Another strain of PRG, Endosafe, has shown better survival response in places dominated with black beetles but with decreased biomass production compared to AR1 [119].

Adaptive Symbiotic Technologies in Seattle, USA, have developed several products under the brand name BioEnsure® using a combination of beneficial endophytes. The products are able to induce tolerance in crops to drought, high salt concentration and temperature; it improves water utilization by plants and is fairly stable in different climate and soil types. The microbial formulations can easily be applied to fields along with other agriculture inputs and are noncompetitive against other normal microbial flora of the soil. The products have a viability of more than two years at 4 °C. BioEnsure® products not genetically modified are classified as organic products by Organic Material Review Institute, Eugene, USA [120]. Muscodor albus isolated from cinnamon tree, has shown properties related to bio-fumigation and it may replace the use of methyl bromide for fumigation of soils [121]. Though the effects of endophytes cannot substitute chemical inputs altogether, combination of different methods and suitable endophyte-plant combinations could be considered for integrated pest management programs [122].

5. Constrains in commercialization of endophytes

There have been numerous reports on the production of plant secondary metabolites by endophytes outside its host but there are no products as such that have successfully been produced in mass scale and commercialized. Production of Taxol by endophytic fungus in the early 90s was thought to develop the process of obtaining metabolites from endophytes with eventual decrease in over-use of plants. However, apart from the use of few endophytes in agricultural system, not a single product from

endophytes has made it to the market with a significant advance in secondary metabolite industry [123]. The reason for the production of host metabolites by the endophytes could be hidden in their genes that must have undergone genetic recombination during the time of their evolution [124]. Our inability to understand the mechanisms by which these endophytes function inside the host, and as stated by Bailey et al their evolutionary significance [125] has limited our knowledge. Some of the constraints involved in the production of secondary metabolites under laboratory conditions include: the low-yield of secondary metabolites, optimization of growth-conditions involving variety of abiotic factors and silent gene clusters, synthesis of metabolites with unidentified functions, unclear understanding of pathways involved in the production of metabolites, role of secondary metabolites in different pathways and lack of a complete knowledge on secondary metabolites [110]. The cellular relationship between the host and its endophyte limits our ability to understand the mechanism of host-secondary metabolite production by an endophyte, and the eventual reduction in synthesis when outside its host in vitro system.

6. Future perspectives

Endophytic microorganisms have convincingly demonstrated their remarkable ability to typically produce an abundance of pharmacological metabolites with possible usage in drug manufacturing. Extensive search for newer metabolites is important to deal with multi-drug resistant microorganisms and to find alternative therapeutic drugs for several diseases. Secretion of plant growth-promoting factors and antagonistic agents against phytopathogens could easily substitute chemical inputs in sustainable agriculture practices with suitable endophytes. Novel enzymes with better specific-activity obtained from endophytes could be valuable in fermentation industries. However, most of the published findings are from controlled experiments and similar results from in vivo trials could satisfactorily establish the practical possibility of endophytes commercialization. Specific mechanisms involved in the complex interactions, types of selection pressures that properly govern the crosstalk between endophytes and their suitable host, efficient production of host secondary metabolites, and possible ways to effectively manipulate the biochemical-pathways, would undoubtedly require comprehensive understanding before the successful commercialization of bioactive metabolites from endophytes.

7. Conclusions

It is estimated that there are more than quarter million species of plants in this planet with a possibility of obtaining more than one million endophytes from these plants. Very few of these microorganisms from their diverse group have been isolated and studied so far. Apart from producing array of metabolites and functions advantageous to its host plant, these microbial resources have proven to secrete similar secondary metabolites even outside its host using in vitro systems. These properties of endophytes not only make them suitable candidates for exploring their ability to produce various bioactive compounds, enzymes, and biopigments, etc., but it may also reduce the dependency of humans on endangered plant species for their secondary metabolites, thus resulting in sustainable use of plant-based bio-resources. The necessary factors controlling growth of endophytes for biosynthesis of host secondary metabolites in vitro, are required to be optimised for commercial-scale production of plant-derived natural compounds employing these endophytes.

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Conflict of interest

All authors declare no conflicts of interest in this review article.

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Plasmid-mediated quinolone resistance genes transfer among enteric bacteria isolated from human and animal sources

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ABSTRACT

This research investigates the transferability of plasmid-mediated quinolone resistance (PMQR) genes among enteric bacteria isolates in human and animal samples, as well as its implication on resistance of recipient cells. A total of 1,964 strains of five different enteric bacteria species (Escherichia coli, Salmonella sp., Shigella sp., Klebsiella sp. and Aeromonas sp.) were screened for plasmid-mediated quinolone resistance (PMQR) genes from a population of quinolone resistant (Q-r) isolates. Screening for PMQR isolates was achieved by plasmid curing using sub-lethal concentration of Sodium Dodecyl Sulphate and PMQR genes (qnrA, qnrB, qnrS, Aac(6')-Ib-crand Qep A) were detected by polymerase chain reaction (PCR). Conjugation and transformation experiments were attempted to ascertain transfer of genes from the Q-r isolates to a susceptible, standard recipient, E. coli J53-2. The minimum inhibitory concentration (MIC) was determined before and after gene transfer, using E-test strips. Results indicate that percentage resistance to the quinolones (Qs): Nalidixic acid, Ciprofloxacin, Pefloxacin and Ofloxacin determined by agar plate diffusion technique stood at 52.6, 47.3, 50.5, 70.6 and 46.0% for Escherichia coli, Salmonella sp., Shigellasp., Klebsiella sp. and Aeromonas sp. respectively. Analysis of variance indicated the occurrence of significant differences (F, 46.77–613.30; 0.00) in the resistance to each tested Qs. Generally, Human isolates showed greater resistance than Animal isolates (57.4 vs 47.2%). Investigation with specific primers indicated 11, 15, 7, 1 and 0 for qnrA, qnrB, qnrS, qepA and Aac(6')Ib-cr genes respectively, out of 1018 Q-r and 29 PMQR isolates. Gene transfer experiments indicated the transfer of all genes except qepA either by conjugation or transformation. The MIC of tested Qs on recipient bacterium before gene transfer greatly increased from 0.0625 to 0.25 µg/mL, after transfer. This study demonstrates that PMQR genes amongst enteric bacteria in the Niger delta of Nigeria were transferable and transfer conferred a higher Q- resistance on recipient bacterium.

Keywords: enteric bacteria; quinolones; gene transfer; minimum inhibitory concentration

1. Importance of medicinal plants

Pathogenic enteric bacteria are the major cause of food and water borne gastrointestinal illnesses in both humans and animals and remain a major public health problem the world over. Quinolones are the choice antibiotics for the treatment of various infections caused by pathogenic enteric bacteria [1]. However, widespread use of these antibiotics in human and veterinary medicine has led to the emergence of antibiotics resistance. Resistance to quinolones can lead to treatment failure and it is of public health

concern [2]. Bacteria acquire resistance to quinolones by either chromosomal mutation-which occurs relatively slowly, confers high–level resistance and transmitted vertically, or by acquisition of plasmids containing genes for quinolone resistance. Plasmid-mediated quinolone resistance (PMQR) confers low- level resistance, but can be transmitted horizontally among members of the same and different species making their spread much faster than that of chromosomal mutation [3]. Plasmid-mediated quinolone resistance genes have been found in variety of Enterobacteriaceae especially Escherichia coli, Enterobacter sp., Klebsiella sp. and Salmonella sp [4]. Resistance to nalidixic acid and ciprofloxacin was reported as extremely high in isolates from broilers in European countries [5]. Transfer of antibiotics resistance genes (ARGs) plays an important role in the development of multidrug resistance (MDR) in bacteria [6]. There are three mechanisms of horizontal gene transfer in bacteria: conjugation, transduction and transformation [7]. Different studies have demonstrated that resistance genes could be transferred from one bacterium to the other both in vitro and in vivo. Transfer of resistance genes between Shigella sp. and E.coli [8,9], in vitro intra and inter–species gene transfer of ampicillin resistance genes among enteric bacteria of diarrhea origin [10], transfer of tetracycline resistance determinants among bacteroids and other enteric bacteria in the human colon [11] have all being reported. PMQR provide a low-level of quinolone resistance not reaching the clinical breakpoints defined by the Clinical and Laboratory Standard Institute criteria [12].

However, PMQR is clinically important because it facilitate the selection of higher levels of resistance. The combination of plasmid-mediated resistance with chromosomally encoded resistance mechanisms of drug classes now result in strains that are resistant to all of the main classes of commonly used antimicrobial drugs. Enteric pathogenic bacteria are of particular interest because of their resistance to multiple antibiotics and their ability to transfer plasmids to other species and bacteria genera. It is therefore important to conduct a study on the transfer of plasmid mediated quinolone resistance gene among enteric bacteria isolated from human and animal sources. The aim of this study is to demonstrate the in vitro transferability of quinolone resistance determinants among enteric bacteria isolated from human and animal sources in Delta State, Nigeria where Self-medication and abuse of antibiotics are common,

2. Methods

2.1. Isolation of plasmid-mediated quinolone resistant enteric bacteria

A total of 1,964 strains of five different enteric bacteria species (Escherichia coli, Salmonella sp., Shigella sp., Klebsiella sp. and Aeromonas sp.) isolated from a previous study composed of 720 human and animal samples and comprises 180 diarrheal stools of patients attending public hospitals, 180 diarrheal stools of patients attending private hospitals, 180 poultry litters and 180 fish pond water samples collected from various hospitals patients, poultry and fish farms in major cities in Delta State, Nigeria. The isolates were screened for PMQR genes from a population of quinolone resistant (Q-r) isolates. The number of strains isolated from each source is shown in Table 1 [13]. Screening for PMQR isolates was achieved by plasmid curing using sub-lethal concentration of Sodium Dodecyl Sulphate and PMQR genes (qnrA, qnrB, qnrS, Aac(6')-Ib-cr and Qep A) were detected by PCR [13]. All bacteria strains were isolated between September 2017 and December 2018.

2.2. Transfer of Quinolone Resistance

Conjugation and transformation experiments were performed on all 29 PMQR isolates to examine the ability to horizontally transfer resistance to quinolones conferred by qnrA, qnrB, qnrS, aac(6')-Ib or qepA genes. Conjugation experiments involving the PMQR-positive isolates detected in this study were performed by the liquid mating assay [14]. Rifampicin resistant E. coli J53-2, azide-resistant Az ^{r} was used as the recipient strain and Luria-Bertani (LB) agar plates containing rifampicin 100 µg/mL, sodium azide 100 µg/mL, ciprofloxacin 20 µg, or nalidixic acid 20 µg/mL (Sigma-Aldrich LP, USA) were used for selection as required. Transformation experiments were equally performed for PMQR-positive isolates. Plasmid DNA was extracted from the donor strains and introduced into chemically competent E. coli J53-2Az^{r} (Invitrogen, USA).Transformants were selected on Luria-Bertani plates supplemented with sodium azide 100 µg, rifampicin 100 µg/mL and nalidixic acid 20 µg/mL for selection as required.

2.2.1 Conjugation experiment

The donor and recipient cells, both previously grown to exponential phase, were mixed in a ratio 1:9 (0.5 mL of the donor was added to 4.5 mL of the recipient in LB broth) and the incubation was continued at 37 °C, with very slow shaking (50 rpm). After 60 minutes, samples were withdrawn and diluted in 0.9% saline solution to 10^{-1}, 10^{-2}, 10^{-5}, and 10^{-6}. One hundred (100) µL aliquots from dilutions 10^{-1} and 10^{-2} were spread on LB agar plates selecting for transconjugants and 100 µL aliquots from dilutions 10^{-5} and 10^{-6} were spread on LB plates selecting for donors and on LB plates selecting for recipients. Plates were incubated at 37 °C over night and colonies were counted and thereafter, conjugation frequencies were calculated by dividing the number of transconjugants by the number of donors. Transconjugants were inoculated on LB agar plates containing ciprofloxacin/nalidixic acid and sodium azide/rifampicin, to screen for transconjugants that co-acquired FQ and sodium azide resistance.

2.2.2 Transformation experiment

For each transformation, one 50 µL vial of One Shot® E. coli J53-2 cells (Invitrogen, USA) was thawed on ice. Then, 2 µL of isolated plasmid was pipetted directly into the vial of competent cells and mixed by tapping gently. The vial was incubated on ice for 30 minutes. The vial was then incubated for 30 seconds at 42 °C in Eppendorf thermomixer device and 250 µL of pre-warmed S.O.C medium was added to the vial and subsequently incubated at 37 °C for 1 hour with shaking at 225 rpm. 50 µL from the transformation vial was spread on labeled LB agar plate supplied with the selecting antibiotics. The plate was incubated at 37 °C overnight and the number of colonies growing on the overnight plate was counted. Transformants were inoculated on LB-plates containing ciprofloxacin/nalidixic acid and sodium azide/rifampicin to screen for transformants that co-acquired ciprofloxacin and sodium azide resistance.

2.3. Sequencing the PMQR-positives

Sequencing was performed in this study by using the BigDye v3.1 sequencing chemistry (Applied Biosystems, USA). To confirm the identification of PMQR genes detected by PCR screening, same primers used in the multiplex and simple PCR screening were used for sequencing both strands of the detected genes [15].

Procedure:
The positive PCR products were purified and used as template for sequencing. Sequencing master mix:

2.4. Reagent amount

Big-Dye v3.1 (Applied Biosystems, USA): 2 µL
Sequencing buffer (Applied Biosystems, USA): 3 µL
Primer 3.2 pmol/µL: 1 µL
ddH_2O: 12 µL

For each reaction, 2 µL template was added to 18 µL master mix and the PCR thermocycler was programmed. Nucleotide sequencing was performed at the Sequencing core facility of the International Institute for Tropical Agriculture (IITA), Ibadan. Searching for nucleotide sequence homology was performed using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). Using the BLAST, the ultimate confirmation of gene sequences was made. First, an ExPasy convert to the amino acid sequence was done. All base and amino acid sequences were then used for a search in the database for homology of sequences. The BLAST-n and BLAST-p were used for nucleotide and amino-acid alignments, respectively.

2.5. MIC determination

The Minimum Inhibitory Concentration (MIC) of tested Qs on recipient bacterium before, and after gene transfer was equally determined using e-test strips.

3. Results

There is a significance difference in the occurrence of quinolone resistance among the isolates from the different sources. Except for Klebsiella sp., Q-resistance was generally higher amongst isolates from Public Hospitals (67.2%) than Private Hospitals (45.0%). Also, with the exception of Salmnonella sp., Q-resistance was generally higher amongst isolates from Poultry Litter (54.2%) than from Fish-pond Water (40.9%). As a composite, human isolates had a higher Q-resistance (57.4%) than Animal isolates (47.2%) and only 1018 of the 1964 isolated enteric bacteria strains were quinolone resistant as shown in Table 1.

Table 1. Number of Q-resistant strains (%) from each site according to species [13].

Species	Public Hospitals	Private Hospitals	Poultry Droppings	Fish Pond Water	TOTAL
E.coli	123/168	67/177	101/165	61/159	352/669
	(73.2)	(37.9)	(61.2)	(38.4)	(52.6)
*Salmonella*sp.	87/126	43/87	65/177	36/98	231/488
	(69.0)	(49.4)	(36.7)	(36.7)	(47.3)
*Shigella*sp.	77/131	38/76	29/46	18/68	162/321
	(58.8)	(50.0)	(63.0)	(26.5)	(50.5)
*Klebsiella*sp.	28/44	19/27	48/61	47/69	142/201
	(63.6)	(70.4)	(78.7)	(68.1)	(70.6)
*Aeromonas*sp.	21/31	11/29	31/56	68/169	131/285
	(67.7)	(37.9)	(55.4)	(40.2)	(46.0)
Total	336/500	178/396	274/505	230/563	1018
	(67.2)	(44.9)	(54.2)	(40.9)	

Table 2. Occurrence of PMQR amongst Q-resistant isolates from human and animal sources [13].

Species	Public Hospitals	Private Hospitals	Poultry Droppings	Fish Pond Water	TOTAL
E.coli	1/123	1/67	5/101	2/61	9/352
	(0.8)	(1.5)	(5.0)	(3.3)	(2.6)
*Salmonella*sp.	0/87	0/43	4/65	3/36	7/231
	(0.0)	(0.0)	(6.2)	(8.3)	(3.0)
*Shigella*sp.	0/77	0/38	1/29	2/18	3/162
	(0.0)	(0.0)	(3.4)	(11.1)	(1.9)
*Klebsiella*sp.	1/28	1/19	4/48	1/47	7/142
	(3.6)	(5.3)	(8.3)	(2.1)	(4.9)
*Aeromonas*sp.	0/21	0/11	2/31	1/68	3/131
	(0.0)	(0.0)	(6.5)	(1.5)	(2.3)
Total	2/336	2/178	16/274	9/230	29
	(0.6)	(1.1)	(5.8)	(3.9)	

Table 3. Resistance profile of plasmid-mediated quinolone resistant (PMQR) before and after plasmid curing.

PMQR Isolates	Resistance Profile Before Plasmid Curing				Resistance Retained After Curing
	NalidixicAcid (NA)	Ciprofloxacin (CPX)	Pefloxacin (PEF)	Ofloxacin (OFL)	
E.coli A067	R	R	R	R	NIL
E.coli A023	R	R	R	R	NIL
E.coli A031	R	R	R	R	NIL
E.coli A078	R	R	R	R	NIL
E.coli A033	R	R	R	R	NIL
E.coli A041	R	R	R	R	OFL
E.coli A051	R	R	R	R	OFL
E.coli H022	R	R	R	R	OFL
E.coli H015	R	R	R	R	NIL
Salmonella A010	R	R	R	S	NIL
Salmonella A089	R	R	R	S	NIL
Salmonella A035	R	R	R	R	NIL
Salmonella A030	R	R	R	R	NIL
Salmonella A022	R	R	R	R	NIL
Salmonella A001	R	R	R	S	NIL
Salmonella A031	R	R	R	R	NIL
Shigella A015	R	R	R	R	OFL
Shigella A031	R	R	R	R	NIL
Shigella A001	R	R	R	R	NIL
Klebsiella A029	R	R	R	S	NA
Klebsiella A022	R	R	R	S	NIL
Klebsiella A012	R	R	R	S	NA
Klebsiella A023	R	R	R	R	NIL
Klebsiella A033	R	R	R	R	OFL
Klebsiella H012	R	R	R	S	NIL
Klebsiella H022	R	R	R	R	OFL
Aeromonas A029	R	R	R	S	NA
Aeromonas A022	R	R	R	S	NIL
Aeromonas A012	R	R	R	S	NA

Key: NIL-Non-existence, OFL-Ofloxacin, NA-Nalidixic acid, R-Resistant, S-Susceptible

The ability to successfully transfer PMQR genes into the standard E.coli$^{Az\text{-}r}$ J53-2 recipient by conjugation and transformation was investigated. This was to ascertain whether the PMQR gene(s) is/are borne on transferable plasmids. This is crucial to the spread of resistance among bacterial population. Among the 5 E.coli strains detected with at least one of the PMQR gene (Table 4), conjugational transfer of at least a gene, was possible in four (80%). However, transfer by transformation of the competent recipient, was possible in all five transformation experiments.

All 7 of the Salmonella strains with PMQR genes in this study, were subjected to inter-generic transfer of resistance genes. Four (57.1%) of the transfer was possible by conjugation (Table 4), while six (85.7%) PMQR gene transfers were possible by transformation. The only qepA gene detected in this study in Salmonella A030, was not successfully transferred either by conjugation or transformation. Inter-generic transfer of PMQR genes was equally attempted for the 2 Shigella sp investigated in this study. Gene transfer by conjugation was not successful though the recipient was successfully transformed with at least a PMQR gene being transferred (Table 4).

The 3 Klebsiella sp. positive for at least a PMQR gene studied, were equally subjected to inter generic gene transfer by conjugation and transformation. None of the conjugation experiments was successful, but all three transformation experiments positively transferred at least one of the PMQR genes (Table 4). Also attempted, was the inter-generic transfer of PMQR genes from the 2 Aeromonas sp. harboring at least a gene (Table 4). It was observed here again, that transfer by conjugation was not successful although at least a PMQR gene was positively transferred by transformation. Transformation was possible in 18/19 of the resistance transfer experiments while conjugation was only possible in 8/19 cases (Table 4). Generally, the qnrB and qnrS genes were always jointly transferred either by conjugation or transformation.

Table 4. Transfer of Plasmid bearing PMQR gene using E. coli J53-2 as recipient.

PMQR Donors	*PMQR Gene In Isolate	Gene(s) Transferred by	
		Conjugation	Transformation
E.coli A 067	QnrA	-	QnrA
E.coli A 023	QnrA, QnrS	QnrA	QnrA
E.coli A 031	QnrB, QnrS	QnrB, QnrS	QnrB, QnrS
*E.coli*A 078	QnrA, QnrB, QnrS	QnrB, QnrS	QnrB, QnrS
E.coli H 015	Qnr A, Qnr B	Qnr B	Qnr B
Salmonella A 010	QnrS	QnrS	QnrS
Salmonella A 089	QnrA, QnrB	QnrB	QnrB
Salmonella A 035	QnrB, QnrS	-	QnrB, QnrS
Salmonella A 030	QepA	-	-
Salmonella A 022	QnrB	QnrB	QnrB
Salmonella A 001	QnrA, QnrB	QnrB	QnrB
Salmonella A 031	Qnr B, Qnr S	-	QnrB, QnrS
Shigella A 031	Qnr A, Qnr B	-	Qnr B
Shigella A 001	Qnr B, Qnr S	-	Qnr B, Qnr S
Klebsiella A 022	Qnr A, Qnr B	-	Qnr B
Klebsiella A 023	Qnr A, Qnr B	-	Qnr A, Qnr B
Klebsiella H 012	Qnr B	-	Qnr B
Aeromonas A 041	Qnr A, Qnr B	-	Qnr B
Aeromonas A 033	Qnr A, Qnr B	-	Qnr A, Qnr B
Total 19	19	8	18

The Minimum Inhibitory Concentration (MIC) of Nalidixic acid(NA), Ciprofloxacin (CPX), Pefloxacin (PEF) and Ofloxacin (OFL) on the recipient bacteria (E. coli J53-2) lacking any PMQR gene/plasmid, was determined. The MICs were 0.25 µg/mL and 0.0625 µg/mL for NA and CPX respectively, and 0.0625 µg/mL and 0.031 µg/mL for PEF and OFL respectively. The respective PMQR gene was subsequently transferred to the recipient by transformation, and the MIC again determined.

This was to ascertain the level of resistance conferred by the acquisition of PMQR plasmid(s).

Table 5. Minimum Inhibitory Concentration (MIC) of nalidixic acid on recipient bacteria, before and after PMQR gene transfer.

Isolates	PMQR Gene Transferred	MIC (µg/mL)		
		Donor	Recipient (Before Gene Transfer)	Recipient (After Gene Transfer)
Aeromonas A-041	Qnr B	64	0.25	4.0
Aeromonas A-033	Qnr A, Qnr B	128	0.25	16.0
E.coli A-067	Qnr A	32	0.25	2.0
E.coli A-023	Qnr A	128	0.25	16.0
E.coli A-031	Qnr B, Qnr S	128	0.25	16.0
E.coli A-078	Qnr B, Qnr S	128	0.25	16.0
E.coli H-015	Qnr B	64	0.25	4.0
Klebsiella A-022	Qnr B	64	0.25	4.0
Klebsiella A-023	Qnr A, Qnr B	64	0.25	16.0
Klebsiella H-012	Qnr B	32	0.25	4.0
Salmonella A-010	Qnr S	64	0.25	4.0
Salmonella A-089	Qnr B	64	0.25	8.0
Salmonella A-035	Qnr B, Qnr S	128	0.25	16.0
Salmonella A-022	Qnr B	32	0.25	4.0
Salmonella A-001	Qnr B	64	0.25	4.0
Salmonella A-031	Qnr B, Qnr S	128	0.25	16.0
Shigella A-031	Qnr B	64	0.25	8.0
Shigella A-001	Qnr B, Qnr S	128	0.25	16.0

Table 6. Minimum Inhibitory Concentration (MIC) of ciprofloxacin on recipient bacteria, before and after PMQR gene transfer.

Isolates	PMQR Gene Transferred	MIC (µg/mL)		
		Donor	Recipient (Before Gene Transfer)	Recipient (After Gene Transfer)
Aeromonas A-041	Qnr B	4.00	0.0625	2.00
Aeromonas A-033	Qnr A, Qnr B	8.00	0.0625	4.00
E.coli A-067	Qnr A	1.00	0.0625	1.00
E.coli A-023	Qnr A	2.00	0.0625	2.00
E.coli A-031	Qnr B, Qnr S	8.00	0.0625	4.00
E.coli A-078	Qnr B, Qnr S	8.00	0.0625	8.00
E.coli H-015	Qnr B	2.00	0.0625	1.00
Klebsiella A-022	Qnr B	2.00	0.0625	1.00
Klebsiella A-023	Qnr A, Qnr B	8.00	0.0625	8.00
Klebsiella H-012	Qnr B	2.00	0.0625	1.00

Continued on next page

Isolates	PMQR Gene Transferred	MIC (µg/mL)		
		Donor	Recipient (Before Gene Transfer)	Recipient (After Gene Transfer)
Salmonella A-010	Qnr S	1.00	0.0625	1.00
Salmonella A-089	Qnr B	2.00	0.0625	2.00
Salmonella A-035	Qnr B, Qnr S	8.00	0.0625	4.00
Salmonella A-022	Qnr B	1.00	0.0625	1.00
Salmonella A-001	Qnr B	2.00	0.0625	1.00
Salmonella A-031	Qnr B, Qnr S	8.00	0.0625	4.00
Shigella A-031	Qnr B	2.00	0.0625	2.00
Shigella A-001	Qnr B, Qnr S	8.00	0.0625	8.00

Table 7. Minimum Inhibitory Concentration (MIC) of pefloxacin on recipient bacteria, before and after PMQR gene transfer.

Isolates	PMQR Gene Transferred	MIC (µg/mL)		
		Donor	Recipient (Before Gene Transfer)	Recipient (After Gene Transfer)
Aeromonas A-041	Qnr B	4.00	0.0625	4.00
Aeromonas A-033	Qnr A, Qnr B	8.00	0.0625	2.00
E.coli A-067	Qnr A	1.00	0.0625	1.00
E.coli A-023	Qnr A	2.00	0.0625	4.00
E.coli A-031	Qnr B, Qnr S	8.00	0.0625	4.00
E.coli A-078	Qnr B, Qnr S	8.00	0.0625	8.00
E.coli H-015	Qnr B	2.00	0.0625	2.00
Klebsiella A-022	Qnr B	2.00	0.0625	2.00
Klebsiella A-023	Qnr A, Qnr B	8.00	0.0625	4.00
Klebsiella H-012	Qnr B	2.00	0.0625	2.00
Salmonella A-010	Qnr S	1.00	0.0625	2.00
Salmonella A-089	Qnr B	2.00	0.0625	4.00
Salmonella A-035	Qnr B, Qnr S	8.00	0.0625	4.00
Salmonella A-022	Qnr B	1.00	0.0625	2.00
Salmonella A-001	Qnr B	2.00	0.0625	4.00
Salmonella A-031	Qnr B, Qnr S	8.00	0.0625	4.00
Shigella A-031	Qnr B	2.00	0.0625	2.00
Shigella A-001	Qnr B, Qnr S	8.00	0.0625	4.00

Table 8. Minimum Inhibitory Concentration (MIC) of ofloxacin on recipient bacteria, before and after PMQR gene transfer.

Isolates	PMQR Gene Transferred	MIC (µg/mL)		
		Donor	Recipient (Before Gene Transfer)	Recipient (After Gene Transfer)
Aeromonas A-041	Qnr B	2.00	0.031	1.00
Aeromonas A-033	Qnr A, Qnr B	4.00	0.031	2.00
E.coli A-067	Qnr A	1.00	0.031	1.00
E.coli A-023	Qnr A	2.00	0.031	1.00
E.coli A-031	Qnr B, Qnr S	4.00	0.031	2.00
E.coli A-078	Qnr B, Qnr S	4.00	0.031	2.00
E.coli H-015	Qnr B	2.00	0.031	2.00

Klebsiella A-022	Qnr B	2.00	0.031	2.00
Klebsiella A-023	Qnr A, Qnr B	2.00	0.031	1.00
Klebsiella H-012	Qnr B	1.00	0.031	1.00
Salmonella A-010	Qnr S	1.00	0.031	1.00
Salmonella A-089	Qnr B	2.00	0.031	1.00
Salmonella A-035	Qnr B, Qnr S	4.00	0.031	2.00
Salmonella A-022	Qnr B	1.00	0.031	1.00
Salmonella A-001	Qnr B	2.00	0.031	1.00
Salmonella A-031	Qnr B, Qnr S	4.00	0.031	4.00
Shigella A-031	Qnr B	2.00	0.031	2.00
Shigella A-001	Qnr B, Qnr S	4.00	0.031	4.00

It was observed (Table 5) that transfer of PMQR plasmid resulted in an increase in MIC of NA from 3-(0.25–2.00 µg/mL), to 6-times (0.25–16.00 µg/mL) in the recipient bacterium. Also, PMQR gene transfer resulted in an increase in MIC of CPX and PEF from 4-(0.0625–1.00 µg/mL), to 7-fold (0.0625–8.00 µg/mL) in the recipient bacterium (Tables 6 and 7). Furthermore, transfer of PMQR plasmid to the recipient bacterium resulted in an increase in MIC of OFL from 5-(0.031–1.00 µg/mL), to 7-fold (0.031–4.00 µg/mL) as observed in Table 8 Presented in figures 1 and 2 are the plasmid bands of purified PCR products of all 19 isolates harboring PMQR genes. Plasmid sizes of bands were compared with the standard molecular weight ladder (M) at the extreme left of the plate. The qnrA gene can be observed in lane 1 while lanes 3 and 16 display the qnrB gene. Along lane 2 is the qnrS gene, while the only qepA gene is located on lane 9. The corresponding approximate sizes of the qepA, qnrA, qnrB and qnrS genes are 199, 417, 469 and 516 bp, respectively (Figure 1). Lane 7 harbors three genes- qnrA, qnrB and qnrS while qnrA and qnrS gene bands are located on lane 4.The lanes 5, 8, 18 and 19 harbor both qnrB and qnrS genes. Furthermore, located along lanes 6, 10, 11, 12, 13, 14, 15 and 17 are PCR product bands for qnrA and qnrB genes. Characterization of PMQR genes according to numbers showed that strains from which plasmids were extracted, on lanes 1, 2, 3 and 16 harbored just one (1) PMQR gene. Strains on lanes 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18 and 19 harbored 2 PMQR genes. Only the strains on lane 7 harbored 3 PMQR plasmid bands.

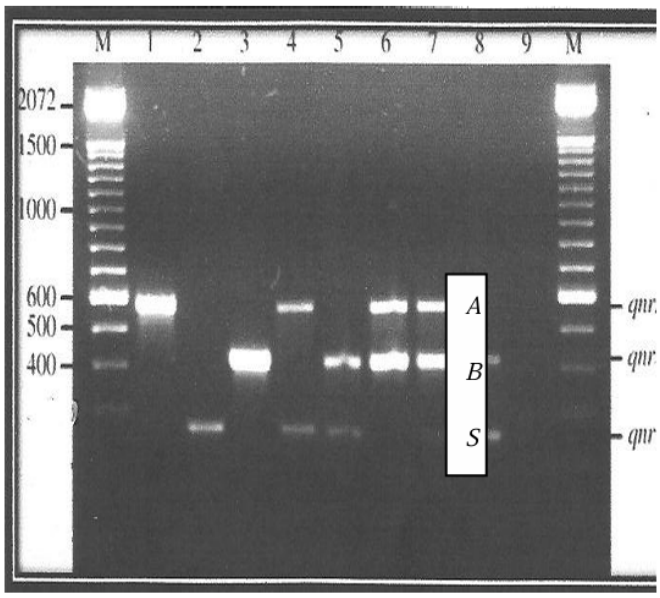

Figure 1. Plasmid bands and their approximate molecular weights of Plasmid-Mediated Resistant Isolates [13].

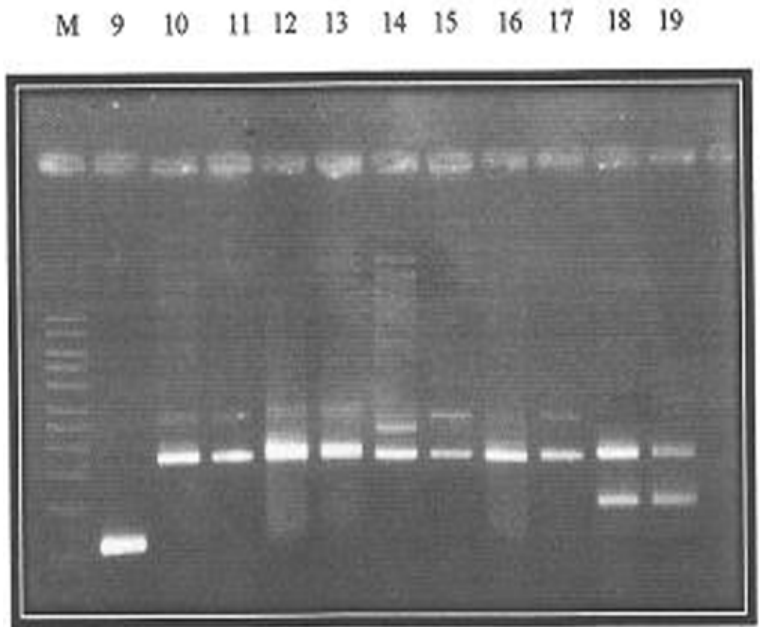

Figure 2. Plasmid bands and their approximate molecular weights of Plasmid-Mediated Resistant Isolates [13].

In this study, a 100% alignment with BLAST-n was observed for qnrA gene, with qnrA database accession numbers KC 414000.1, JN 687470.1, JF 969163.1, JF 773308.1 and HQ 184955.1 belonging to isolates of Enterobacter hormaechei, Providencia stuartii, Vibrio fluvialis, E. coli and Enterobacter cloaca respectively. Furthermore, using the BLAST-p for amino acid alignment, a 99–100 % alignment was observed for qnrA gene in this study with those of Klebsiella pneumoniea isolates with accession numbers ABQ 01122.1, YP 002332855.1, ADO 60956.1, ADO 60962.1 and ADY 17943.1. Similar observations were recorded for the other PMQR determinants amplified in this study, by PCR.

4. Discussion

Results indicate the possibility of a high frequency of transfer of PMQR determinants, both by conjugation and transformation. Generally, transformation was possible in 18/19 of the resistance transfer experiments while conjugation was only possible in 8/19 cases. These results are quite significant to the dissemination of quinolone resistance in the environment. Also worthy of note is that, these transfers were not only intra-generic, but also inter-generic. However, inter-generic transfer by conjugation was unsuccessful in most cases. Success in transferring the qnr genes by conjugation has been frequently reported [16,17]. In addition, transfer of these qnr genes can also be performed by transformation [18]. On the other hand, both of qnrS and qnrB genes have also been detected on non-conjugative plasmids [19,20]. In the study conducted by Jiang et al, southern hybridization indicated that qnrA, aac(6`)-Ib-cr, and ESBL-encoding genes were always located on the same plasmids [21]. However, the association between these genes on same plasmids was not confirmed in the present study. On the other hand, the qepA gene were neither transconjugable nor transformable. The failure in transferring this gene suggests that it is located on a non-transferable plasmid. Nevertheless, a transferable plasmid has previously been demonstrated to carry the qep determinant; a plasmid which co-transfers the rmtB gene [22]. However, in the present study, it is difficult to draw conclusions regarding its non-transferability, because it was only in a single isolate that qep determinant was

detected. Interestingly, chromosomal location for qnr genes has been suggested by some studies [23,19]. This could be the case for Salmonella A-030 in the present study, where repeated conjugation and transformation experiments yielded negative results. However, the observation was that qnrB and qnrS were always jointly transferred either by transformation or conjugation. This suggests that both genes are closely linked, or even occurring on the same plasmid. The acquisition of such plasmids bearing more than one Q-resistance marker could be detrimental epidemiologically. This may result in novel bacteria with multi-drug resistance capability which is not good for management of infectious diseases. The high rate of PMQR gene acquisition by transformation, as observed in this study, is very significant. This is because, though transformation was induced and cells made competent in the present study, natural occurrences of transformation is quite possible [24]. In the natural world, DNA usually becomes available by death and lysis of other cells, but in the laboratory it is provided by the researcher, often as a genetically engineered fragment or plasmid. During uptake, DNA is transported across the cell membrane and the cell wall if one is present. Once the DNA is inside the cell it may be degraded to nucleotides, which are reused for DNA replication and other metabolic functions. Alternatively it may be recombined into the cell's genome by its DNA repair enzymes. If this recombination changes the cell's genotype the cell is said to have been transformed. Hence, for bacteria in diverse microbial pool, antibiotic resistance may so easily be disseminated among the microbial population [7]. It was observed that the transfer of PMQR plasmid resulted in an increase in MIC of Nalidixic acid from 0.25 up to 16.00 µg/mL in the recipient bacterium.

Also, PMQR gene transfer resulted in an increase in MIC of Ciprofloxacin and Pefloxacin from 0.06 up to 8.00 µg/mL in the recipient bacterium. Furthermore, transfer of PMQR plasmid to the recipient bacterium resulted in an increase in MIC of Ofloxacin from 0.031 to 4.00 µg/mL). All observations pre-suppose that acquisition of the PMQR gene resulted in an increased resistance to the respective Q. The significant observation at this point is that, resistance status conferred by the acquisition of resistance plasmids did not at any instance, exceed the original resistance of the donor. This observation suggests that inasmuch as resistance is conferred by PMQR plasmid acquisition, the plasmid alone does not confer maximum resistance status. Other mechanisms of resistance, such as chromosomal mutation synergistically confer the maximum resistance possible. In other words, though plasmid-mediated resistance is important, it works hand-in-hand with chromosomally- mediated resistance, to pose an even more significant resistance status. In general, the acquisition of a qnr-bearing plasmid will not render a wild-type organism quinolone insusceptible according to CLSI clinical breakpoints. The extent to which qnrA protects isolates of Enterobacteriaceae against quinolones has usually been examined by measuring the difference in quinolone MICs for an E. coli strain with and without a qnrA-bearing plasmid. The report by Wang et al., [25,26] showed that the MIC of ciprofloxacin increased from 0.008 µg/mL to 0.25 µg/mL in an E.coli J53 transconjugant with a range from 0.125 µg/mL to 2.0 µg/mL for other qnr plasmid transconjugants of this strain.

One study assessed the quinolone resistance conferred by 17 clinical qnrA-bearing plasmids. Donor bacteria originally harboring these plasmids had exhibited higher levels of resistance to quinolones than the transconjugants, suggesting that additional mechanisms of quinolone resistance frequently coexist with qnrA. There were also differences among transconjugants in the qnrA effect on fluoroquinolone MICs [15]. Although for most agents in previous studies the presence of a qnr plasmid increased their MIC by between 16-fold and 125-fold, this increase was less (16-fold to 32-fold) for ciprofloxacin. The agent for, which the loss of activity was least pronounced was nalidixic acid (twofold to eightfold increases in MIC) [26].

Illustrating this phenomenon, Hopkins et al. found that in non-Typhi Salmonella isolates, a phenotype of reduced susceptibility to ciprofloxacin(MIC > 0.06 μg/mL) but preserved susceptibility to nalidixic acid (MIC ≤ 16 μg/mL) identified qnr-positive strains [27,28]. Also note-worthy is the finding that some qnrA-carrying plasmids from U.S. K. pneumoniae isolates yielded transconjugants with very similar quinolone susceptibilities [26], whereas other qnrA-carrying plasmids from U.S. and Chinese isolates of Enterobacteriaceae varied in ciprofloxacin susceptibilities by 16-fold [25]. There are several reasons for this phenomenon. In some cases these differences resulted from the presence of an additional resistance determinant, aac(6')-Ib-cr, on some plasmids [15,29]. For other strains, the copy number and especially the transcriptional level of the qnr genes affected quinolone resistance [23,29]. qnrS- and qnrB-carrying plasmids confer quinolone resistance that is similar to that conferred by qnrA1. When cloned into a derivative of E. coli DH10B, qnrS1 increased the MICs of nalidixic acid, ciprofloxacin, and ofloxacin 8-, 83-, and 24-fold, respectively. These changes led only to nalidixic acid resistance by CLSI breakpoints [20]. The impact of some qnr allele variants on quinolone MICs has additionally been examined. Overall, the patterns of resistance have been similar, with 2- to 8-fold and 8- to 32-fold increases in MICs of nalidixic acid and ciprofloxacin, respectively [20,30,31] All genes isolated, amplified, sequenced and aligned in this study, were isolated from animal sources, except the two obtained from human sources. These animal sources were largely from poultry which underscores the fact that emergence of quinolone resistance genes is fast spreading via poultry. This is likely due to indiscriminate use of quinolone antibiotics as prophylaxis in poultry feed.

5. Conclusions

In this study, the quinolone resistance determinants- qnrA, qnrB and qnrS, were more implicated in conferring the plasmid-mediated Q-resistance and they were found on transferable plasmids which may be acquired either by conjugation or transformation. Though resistance transfer was more successful by transformation, conjugational transfer was equally possible. The qnrB and qnrS determinants were in most cases, jointly transferred and when they do, convey a higher resistance as compared with a single determinant transfer.

6. Recommendation

Since PMQR determinants are more observed in bacteria from animal husbandry, and these determinants are transferrable with unhealthy consequences, the following recommendations are suggested to check the FQ-resistance menace:

I. The quinolones should not be used in animal husbandry for prophylactic purposes.

ii. Microbes from animals should not be allowed to cross-contaminate materials that will be consumed by man.

iii. If possible, the quinolones generally should temporarily be withdrawn from circulation to restore the potency of the drug.

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Conflict of interest

The authors declare that there is no conflict of interest. The authors certify that they have no link with any organization with any interest in the research. All co-authors have seen and agreed with the contents of the manuscript and there is no financial interest to report.

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