Microbicidal clays: composition, activity, mechanism of action, and therapeutic applications

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Infectious diseases are major causes of death, disability, and social and economic disruption for millions of people throughout the world. However, our ability to treat these infectious pathogens is declining due to the increase in antibiotic resistance and insufficient development of novel antibacterial agents. Clay minerals offer an inexpensive, complementary therapeutic option for topical bacterial infections, yet further research is needed to scientifically validate their efficacy. Clay minerals have a net negative charge allowing exchange of positively-charged ions on their surface. The physical properties of clay minerals have been extensively exploited for therapeutic applications either for use as a carrier of antibacterial agents or to remove toxic substances from the body. In this chapter, we will review the a) historical and current use of medicinal clays, b) prophylactic and therapeutic applications of clays, c) the composition of antibacterial clays, d) microbicidal activity, mode of action, and biomedical use of metal ions, e) generation of chemically-modified antibacterial clays, f) *in vivo* applications and mode of action of natural and modified clays, and g) future perspectives and considerations for biomedical and agricultural uses of clays.

Keywords clays; antibacterial; microbicidal; metal ions

1. Introduction

1.1. Antibiotics, antimicrobials, and microbicides

The discovery and development of antibiotics and similar drugs, collectively called antimicrobial agents, represent one of the most significant medical successes of the 20th century and have substantially reduced illness and death from infectious diseases. By definition, antibiotics are low molecular weight products of microorganisms that kill or inhibit the growth of susceptible microorganisms. In contrast, antimicrobial agents are synthetically-derived or chemically-modified compounds that exhibit antimicrobial activity at low concentrations. Ideally, antimicrobial compounds will disrupt microbial-specific processes or structures so that host toxicity and adverse side effects are minimized [1]. Microbicidal agents are chemical or physical agents that kill microorganisms and viruses and include compounds categorized as disinfectants, antiseptics, and sanitizers. Disinfectants are designed to kill microorganisms and viruses, but should only be used on inanimate objects, while antiseptics and sanitizers kill or neutralize bacteria and viruses on the skin and some mucosal surfaces [2].

1.2. Complementary and alternative medicine

The overuse and misuse of antibiotics over the past 70 years has led to a steady increase in the number of infections caused by antibiotic-resistant bacteria [3, 4]. As the discovery of new antimicrobials has plummeted over the past two decades, the rise of antibacterial resistance has prompted the search for and development of alternative strategies and novel therapeutic treatments to combat bacterial infections. One strategy is to integrate traditional and alternative medicines in the search for effective, low risk, and inexpensive antimicrobial agents.

Complementary and alternative medicine systems have been used throughout history for a diversity of healthcare applications. The US National Institutes of Health National Center for Complementary and Alternative Medicine defines complementary and alternative medicine (CAM) as "a group of diverse medical and health care systems, practices, and products that are not generally considered to be part of conventional medicine." While complementary medicines are commonly *used together* with conventional medicinal practices, alternative medicines *replace* the use of conventional medicines. Ongoing efforts are associated with promoting integrative or integrated medicine, which unites conventional medicine with CAM therapies for which there is evidence of safety and effectiveness. More than 80% of the world's population uses CAM, with recent reports indicating increased use in the US and integration into the US healthcare system. Reports from US national health surveys in 2002 and 2007 revealed that 34% to 42% of American adults or children report using some form of CAM practices or therapies, demonstrating its widespread use and increased acceptance across the population [5-7]. CAM products commonly used by consumers for topical antibacterial applications include herbal products, essential oils, colloidal silver, and clays [5, 7-13].

2. Historical and current use of medicinal clays

2.1. Historical uses of clay – geophagy

Clays have been used for medicinal applications throughout recorded history. The direct consumption of earth or soil-like substances, such as clay or chalk, for medicinal or spiritual purposes is termed *geophagy*. The ancient tablets of Nippur, written approximately 5,000 years ago, listed clays as medicament for healing wounds and stopping "fluxes from the body". The Ebers Papyrus, the world's oldest medical text dated approximately 1600 B.C., lists clay as a mineral remedy for ailments such as diarrhea, dysentery, tapeworm, hookworm, wounds, and abscesses [14]. In the late 19th and early 20th centuries, German physician, Dr. Julius Stumpf, brought attention to the use of fine kaolin for the treatment of cholera [15]. Following an injested bolus of dissolved kaolin clay, cholera patients reported immediate relief from vomiting. Stumpf attributed this relief to "abundantly cover[ing] the bacteria with finely divided inorganic matter [so that] they can no longer multiply; as a result the formation of toxins is stopped, and the disease takes a retrograde course" [15].

2.2. Modern uses of clay – geophagy

Women of the Tiv tribe in Nigeria have been reported to eat clay, particular during and shortly after pregnancy [16]. The Tiv people typically mine clays located two to four feet below the earth's surface, in regions that are below extant or extinct river beds. Analyses of the chemical compositions in these subsurface areas revealed higher concentrations of calcium and magnesium relative to the top soil. Given that the Tiv people are one of the only tribes that do not drink milk (milk-producing animals are susceptible to parasitic infections that are endemic in the area) and that the local top soil is typically deficient in calcium, the dietary intake of calcium through food crops is thought to be minimal. Together, these data indicate that the Tiv geophagy cultural practices likely developed due to actual physiological need [17].

2.3. Historical uses of clay – topical applications

In addition to the widespread practice of geophagy, clays have been deployed for topical uses for centuries. In the first century A.D., Dioscorides, a Greek physician and pharmacologist, published his book, *De materia medica*, an exhaustive encyclopedia of nearly 600 medicinal plants and 1,000 drugs. Dioscorides described rubbing baked clay tiles with vinegar and using them to heal itching, pustules, gout, and goiters [18]. During the late 19th century, clays were used as topical treatments for surgical wounds with demonstrated beneficial effects on pain management, inflammation, putrefaction, and healing processes [19]. Further, Reinbacher [20] describes German physician, Dr. Julius Stumpf's treatment strategy in 1898 of a patient who had long been suffering from a deep and suppurating ulcer of the tibia. This patient refused amputation, so the physician began treatment with a thick layer of a fine clay poultice. The wound immediately stopped producing a malodorous discharge, and after four days of repeated clay application and bandaging, the ulcer healed.

2.4. Modern uses of clay – topical applications

More recently, hydrated clay poultices were used for the treatment of Buruli ulcer, a difficult-to-treat necrotic skin infection caused by the bacterium *Mycobacterium ulcerans* [21, 22]. Two different green clays, which were later demonstrated to be mineralogically identical [22], were applied daily as hydrated poultices to individuals with advanced, necrotic Buruli ulcer disease. After several months of topical treatment, the *M. ulcerans* skin infections resolved, and patients often healed with soft supple scarring and a resumption of normal motor function [21-23]. In Iran, boiled *Caprifoliaceae*, or honeysuckle, flower extract is mixed with clay and applied dermally to treat colds [24]. In Guinea, interviews with over 400 traditional healers revealed numerous occurrences whereby healers mixed plant extracts with clays to generate poultices for topical applications [25].

3. Composition of pharmaceutical clays and prophylactic and therapeutic applications

3.1. Pharmaceutical clays

Clays are natural, fine-grained (<2 micron) particles composed of negatively-charged silicate sheets, commonly referred to as phyllosilicates. In order to balance the charge, the negatively-charged clay surface freely exchanges positively-charged cations or products from the environment [26]. The use of clay minerals and non-silicate minerals as excipients in pharmaceutical and healthcare formulations has been extensively described [27-31]. Phyllosilicates commonly used in pharmaceutical preparations include smectites, palygorskite, sepiolite, kaolinite, and talc, with montmorillonite, saponite, and hectorite comprising the most widely used smectite family clays [30]. Clays harbor unique properties that are important for various pharmaceutical preparations: i) small particle size, ii) large surface reactivity, iii) ion-

exchange behavior, iv) swelling and sorption properties, v) rheological properties, vi) viscosity and flow behaviors, vii) solubility, viii) thermal capacities, ix) plasticity features, and x) optical attributes [27, 30]. Because of their large surface area, ion-exchange behavior, and consistent microporous structure, zeolites are used similarly to clays in pharmaceutical or therapeutic applications [30]. The chemical composition of natural clays used in pharmaceutical and cosmetic preparations varies considerably. While some clays, such as kaolinite, sepiolite, and talc, exhibit small variations, smectites and palygorskite have very broad compositional ranges and variations [29]. Similarly, trace element composition of clays varies extensively. Trace elements, which include both toxic and less hazardous elements, can be embedded within the structure of the clay minerals or adsorbed to the surface, with the latter elements more likely to mobilize and transfer to leaching solutions [29].

3.2. Prophylactic and therapeutic applications

Prophylactic and therapeutic uses of clay minerals largely exploit the physical properties of clay minerals. Minerals have been administered in oral (e.g. antacids, gastrointestinal protectors, antidiarrhoeaics, osmotic oral laxatives, homeostatics, direct emetics, antianemics, etc) and topical (e.g. antiseptics, disinfectants, dermatological protectors, anti-inflammatories, local anesthetics, decongestive eye drops, etc) applications [32]. Considering the adsorptive and absorptive properties of clays, pharmaceutical and therapeutic benefits are commonly linked with the associated physical and physicochemical properties of clay minerals, described above [29].

4. Natural antibacterial clays

4.1. Biotic activities associated with soils and clays

Examples of clays and soils being used for the treatment of cutaneous bacterial infections have been documented around the world throughout history with antimicrobial activity being attributed to biotic and abiotic factors. Red soils from Jordan have historically been used for treating skin infections. In the case of the Jordan red clays, microbicidal activity was due to the proliferation of bacteria naturally present within the soil and their concomitant production of antimicrobial compounds [33]. Since autoclaved red soils failed to kill inoculated bacteria, biotic factors (i.e., the presence of antimicrobial-producing microorganisms in the soil) served as the basis for the antimicrobial activity of Jordan's red soils [33]. Other biotic influences, including protozoan or myxobacterial predation, lytic microorganisms, and bacteriophages, may also be responsible for controlling bacterial populations within soil [34-37].

4.2. Abiotic activities associated with natural antibacterial clays

Due to their net negative charge, clays bind toxic metals to their surface. In a hydrated environment, the ionic species adsorbed to clay surfaces can be exchanged into the surrounding medium in a manner that depends on the ionic strength of the aqueous medium and cation selectivity of the clay. Aqueous clay mixture extracts (leachates) prepared from antibacterial clay minerals maintain antibacterial activity, demonstrating that the *in vitro* antibacterial activity of the natural clay sample is dependent on chemical desorption of specific metal ions from the surface of the clay particles [38, 39]. Haydel et al [23] described the broad-spectrum *in vitro* antibacterial activities of a natural iron-rich clay that was previously used therapeutically to treat patients infected with *M. ulcerans* [21]. Cunningham et al [38] identified two different natural clay mixtures which exhibited broad-spectrum antibacterial activity against *Escherichia coli*, extended-spectrum beta-lactamase *E. coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (MRSA). The authors demonstrate that the antibacterial activity of these clay samples is due to the release of exchangeable metal ions from the surface of the minerals [36]. Hence, antibacterial activity of these natural clays is dependent on the abiotic, microbicidal activities of desorbed metal ions [36, 39].

4.3. Microbicidal activity of metals

While many metals are essential to life, some metals function as abiotic microbicides by affecting the growth, morphology, and biochemical activities of microorganisms [40, 41]. The toxicity of a given metal depends on its speciation, concentration, and chemical properties, as well as the physicochemical characteristics presented by a given environment [40]. While some metal ions may be toxic, the degree of toxicity is a function of the bioavailability and free concentration of the metal, not the total concentration of the metal. Therefore, ion toxicity is directly linked to ion speciation changes influenced by the pH, redox state, ion solubility, osmotic strength, and temperature in the environment and during experimental conditions [42]. However, metal compounds, particularly containing silver, copper, and zinc, metal-based nanoparticles, and products impregnated with these metal ions or metal compounds have been exploited for biomedical and environmental applications and as microbicides for preventing infections [41].

4.4. Silver and silver products – mechanism of antibacterial action

Silver and silver-based nanomaterials exhibit broad-spectrum microbicidal activity against bacteria, fungi, and viruses [11]. While the beneficial antimicrobial effects of silver are well characterized, proposed mechanisms of action vary greatly and include the generation of reactive oxygen species (ROS), cell membrane damage, inhibition of respiration, and most recently, inactivation of iron-sulfur clusters of bacterial dehydratases involved in amino acid biosynthesis [12, 43, 44]. Proteomic analyses of *E. coli* exposed to silver ions and silver nanoparticles revealed an accumulation of envelope protein precursors, destabilization of the outer membrane, and depletion of intracellular ATP, indicative of collapse of the membrane potential [45]. Further studies have shown that silver interacts with the many membrane-bound proteins found in the cytoplasmic membrane, such as transport or respiratory chain enzymes [46]. Su et al [47] showed a significant increase in inner membrane permeability in *E. coli* following a 72 h exposure to silver nanoparticle-clay hybrids as compared to clay-only control experiments. However, in this study, *E. coli* cells were shown to be completely killed after an overnight exposure to the silver-clay nanohybrids. Therefore, it is unclear if the increase in membrane permeability was due to the silver nanoparticles or if membrane permeability was a consequence of bacterial death [47]. Exposure of bacteria to silver ions has also been shown to induce production of large quantities of ROS, thus potentially damaging the cell [43, 46, 47]. However, binding of silver(I) to enzymes in the electron transport chain can uncouple the respiratory transport chain, resulting in the formation of superoxide and hydroxyl radicals [43, 46]. Thus, the generation of ROS is likely a secondary consequence rather than the direct cause of silver toxicity. The silver ion is highly active and can bind strongly to electron donor groups containing sulfur, oxygen, or nitrogen [48]. Very low concentrations of silver inhibited *E. coli* growth and inactivated fumarase A, a member of the dehydratase family [43]. Silver-mediated inactivation of fumarase A occurred *in vitro* and *in vivo* under anaerobic conditions to prevent production of oxygen species. Therefore, silver toxicity in *E. coli* is not mediated by ROS [43].

4.5. Copper ions and copper surfaces – mechanism of antibacterial action

Copper ions catalyze reactions that produce hydroxyl radicals via Fenton and Haber-Weiss reactions and cause oxidative damage to lipids, proteins, and DNA [49-52]. Bacteria express silver, copper, and mercury export systems to pump metals out of the cytoplasm [53-55] and counteract ROS by producing scavenging enzymes [56, 57]. Moreover, since copper ions protected DNA from hydrogen peroxide-mediated oxidative damage [58] and oxidative damage is quickly repaired *in vivo* [43], the underlying influence of oxidative stress in metal-induced antibacterial activity when cells are actively replicating may be indirect and circumstantial [43]. The molecular basis of copper toxicity occurs via attack of bacterial iron-sulfur clusters in cytoplasmic dehydratases [59]. Soft metals, such as copper, bind the sulfur atoms of the cluster with high affinity, subsequently displace the catalytic iron atoms, and rapidly inactivate the dehydratase catalytic cluster [43, 58]. This deprivation of the essential iron ion in the iron-sulfur cluster abolishes the functional activity and ultimately causes death [58]. Moreover, other soft ionic metals, such as silver, mercury, cadmium, and zinc, that are not redox-active, also damage cytoplasmic dehydratases and inhibit bacterial cell growth [43]. Additionally, dry, metallic copper surfaces rapidly and efficiently kill bacteria [60]. *E. coli* contact with copper surfaces caused rapid cellular membrane damage and loss of cellular integrity, but did not cause DNA damage [60]. While copper ions damage exposed dehydratase iron-sulfur clusters *in vitro* and *in vivo*, cells exposed to dry copper surfaces do not replicate [43], so dehydratases required for cellular metabolism are not likely targets for toxicity on dry copper surfaces [60]. Furthermore, supplementation with oxidative stress protectants, such as catalase, superoxide dismutase, or the hydroxyl radical quencher mannitol, delayed killing of *E. coli* cells on dry copper surfaces [60].

4.6. Zinc, zinc oxide, and zinc nanoparticles – mechanism of antibacterial action

Zinc is known to have antibacterial properties, although the precise functional mechanism is unknown. Zinc oxide and zinc oxide nanoparticle suspensions produce ROS, particularly hydroxyl radicals, hydrogen peroxide, and singlet oxygen, which are thought to contribute to antibacterial activity [61-68]. Zinc oxide nanoparticles killed *Campylobacter jejuni*, disrupted the cell membrane, and induced expression of two oxidative stress genes (*katA* and *ahpC*) and a general stress response gene (*dnaK*) [69]. However, Raghupathi et al [70] exposed *S. aureus* to zinc oxide nanoparticles for 1 h and reported minimal to no increased expression of *S. aureus* ROS-responsive genes, suggesting that other mechanisms may contribute to zinc-mediated antibacterial activity. Additional studies indicated that zinc oxide damages the *E. coli* cell membrane, leading to intracellular zinc accumulation and growth inhibition [64, 71], and that zinc oxide nanoparticles bind to the cellular surface and kill bacteria directly via electrostatic forces [66, 72]. Although millimolar levels of zinc did not inhibit *E. coli* growth, micromolar concentrations of zinc decreased fumarase activity by damaging iron-sulfur clusters [43]. Unlike silver- and copper-mediated damage to iron-sulfur clusters of dehydratases, fumarase damage caused by zinc was reversible upon the addition of ferrous iron and dithiothreitol [43]. In *Streptococcus pneumoniae*, zinc competes with manganese for binding to PsaA, a protein that transports manganese into the cell to manage oxidative stress [73]. PsaA-zinc complexes were thermally stable, resulted in decreased manganese uptake, and inhibited *S. pneumoniae* growth *in vitro*. Additionally, cells with stable PsaA-zinc complexes were more sensitive to oxidative stress and more susceptible to killing by polymorphonuclear leukocytes [73].

4.7. Biomedical applications of metal ions and metal compounds

Some common clinical uses of silver include i) topical silver sulfadiazine to prevent burn wound infections (although a recent systematic review reported that topical silver-containing dressings showed significantly worse healing time compared to controls and showed no evidence of effectiveness in preventing wound infections) [74]; ii) silver alloy-coated urinary catheters to prevent bacteriuria [75]; and iii) chlorhexidine- and silver sulfadiazine-impregnated central venous catheters to prevent catheter colonization and bloodstream infections [76]. Until antibiotics became commercially available, inorganic copper formulations were used to treat chronic adenitis, eczema, impetigo, scrofulosis, tubercular infections, lupus, syphilis, anemia, chorea, and facial neuralgia [77]. The spread of antibiotic-resistant bacteria in health care facilities has stimulated a renewed interest in copper and copper alloys as the basis of self-sanitizing, solid antimicrobial material [78]. Therapeutic uses of zinc include i) topical application of zinc oxide to promote wound healing and prevent infection [79, 80], ii) zinc supplementation as an adjunct to oral rehydration therapy for the treatment of acute diarrhea, iii) ionic zinc nasal gel application for shortening the duration of common cold symptoms (when administered within 24 h of symptoms) [82], and iv) oral zinc acetate or zinc gluconate lozenges or syrup (when administered within 24 h of symptoms) for reducing duration and resolving symptoms of the common cold. While the beneficial effects of zinc lozenges vary among controlled trials, a 2011 Cochrane review revealed evidence indicating that zinc lozenges are efficacious in the treatment of the common cold [83, 84]. Given that desorbed, exchangeable copper and zinc are involved in the *in vitro* activity of microbicidal clays [39], investigating and generating chemically-modified clays could be valuable in biomedical applications.

5. Generation of chemically-modified clays to serve as antibacterial agents

5.1. Metal ion-exchanged clays

While some natural clays are antibacterial [22, 23, 38], much of the research associated with antibacterial clay minerals has focused upon ion-exchanged clays. Zhao et al [85] successfully ion-exchanged a phyllosilicate clay mineral, palygorskite, with copper and silver, respectively, without significantly altering the physical clay structure. *In vitro* studies have shown that mixing the ion-exchanged palygorskite in an aqueous suspension resulted in complete killing of *E. coli* and *S. aureus* and adsorption of the bacterial cells to the clay surface [85]. Xia et al [86] evaluated the *in vivo* effects of copper-exchanged montmorillonite (Cu-MMT) on the growth performance, intestinal microflora, and morphology of weanling pigs. Their results showed that supplementation with Cu-MMT improved growth performance of the pigs and reduced the total viable counts of intestinal *Clostridium* and *E. coli*, whereas supplementation with MMT or copper sulfate alone had no effect on growth performance or intestinal microflora as compared with the basal diet-only control [86].

5.2. Nanoparticle-exchanged clays

As a layered phyllosilicate, montmorillonite clay harbors intercalation, swelling, and ion exchange properties [87]. Therefore, the montmorillonite interlayer space can serve as a support for transition metal-nanoparticle synthesis and as an adsorptive layer for cation deposition [88, 89]. Shameli et al [90] exploited these characteristics to synthesize silver nanoparticles in the external and interlamellar space of montmorillonite clay and investigated the effect of several different sizes of silver nanoparticles on antibacterial activity. While the montmorillonite interlayer space was minimally affected by the different sized silver nanoparticles, the smaller-sized silver nanoparticle-montmorillonite composites exhibited significantly higher antibacterial activity against *S. aureus*, MRSA, *E. coli*, *E. coli* O157:H7, and *Klebsiella pneumoniae* [91].

Bagchi et al [92] successfully exchanged montmorillonite clay with copper nanoparticles and performed antimicrobial susceptibility studies. After 12 h exposures, the copper nanoparticle-exchanged montmorillonite exhibited antimicrobial activity against *E. coli*, *S. aureus*, *P. aeruginosa*, and *Enterococcus faecalis*. To determine if these copper nanoparticle-clay composites displayed cytotoxic effects on eukaryotic cells, two different human cell lines were exposed to composite concentrations greater than the determined minimal bactericidal concentration for the microorganisms studied. The copper nanoparticle-loaded montmorillonite clay displayed minimal adverse effects on the two eukaryotic cell lines, thus demonstrating promise for the use of clays in therapeutic and medicinal applications [92].

5.3. Nitric oxide-releasing, zinc-exchanged zeolite

Nitric oxide (NO) is synthesized throughout the body by endothelial cells, macrophages, neutrophils, fibroblasts, and keratinocytes. NO serves as an intercellular messenger and as a toxic defense molecule for nonspecific immune responses to infectious pathogens [93]. Physiologically, NO also plays a key role in wound healing with beneficial effects attributed to angiogenesis, inflammation, cell proliferation, matrix deposition, and remodeling [94]. NO-related antimicrobial activity has been demonstrated *in vitro* against a broad range of pathogenic microorganisms, including

viruses, bacteria, fungi, and parasites [95]. Continuous exposure to 160 ppm gaseous NO for 5 h caused an 84% and 98% reduction of *P. aeruginosa* and *S. aureus* viability, respectively [96]. However, since NO is an extremely reactive gas, it is difficult to administer for therapeutic purposes. Therefore, recent research has focused on the development of suitable vehicles for NO storage and delivery [96]. Fox et al [97] recently demonstrated *in vitro* bactericidal activity of NO-releasing, zinc-exchanged zeolite against *P. aeruginosa*, *S. aureus,* MRSA, and *Clostridium difficile*. The NO-loaded Zn^{2+}-exchanged zeolite exhibited significantly higher bactericidal activity as compared to the NO-free Zn^{2+}-exchanged zeolite and thus, the observed bactericidal effect was associated with the release of high concentrations of NO [97].

5.4. Clays modified to deliver antimicrobials

Clays have been further manipulated to serve as topical delivery agents for various antimicrobial products. Zinc is an essential trace element that plays a significant role in wound management [79, 98]. Topical application of zinc oxide promotes wound healing by enhancing autodebridement and epithelialization and decreasing inflammation and bacterial growth [80, 99]. The administration of zinc reduces or delays the development of erythromycin resistance by *Propionibacterium acnes*, the normal flora bacterium commonly implicated in acne vulgaris, folliculitis, and other skin disorders [100]. Since erythromycin is unstable in solution and insoluble in water [101, 102], various strategies to improve the stability of the macrolide antibiotic in solution have been proposed. Chemical manipulation of an inorganic material could serve as a carrier for erythromycin and zinc and an exchangeable medium that would promote stability. Cerri et al [103] exchanged a natural zeolite with inorganic Zn^{2+} and subsequently charged the micronized composite with erythromycin to investigate its antimicrobial efficacy against erythromycin-resistant *Propionibacterium* strains. HPLC determinations showed that 85% of the drug contacted with the carrier was loaded, and the simultaneous release of zinc and erythromycin was demonstrated with 82% percent of the loaded antibiotic being released after 30 min [103]. After 3 h of contact within a suspension of the zinc-carrier-erythromycin system, a 99.5% reduction in *P. acnes* viability was observed, compared to an erythromycin solution which reduced *P. acnes* viability by ~40% [104]. While delivery of the antimicrobial is important for treating cutaneous bacterial infections, the ability of the clays to physically adsorb and remove bacterial cells, toxins, and debris from the wound may provide additional benefits in regards to wound healing.

6. *In vivo* applications and mode of action of natural and modified clays

Existing anecdotal evidence from historical practices supports the *in vivo* use of hydrated clay minerals for the treatment of cutaneous bacterial infections [21]. Data demonstrating the *in vitro* antibacterial activity of clays and clay mixtures complement the historical observations; however, further studies are needed to assess the *in vivo* efficacy, practical use, and safety of microbicidal clay applications. While studies have been conducted to characterize the *in vitro* mechanism of action [38, 105], it is entirely possible that the *in vivo* mechanism of action is different and that the beneficial effects associated with wound healing are independent of *in vitro* antibacterial activity.

6.1. Clay dietary supplements

Multiple studies have confirmed the decontaminating and detoxifying properties of clay minerals. Aflatoxins belong to a group of naturally-occuring, potent toxins of fungal origin. These toxins, produced by fungi in or on food and feeds, have been implicated in death and disease in humans, domestic animals, and livestock [106, 107]. Schell et al [108] demonstrated that feeding certain clays to recently weaned piglets can effectively prevent some of the negative effects associated with aflatoxin-contaminated food products. In this study, piglets fed aflatoxin-contaminated corn and calcium bentonite had the highest average daily gains as compared to pigs fed aflatoxin-contaminated corn and other clays. In contrast, pigs fed aflatoxin-contaminated corn and a hydrated sodium calcium aluminosilicate showed intermediate levels of performance, while the performance of pigs that were fed aflatoxin-contaminated corn and the palygorskite remained poor. These data demonstrate that the inclusion of particular clays with an aflatoxin-contaminated diet promotes marked improvements in performance [108]. Since clays bind aflatoxin in aqueous solution [109], it is presumed that the *in vivo* mechanism of the clay is to bind the toxin and prevent intestinal absorption of the toxin, thereby generating a prophylactic effect [108].

6.2. Clay-mediated physical adsorption of toxins

Phillips et al [109] demonstrated the *in vitro* chemisorption of aflatoxin to different clay minerals, with the adsorption capacities and the stability of the aflatoxin-clay adsorption complex exhibiting marked variabilities. The authors proposed that the *in vivo* mechanism of detoxification is due to the selective chemisorption of the clays to the aflatoxin in the gastrointestinal tract, resulting in a clinically significant decrease in toxin bioavailability and diminished aflatoxicosis in young animals [109]. Since these minerals do not display *in vitro* antibacterial activity, the positive *in vivo* outcome is likely due to the physical activity of the clays rather than the desorption of an unrealized antimicrobial

component. Afriyie-Gyawu et al [110] evaluated the long-term toxicity effects of NovaSil clay, a common anti-caking agent in animal feeds, by feeding rats the clay over the course of 28 weeks. No overt toxic effects were observed, even at the highest administered dosage (2% w/w), supporting the safety of *in vivo* use of clays to detoxify toxin-related diseases [110]. Weese et al [111] performed *in vitro* assays to demonstrate the binding of *C. difficile* toxins A and B and *Clostridium perfringens* enterotoxin by a smectite clay. While bacterial growth was present in the positive control (bacteria and clay) experiments, no bacterial growth occurred during metronidazole and smectite co-incubations, indicating that smectite had no adverse effect on the bactericidal activity of metronidazole [111]. Combined, these data indicate that the prophylactic *in vivo* mechanism of action is due to the physical adsorption of toxins rather than antimicrobial activity.

Clays have also been employed to counteract poisons and chemical warfare toxins. Paraquat and diquat are non-selective, highly toxic herbicides widely used in agriculture for weed control. While these toxic chemicals can damage the skin, paraquat or diquat ingestion causes life-threatening effects on the gastrointestinal tract, kidney, liver, heart, and other organs and is often fatal. Treatments that will counter the fatal outcome of paraquat or diquat ingestion include immediate gastrointestinal decontamination via administration of Fuller's Earth (an attapulgite clay), bentonite clay, or activated charcoal [112]. Tsai and Lai [113] demonstrated the high adsorption affinity of paraquat to clay, and the *in vivo* efficacy of clay-paraquat adsorption was confirmed by Idid and Lee [114] in an animal study. The World Health Organization also recommends the use of clays as adsorbents for exposure to chemical warfare agents, such as mustard gas.

6.3. Wound debridement

Debridement is the removal of devitalized tissue, particulate matter, or foreign materials from a wound and is often the first goal of wound care. Debridement can be accomplished surgically, chemically, mechanically, or by means of autolysis [115]. Clay minerals represent a potential therapeutic for use as a mechanical debridement tool due to their physical adsorptive and absorptive properties. However, limited scientific data and no controlled clinical trials exist to assess the use of clays for wound debridement. As described above, topical application of two different hydrated iron-rich illite and montmorillonite clays have been used to treat *M. ulcerans* infections (in uncontrolled trials). A humanitarian carefully observed and documented the debridement of the Buruli ulcer wounds, recording "that seeing, with your own eyes, the débridement of the ulcer while it is taking place, in all its phases, is very enlightening, particularly when accompanied by regeneration of tissue in other areas of the ulcer (a phenomenon which happens frequently with this method of treatment)" [21]. Data and observations collected thus far suggest that the therapeutic *in vivo* mechanism of action, whether clays are ingested or applied topically, is due to the physical, adsorptive and/or absorptive properties of the minerals. However, future studies and controlled trials are needed to define the precise *in vivo* mechanism(s) of action and to validate the wound debridement observations.

7. Future perspectives and considerations

7.1. Safety precaution – consumption

The US Food and Drug Administration (FDA) lists bentonite and kaolinite clays as "generally recognized as safe (GRAS)" products. Therefore, these products are not subject to premarket review and approval by the FDA because it is generally recognized, by qualified experts, to be safe under the intended conditions of use regulated by the FDA. However, the use of clays for therapeutic applications does not come without potential negative health consequences. Consumption of geophagic clayey soils, for example, has been associated with nematode infections [116], anaemia, microbial infections, helminthiasis, intestinal obstruction, dental abrasion, and heavy metal poisoning [117-119].

7.2. Safety precaution – consistency

While natural clays hold great potential as topical antibacterial or therapeutic agents, not all clays have equivalent *in vitro* antibacterial activity. Otto and Haydel [39] collected four independent natural clay samples from the same source that produced one of the aforementioned antibacterial clay mixtures [38]. While the four clay mixtures were nearly mineralogically identical, only two of the four samples demonstrated *in vitro* antibacterial activity against *E. coli* and MRSA [39]. More stringent analyses demonstrated that the variability in the *in vitro* antibacterial activity of the clay mixtures was due to varying concentrations of exchangeable metal ion species on the surface of the minerals. The authors assert that because of this variability across natural mineral samples and the correlated variability in antibacterial activity, efforts must be taken to standardize the composition and antibacterial efficacy of clays if these clay mixtures or developed clay minerals are to be used therapeutically against topical bacterial infections [39].

7.3. Safety precaution – heavy metal exposure

Because clays can bind metal cations, safety precautions must be taken to minimize the potential exposure to toxic metal ions. Exposure or ingestion of large amounts of some metals, such as arsenic, cadmium, lead, and mercury, can cause acute and chronic toxicity and impose significant damage to the human body. The European Pharmacopea [120] requires that pharmaceutical clay preparations have ≤50 ppm heavy metals; however, the FDA does not specify any such requirements. Furthermore, because clays are a GRAS product, minimal regulation of natural therapeutic clays exists to minimize exposure to toxic metal ions. Tateo et al [121] showed that ions, specifically Li, Sr, B, I, Rb, Br, Ba, Na, Cl, Se, and Ca, bound to pelotherapy clays were released from the mineral surface and penetrated human skin. Mascolo et al [122] fed mice one of three different clay samples and detected increased levels of arsenic, nickel, and selenium in urine samples, thus indicating that desorptive processes occur after clay ingestion. While some elements are essential, caution must be taken to ensure that the desorbed ion concentrations do not reach hazardous levels. Furthermore, precautions must be taken to characterize clays used for therapeutic applications with the goal of minimizing exposure to toxic ions.

In a 2008 study, Ferrell [123] described traditional clay geophagy practices in Chimayo, New Mexico. In this study, various clays were exposed to 0.12 M HCl, a pH environment similar to that of the human stomach, to determine the concentrations of different ions extracted from the clays. Data from this study demonstrated that in this highly acidic environment, the concentrations of sodium, chromium, antimony, and arsenic exceeded the recommended daily dietary intake values. As a result, long-term, daily consumption of clays could potentially lead to heavy metal toxicity. Furthermore, excessive intake of clays can lead to intestinal obstructions, which may require surgery to remove the obstruction and sections of the damaged intestine [123, 124]. To circumvent these potential health problems, it was recommended that safety precautions be established to minimize these negative potential health concequences of geophagy [123].

7.4. Potential therapeutic and prophylactic applications

Numerous potential applications exist for antibacterial clay minerals. For human clinical therapeutic applications, clays could be employed to treat acne vulgaris caused by *P. acnes*, skin infecions caused by *S. aureus* or MRSA, *P. aeruginosa* infections in burn victims, or as a cover for diabetic skin ulcers, just to name a few. However, because data thus far has demonstrated that the *in vivo* therapeutic activity of clay minerals is largely due to the physical adsorptive nature of clays, the breadth of applications extends beyond antimicrobial applications. However, as mentioned above, the safety and efficacy must be tested and guaranteed prior to implementation in therapeutic applications. The majority of *in vivo* applications of clay minerals have been investigated for agricultural purposes, with clays yielding positive therapeutic or prophylactic effects as food supplements or as litter additives. Given appropriate efficacy testing, natural or chemically-modified clays have great potential for continued use in the agricultural industry.

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