The anti-microbial properties of slate particles used in a traditional balm and the effect on the growth of Staphylococcus aureus

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The anti-microbial properties of slate particles used in a traditional balm in the growth of *Staphylococcus aureus*

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Highlights

Culturing using viable plate counting and microplate MPN showed similar results where *S. aureus* population decreased with horizontal shaking at 300 rpm, but increased with vertical shaking at 40 rpm. The results show that slate particles showed no antimicrobial activity although extractives from the slate particles showed limited antimicrobial activity lasting a few hours. However, slate particles had a deleterious effect on bacteria compared with silica at higher rotational speeds, indicating potential abrasive properties.

Traditionality

A traditional balm was developed in the early 19th century in Ynys Mons, North Wales, UK using available materials within the local region and has survived in regular use until the present day. The balm has been used to treat a variety of skins problems such as minor burns, minor wounds, eczema and dermatitis.

Abstract

Objective

The study investigated the growth *S. aureus* on slate particles that are an important component in a traditional balm used in the treatment of sores and burns. *S. aureus* is one of a variety of opportunistic pathogens arising from burns.

Methods

The initial experiments were performed by culturing *S. aureus* in dilute liquid medium with slate particles using horizontal shaking at 300 rpm. It was considered that the results may have arisen due to an artefact of the experiment and culturing was performed differently to address potential problems. Therefore, culturing was performed using vertical end-over-end rotation at much lower speeds of 40 rpm.

Different culturing strategies were also explored using spread plating and microplate MPN dilutions that were monitored using a microplate reader. In addition, extractives were recovered from the slate particles and these were assessed for anti-microbial activity.

Results

Viable culturing was assessed using spread plates and a microplate MPN method, which yielded similar results. Significant differences were found between different culturing techniques revealing that the population of *S. aureus* declined by 98% when grown under horizontal shaking conditions and slightly increased when grown under vertical rotation at 40 rpm.

Conclusion

These results show that significantly different conclusions can be obtained using slightly modified methods that are involved different rotational direction and speed of rotation. *S. aureus* populations dramatically declined at the higher speeds whereas there was a slight increase at lower speeds. The recovery of extractives showed some inhibitory activity during the initial stages of growth and these results are discussed.

Keywords

mixing; bacterial inhibition; MPN; microplate; metal oxide

List of Abbreviations

S. aureus Staphylococcus aureus

XRDF x-ray diffractometer

LB Luria broth

1. Introduction

There is evidence that some clays may possess anti-microbial activities and ~ 5% of clays worldwide, mostly of volcanic origin, exhibit antibacterial resistance due to the presence of reduced iron minerals, which cause oxidative stress to the cells [1,2,3]. The antibacterial activity of clay against *Escherichia coli* has also been reported [4] along with the growth inhibition of bacterial biofilms on goethite [5]. However, the majority of clay particles have beneficial effects on microorganisms, particularly montmorillinite, an expandable clay with a structure that enables exchange of essential metal nutrients, absorption of microbial by-products and protection from desiccation and extremes of pH [6].

Slate has been an important component used in traditional medicine in North Wales, UK for over the past two hundred years, where it was prepared and used successfully for treating and healing a victim with substantial burns to the skin. The formulation is made from unique ingredients, found only in Snowdonia, and has been used by local people on the island for generations. The product is applied externally as a cream and is used to treat a range of skin conditions, including burns, wounds, eczema and dermatitis. It leaves no visible marks or blemishes, and also gives instant pain relief [7]. While complementary medicines account for ~ 7% of the total medical market in Europe, the majority of treatments are based on the use of acupuncture and homeopathy in the UK, rather than traditional medicine [8]

If slate particles have anti-microbial activity as shown with many other clays, then it could be used in limiting bacterial growth that may occur during healing of wounds. There is a need for new anti-microbial therapies due to increasing antimicrobial resistance occurring caused by overuse of antibiotics and the lack of medications to treat fungal infections occurring in increasing numbers of people having diabetes [9].

Only a few studies have examined bacterial association with slate and all of these studies have investigated black slate that contains a small percentage of carbon unlike other types of slate. One of these determined that sulphate reducing bacteria were responsible for depositing sulphide in slate 2.75 billion years ago [10]. Another study showed that Gram-positive bacteria were more likely to be involved in the

weathering of black slate, especially in the presence of nutrients and absorbing 22% of available carbon present in slate [11]. Another report indicated that the multi-copper enzymes, laccases, which are usually involved in degrading lignin were responsible for causing erosion of black slate in order to access the organic carbon [12]. Both studies examined the accessibility of microorganisms to carbon fixed within black slate which is not present in other coloured slates. It is remarkable that black slate remains resistant to microbial attack considering black slate is one of the few rocks with a considerable carbon content in contrast to other rocks. Why slate was chosen to be a constituent in the original balm 200 years ago will always remain a mystery although the knowledge into the preparation of the balm has been passed down through the generations. Currently, it is proposed that the high levels of Fe and Al in Welsh slate [13] may contribute to oxidative bacteria cell death through a process of forming hydroxyl radicals that attack cell proteins when leached from clay particles [2].

The aim of this study was to establish the appropriate method to study the anti-microbial effect of slate particles on bacteria that could be involved in wound infections. Bacterial growth studies were conducted in a suspension with adequate mixing to facilitate contact between bacteria and the slate particles, facilitating the diffusion of minerals from slate into suspension and to sustain an oxygenated environment. The growth of *Staphylococcus aureus* 9518 on slate particles was compared with other similar materials such as Celatom [diacotameous earth], silica gel and Cel fine, which acted as controls.

2. Materials and methods

Purple slate from North Wales and supplied by Mon Naturals Ltd, was milled into a fine powder using a rock mill with the majority <0.2 mm diameter. Celatom, silica gel and Cel Fine were purchased from Sigma with the intention of using these as negative controls. The anti-microbial properties of these particles were examined against *Staphylococcus aureus* NCIMB 7518, a human pathogen which is regularly used in anti-microbial testing. Horizontal shaking and temperature control of the

samples was achieved using an AccuTherm shaker (Labnet International Inc) and vertical rotation of the samples was achieved using a Stuart rotator SB3 that was placed into a LMS incubator set at the desired temperature. The experiments to evaluate the effect of horizontal shaking were performed in triplicate by weighing 100 mg of each particulate material into Eppendorf tubes and these tubes were sterilized by autoclaving at 121 °C (15 min). Once cooled, 400 µl of *S. aureus* was added, which had been grown for 24 h in LB at 30°C and 200 rpm, and then diluted 1/200 in LB to about 1 x 10^7 cells per ml. The total direct cell counts were determined directly under a microscope using a haemocytometer. The re-suspended particulate materials were incubated at 30°C with horizontal shaking an angular velocity of 31.42 rad/s for 24 h, in order to ensure that the particles remained suspended during incubation.

The experiments to evaluate the effect of extractives from slate powder were determined by vigorously shaking the powder in water. Slate powder (100 mg) was weighed into Eppendorf tubes and autoclaved at 121°C for 15 min and then 0.9 ml sterile distilled water was added to each tube. The tubes were shaken at 300 rpm, 30 °C for 24 h and then centrifuged at 13000 rpm for 5 min. An aliquot of 100 µl of supernatant was placed into a fresh sterile Eppendorf tube along with 100 µl LB culture of *S. aureus* that had been diluted 50-fold. A similar concentration of *S. aureus* was added to the controls containing saline solution. These cultures were incubated at 30°C with horizontal shaking at an angular velocity of 31.42 rad/s for 24 h.

The experiments to evaluate the effect of rotational mixing on the growth of *S. aureus* was performed by weighing 0.5 g of each particulate material into 50 ml Falcon tubes that were autoclaved. Afterwards, 2 ml 500-fold diluted *S. aureus,* which had been grown in LB for 24 h, with shaking at 200 rpm, was added. The tubes were placed onto a vertical rotation device and were incubated at an angular velocity of 4.19 rad/ s and at 25°C or 30°C.

At end of the experiments, the suspension was allowed to settle for <1 min and 100 µl was removed. The colony forming units (CFU) were determined by ten-fold serial dilution in saline solution (0.85% (w/v) NaCl), vortex mixed for 20 s and

plated onto LB agar. The plates were incubated at 30 °C for 2 days and then colonies were counted. In addition, most probable number was determined using the microplate whereby 11.1 µl of cell suspension was ten-fold serially diluted in 100 µl of LB medium. A fresh pipette tip was used after each dilution to ensure no carryover of cells that might become attached to the tip. From the final sample within the dilution series, 11.1 µl of cell suspension was discarded to ensure the volumes within each well remained at 100 µl. The microplates were incubated at 30°C either with shaking within the microplate reader or without shaking and were measured after 24 h at 600 nm. Only values with an optical density greater than 0.3 were scored as positive in order to exclude false positive results. The populations were calculated as for Most Probable Number (MPN).

Statistical analysis was performed using the tTest on Log_{10} viable counts.

3. Results

The particle size of the majority of slate studied, after milling, was <0.2 mm (Table 1), which is about 1000-fold larger than typical bacterial cells, with a diameter of 1 µm. The oxide content of the different slates, as determined using x-ray diffractometer (XRDF), indicates that there are significant quantities of iron and aluminium oxides, which could negatively affect bacterial survival (Table 2). The control materials contained much lower concentrations of these oxides. Compositional comparison between purple slate and heather blue slate appeared to show that the concentrations of titanium and aluminium oxides were slightly higher in purple slate.

The effect of shaking conditions revealed that the *S. aureus* viable population declined when shaken horizontally but increased under slow rotation (Fig. 1). It would appear that the physical interaction between the slate particles and bacterial cells at the higher angular velocity during horizontal shaking, reduced the viable cell population by 98%.

The experiment, repeated under slow rotational mixing over 48 h, revealed that the population rapidly increased when associated with slate particles after 24 h but showed no further increase (Fig. 2). The population associated with the controls

continued to increase after 24 h but remained lower than the population associated with slate particles.

A comparison between two different techniques for determining viable populations, colony counting and MPN, appeared to be similar with higher populations associated with Celatom and silica gel compared with slate (Fig. 3). However, statistical analysis revealed that only results obtained with colony counting were statistically different. A high cut-off value of 0.3 was used in order to eliminate potentially erroneous results, perhaps due to the presence of minor insoluble components present in the medium, that could lead to differences between wells where no growth was observed.

The extractives recovered from slate particles appeared to have a minor transitory effect during the latter stage of the log phase growth curve of *S. aureus* (19 h) when the cells were incubated under stationary conditions (Fig. 4). However, the populations containing the slate extractives became similar to the controls as the populations reached stationary phase after prolonged growth. Under shaking conditions, the population of *S. aureus* at 24 h showed a similar trend to the populations under stationary conditions, where the population containing no extractives appeared to be higher than the population containing extractives. It is possible that the bacteria form biofilms on the slate surface under stationary conditions whereas the majority of these cells remain as planktonic cells during shaking conditions. Statistical analysis revealed that only the sample at 19 h was significantly higher with the control compared with the slate sample under stationary conditions ($P = 0.016$). More rigorous methods to remove the biofilms using the stomacher might have revealed higher bacterial populations.

4. Discussion

The concentrations of iron and aluminium oxides that were associated with the slate particles were similar to those previously described for purple slate from the same region [14]. Consequently, the concentration of iron oxides in slate was present at higher concentrations to clays showing anti-microbial activity. More

specifically, the higher concentration of titanium oxide associated with purple slate compared with heather blue slate may have bacteriocidal activity based on the findings in a previous study using titanium dioxide coated nanoparticle [15]. However, another study showed that anti-microbial activity was found to be associated with particles smaller than 0.2 μm rather than with larger particles [3] and size determination of the slate particles appeared to be larger.

The initial results indicated that the slate particles showed anti-microbial activity against *S. aureus* when incubated under horizontal shaking. However, it was considered that the bacteria had become physically disrupted based on the physical hardness of slate compared with clay. As a comparison, another study revealed that higher agitation speeds of 503 rad/s (4800 rpm) for 1 min using zirconia/silica beads showed that *Bacillus subtilis* spores were lysed [16]. It must be assumed that the disintegration of bacterial spores may be more difficult to achieve in comparison to a laboratory grown *S. aureus* on complex medium. In our experiment, shaking occurred over 24 h although it is uncertain whether the reduction in bacterial counts occurred much sooner. It is also possible that cells had remained intact and become non-viable because no direct imaging technique, e.g. *Bac*Light Live/ Dead with fluorescent microscopy, was used to assess the remaining population.

The MPN results provide an approximate estimate of populations with values occurring at 3 distinct values when analysed in triplicate. More accurate values would be obtained by increasing the number of replicates leading to improved accuracy. In addition, it is possible that the high cut-off value of 0.3 could be reduced by using a soluble clear minimal medium or by filtering the LB medium before or after autoclaving in order to reduce media specific effects. The advantage of using the MPN is that fewer materials are used, it is rapid to set up and the effects can be monitored continuously (Fig. 3) whereas the advantage of using colony counting is accurate results can be obtained within a specific timeframe.

The assessment of extractives released from slate on populations growing under shaking conditions after 24 h revealed these were higher compared to the population growing under stationary conditions, perhaps due to oxygen transport constraints. Previous studies have shown with anti-bacterial clays that the

extractives showed a dramatic decrease in bacterial populations, particularly under slightly acidic or alkaline conditions where potentially inhibitory metal oxides become soluble [1, 2, 3]. However, some clays, such as bentonite, may have a stimulatory or inhibitory effect on respiration of growth of bacteria depending on the concentration of the clay [16]. Although different quantities of slate particles were used in each of the experiments, the concentration of 0.25 g slate per ml culture was similar. It would appear that the slate particles contain inhibitory compounds that limit bacterial growth during the initial growth stages but the concentration of these compounds decrease when they are absorbed into the dead bacterial detritus. A comparison between the experiments revealed that the concentration of LB was slightly higher in the experiments where shaking was used, compared with rotational mixing that could result in higher populations. However, in the natural environment on the surface of the skin, the readily availability of easily metabolizable nutrients is likely to be lower and instead resident *S. aureus* populations would be slowly degrading dead skin cells. Therefore, assuming titanium oxide is the inhibitory compound, it is possible that the concentration of this oxide is sufficiently enough to limit a rapid growth of opportunistic bacteria but allow resident slow growing bacteria to continue to proliferate. Furthermore, it would be expected that the inhibitory effect of the balm would be greater due to the higher concentration of slate particles compared with the slate particles that were diluted in the growth experiments.

Previous work has shown that clays with anti-bacterial properties may contain other reduced ferrous minerals (e.g. pyrite) that drive production of reactive oxygen species, including hydrogen peroxide and hydroxyl radicals, which damage the bacterial cell membranes and intracellular proteins [3]. Therefore, it is possible that this is also occurring with slate due to the increased bacterial population, but there is insufficient reduced iron to negatively affect the bacteria. It would appear that kaolinite had a similar effect in increasing the populations of *Escherichia coli* with a concomitant decrease in bacterial cell sizes [18]. The authors concluded that catabolism was down-regulated while the metabolism of acetate was upregulated, favouring cell division over cell maintenance. The cell sizes of *S. aureus* are

considerably smaller than other microorganisms that were examined and the higher surface area may result in more notable differences being observed.

5. Conclusions

This study revealed that mixing conditions have a profound effect on the growth of *S. aureus* with slate particles and growth under low rotational speeds did not reveal any antimicrobial activity. However, the volume of liquid and the transient contact between bacteria and slate particles might limit assessing potential antimicrobial activity. In the natural environment on skin, the contact between slate particles and microorganisms on the skin surface would be constant with considerably lower quantities of free water. Extracts from slate particles appeared to show some antimicrobial activity at the start but this activity began to decline as bacterial populations increased.

Conflicts of Interests

The authors declare they have no conflicts of interests

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Table 1 Distribution of particle slate particle sizes after ball milling using the Kajaani FS-200

Particle size	Percentage
0.5-1.2 mm	0.93
0.2-0.5 mm	1.05
<0.2 mm	98.01

Table 2 Percentage compositional of oxide content in slate compared with controls which was determined using XRDF

Oxide	Purple slate	Heather blue slate	Celatom	Cel Fine	Silica gel
SiO_2	57.96	58.3	88.79	91.1	100
TiO_2	0.98	0.9	-	-	-
Al_2O_3	19.81	19.9	4.58	4.0	-
Fe_2O_3	9.96	8.5	1.50	1.3	-
MgO	2.25	2.3	0.17	-	-
CaO	-	-	1.16	0.5	-
NaO/ KO	-	-	-	1.1	-
Others	9.04	10.1	3.80	2.0	-

Figures

Fig. 1 The growth of *S. aureus* for 24 h on diluted LB medium under horizontal shaking at 30°C and also under vertical rotation at 25°C. The bacterial population was determined using plate counting. Statistical analysis at $P<0.05$ are described along with actual values. The population under shaking were significantly lower with slate compared with Celatom ($P = 0.009$) and silica gel (0.014). In contrast, the population under rotation was significantly higher with slate compared with Celatom and Cel Fine, P at 0.028 and 0.039, respectively.

Fig. 2 The growth of *S. aureus* on diluted LB medium under vertical rotation at 40 rpm and 30°C over time. The population associated with slate was significantly higher compared with Cel Fine at 24 h ($P = 0.0015$) and 48 h (0.0068). The population associated with Cel Fine significantly increased ($P = 0.026$), whereas the population associated with slate remained unchanged.

Fig. 3 The growth of *S. aureus* on LB medium over 24 h under horizontal shaking and under stationary conditions. Growth was assessed using different methods using plate counting of colonies (CFU) and Most Probable Number (MPN). CFU count was significantly lower with slate compared to Celatom ($P = 0.009$) and silica gel (0.014). MPN was significantly lower with slate compared to silica gel ($P = 0.016$).

Fig. 4 The activity of extractives from slate powder was assessed by growth of *S. aureus* on LB medium under stationary and shaking conditions at 30°C for 24 h. The extractives appeared to have an initial effect in limiting *S. aureus* growth and was significantly lower at 19 h under stationary conditions ($P = 0.016$).