THE EFFECT OF *STAPHYLOCOCCUS EPIDERMIDIS* ON THE pH AND DISSOLUTION OF SUBSTITUTE BONE GRAFT MATERIALS

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A thesis submitted to the faculty of medicine and dentistry for the degree of master of philosophy

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ABSTRACT

Objective: To determine the effect of *S. epidermidis* 11047 on the pH and dissolution of 5 different bone substitute materials.

Method: Part 1 – Measured the pH, calcium and phosphate of the culture medium in the presence and absence of the 5 bone substitute materials before and after autoclaving. Part 2 – Measured the pH of the culture medium in the presence and absence of the 5 bone substitute materials before autoclaving and measurement of pH, calcium and phosphate 24 hrs later following incubation in the presence or absence of *S. epidermidis*.

Results: There was no clinically significant difference in pH, calcium or phosphate measurements following autoclaving therefore part 2 of the experiment could proceed despite some statistically significant differences. In part 2, the pH of the test samples (with bacteria) after 24 hrs showed a statistically and potentially clinically significant reduction in pH. Although the pH was maintained just above the 'critical pH' of 5.3 in all cases. Despite some statistically significant differences in the calcium and phosphate measurements in the test and control groups, there is a lack of consistency and only small differences in calcium or phosphate measurements. Cerasorb® M (H4) underwent physical degradation, which

was obvious to the naked eye and could be measured using SEM images. The degradation resulted in fragments up to 7 times smaller in the test sample as compared with the control and untreated sample.

Conclusions:

Part 1

- Autoclaving does not have a clinically significant effect on pH, calcium or phosphate levels of a culture medium in the presence or absence of the 5 bone substitute materials for the purposes of this study
- There was a statistically significant reduction in pH, with a maximum decrease of 0.09

Part 2

- There was a statistically and potentially clinically significant reduction in pH caused by the presence of *S. epidermidis* in all test samples
- Cerasorb® M (H4) is degraded in the presence of *S. epidermidis*

ACKNOWLEDGEMENTS

I would like to thank the following people for their assistance in the completion of this thesis:

My supervisor Dr Rachel L. Sammons, for her kind assistance, encouragement and patience through out my research

Mrs Sue Fisher for her expertise in guiding me through the use of the scanning electron microscopes

Dr Pamela Walsh for her much appreciated help with journals as well as providing excellent contacts

Dr P. Rock for his help and guidance in getting my research set up

Dr A. Dhopatkar for his assistance in the final stages of my write up

My family for their constant encouragement, with special thanks to Grainne for making sure my write up was handed in before the submission deadline!

And especially my husband Brian for his support through it all

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LIST OF ABBREVIATIONS

Bacteria	S
BioOss®	H3
Bonesave	H5
Broth	B
Cerasorb®	H1
Cerasorb® M	H4
Hydroxyapatite	HA
Optical density	OD
Ossbone	H2
Scanning electron microscope	SEM
Staphylococcus *aureus*	*S. aureus*
Staphylococcus *epidermidis*	*S. epidermidis*
Tryptone soya broth	TSB
Tri-calcium phosphate	TCP

1. INTRODUCTION

1.1 Bone

Bone is a complicated and specialised form of connective tissue that provides mechanical support to the body as well as serving as a reservoir for minerals including calcium and phosphate. Although bone is a dynamic tissue with the ability to remodel and even regenerate there are many instances were bone grafting is required (Murugan and Ramakrishna, 2005). The presence of extensive local bone loss regardless of the cause presents a considerable clinical challenge. Replacing missing tissue within the body is fraught with problems and consideration must be given to the nature of the graft tissue and its biocompatibility, the availability of appropriate material and its acquisition (Burchardt, 1987). The ideal bone graft would possess all the qualities of the bone it is replacing and should exhibit:

(i) osteointegration, the ability to chemically bond to the surface of bone without an intervening layer of fibrous tissue.

(ii) osteoconduction, the ability to support the growth of bone over its surface.

(iii) osteoinduction, the ability to induce differentiation of pluripotential stem cells from surrounding tissue to an osteoblastic phenotype.

(iv) osteogenesis, the formation of new bone by osteoblastic cells present within the graft material. (Moore *et al*., 2001)

1.1.1 Bone Grafting

Only an autogenous bone graft (bone harvested from the same individual) has the potential to fulfil all of the above characteristics, which makes it an ideal graft material. However, the disadvantages of autogenous grafts include, increased operative time, limited availability and morbidity related to blood loss, wound complications, local sensory loss and pain (Kurz *et al*, 1989).

Allografts (bone harvested from a donor from the same species) address the issues of availability and avoidance of morbidity. They are of use when there are large bony defects, which require structural support or when there is an inadequate volume of autogenous bone available. Although an allograft does possess osteoinductive properties, this may only be in the demineralised form (Moore *et al*, 2001). Complications associated with allografts include fracture, non-union and infection, with greater infection risks with increased size of the graft. There is also a potential risk of viral transmission, which requires the screening of all donors (Garbuz *et al*, 1998).

Xenografts (bone harvested from another species) are a more readily available source of graft material but have similar complications to allografts, in addition to an increased risk of rejection via an immune response. Xenografts are treated to reduce the antigenicity and and hence reduce the immune response. This resultant matrix retains the structural properties of cancellous bone but the graft has no osteoinductive capacity (Elsinger and Lead, 1996). Thompson *et al.* (2002) showed 5 cases in which bovine xenografts to the ankle and hind foot failed due to non-union. This was followed by successful clinical and radiological union when secondary surgery was carried out using

iliac crest autografts in each case. The conclusions of this paper were based on experience but suggested that a deproteinated xenograft can incite a significant foreign body reaction, leading to failure of graft incorporation and ultimately non-union. It was suggested that this type of xenograft should be avoided in the ankle and hind foot but did not rule out its effectiveness elsewhere.

The alternative to using donor bone is to use a synthetic (alloplastic) graft material. The benefits of this alternative include availability, sterility, reduced cost, and reduced morbidity. However, there are disadvantages to the many types of synthetic bone substitute materials available, which explain why such therapy is not usually the first choice. Each case requiring a bone graft must be assessed individually in terms of the nature of the bony defect and the characteristics of the available bone grafts (Murugan and Ramakrishna, 2005).

One way of providing a strong, long lasting interface between a bone replacement implant and the surrounding tissue involves the use of biomaterials. These materials mimic the behaviour of natural bone, and in some cases osteoclasts resorb them and replace them with natural bone. Such materials include hydroxyapatite (HA), Bioglass® and Plaster of Paris (calcium sulphate dihydrate) (Narayan, 2004).

1.2 Biomaterials

1.2.1 Synthetic bone substitutes

Biomaterials are alternatives to the use of human or animal bone. A biomaterial is defined as 'any synthetic material that is used to replace or restore function to a body tissue and is continuously or intermittently in contact with body fluids' (Agrawal, 1998). Since biomaterials are placed inside the body there are very important restrictions on the properties of such materials, they must be biocompatible, non-toxic and non-carcinogenic. One of the most prominent areas for application of biomaterials is that of orthopaedic implant devices (Davis, 2003). Biomaterials have been used for many years and were developed on a 'trial and error' basis with no specificity to tailor them to their individual use. By coupling biomaterials with new developments in nanotechnology and molecular biology, it is hoped that engineered surfaces can be synthesised to enable specific surface bioreactions and ultimately integration of the biomaterial into the living system (Ratner, 1993).

1.2.2 Composition

Synthetic bone grafts possess up to two of the characteristics of an ideal bone graft, osteointegration and osteoconduction. Synthetic materials that demonstrate suitable characteristics for bone grafting include those composed of calcium, silicon or aluminium (Moore *et al*, 2001).

Synthetic bone graft materials may be either single or multi phase. Single-phase materials do not always provide all the essential features required for bone growth. Multiphase materials (composites) have a structure and composition similar to natural bone. Nano-composites, particularly HA and collagen-based materials have gained much

recognition not only due to their composition and structural similarity to natural bone but also their unique functional properties such as larger surface area and superior mechanical strength compared with their single phase counterparts (Murugan and Ramakrishna, 2005). Second generation bioactive implants promote regeneration of the surrounding tissue through the production of components that elicit a controlled action and reaction. Third generation biomaterials are biocompatible, restorable and bioactive and they are also being designed to activate genes that stimulate living tissues. There is now a goal to produce a bone-filling biomaterial with architectural and mechanical properties that enable osteogenesis through a controlled release of bio-active molecules at the living tissue graft interface (Palazzo *et al*., 2005).

1.2.3 Bioceramics

Ceramics that are highly biocompatible and tissue responsive may also be referred to as bioceramics. They can be categorised into three types according to their elicited tissue response (Murugan and Ramakrishna, 2005):

i. Nearly inert e.g. alumina and zirconia
ii. Bioactive e.g. HA and bioglass
iii. Bioresorbable e.g. tri-calcium phosphate (TCP)

The bioactive group elicits a strong interfacial interaction with the host tissue and they are considered to provide osteointegrative stimuli. They are however much less resorbable than TCP, which is widely used as a bioresorbable bone graft (Murugan and Ramakrishna, 2005).

1.2.4 Calcium phosphate compounds

Many forms of calcium phosphate have been used in the development of bone substitute materials and some of the main compounds are summarised in table 1.1 (Kamitakahara *et al.,* 2008).

Table 1.1: Main Calcium phosphate compounds (Kamitakahara *et al*., 2008)

Ca/P molar ratio	Compound	Formula	Symbol
0.5	Monocalcium phosphate monohydrate	$Ca(H_2PO_4)_2\ H_2O$	MCPM
0.5	Monocalcium phosphate anhydrous	$Ca(H_2PO_4)_2$	MCPA
1.0	Dicalcium phosphate dihydrate	$CaHPO_4 \cdot 2H_2O$	DCPD
1.0	Dicalcium phosphate anhydrous	$CaHPO_4$	DCPA
1.33	Octacalcium phosphate	$Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$	OCP
1.5	Tricalcium phosphate	α-$Ca_3(PO_4)_2$	α-TCP
1.5	Tricalcium phosphate b	β-$Ca_3(PO_4)_2$	β-TCP
1.67	Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	HA
2.0	Tetracalcium phosphate	$Ca_4(PO_4)_2O$	TTCP

Tricalcium phosphate (TCP) ceramics resorb during bone regeneration and can be completely substituted by the bone tissue after stimulation of bone formation. Therefore TCP ceramics are considered as a potential scaffold material for supporting bone regeneration (Kamitakahara *et al*., 2008).

Biphasic calcium phosphate (BCP) bioceramics are bone substitute materials consisting of a mixture of HA and beta-tricalcium phoshate (β-TCP) of varying ratios. The β-TCP is preferentially dissolved and the bioreactivity is inversely proportional to the HA/ β-TCP ratio as well as dependant on the crystallinity of the β-TCP. Recommended use includes an alternative or additive to autogenous bone in both dental and orthopaedic treatment (Legeros *et al*., 2003).

1.2.5 Hydroxyapatite

HA is a calcium phosphate-based bioceramic. Stoichiometric HA has a chemical composition of:

$$Ca_{10}(PO_4)_6(OH)_2$$

It is frequently used as a bone graft substitute owing to its chemical and structural similarity to natural bone mineral. HA derived either from natural sources or made synthetically is regarded as a bioactive substance, since it forms a strong chemical bond with the host bone tissue. It is also osteoconductive, non-toxic, non-immunogenic and its structure is crystallographically similar to that of bone mineral, with a comparable amount of carbonate substitution (Murugan and Ramakrishna, 2005). HA is the most commonly used calcium phosphate in bone graft fabrication (Vallet-Regis and Gondalez-Calbet, 2004)

HA was first synthesised by Daubree (1851) but it was not until 1951 that a synthetic HA suitable for repair of bone defects was developed (Ray and Ward, 1951). Since that time many companies have commercially developed and sold HA for clinical use. Although

HA is excellent as a bone graft material, it is limited in its use in orthopaedic applications due to its inherent low fracture toughness. (Murugan and Ramakrishna, 2005)

Chu *et al*. (2002) studied the mechanical and in vivo performance of HA implants with controlled architictures and demonstrated that the internal architecture of HA implants has an impact on mechanical and biological behaviour. The results of this study showed that it is possible to control the shape of newly formed bone tissue via the internal architecture of the implant. HA implants with two different architectural designs (orthogonal and radial channels) were implanted in the mandibles of minipigs. Although normal bone regeneration occurred in both groups, the shape of the regenerated bone was significantly different. In the samples with the orthogonal channels, bone and HA formed an interpenetrating matrix, whereas in samples with radial channels the newly formed bone formed an intact piece at the centre of the implant. This would suggest that the geometry as well as the composition of the HA implant is important in the clinical outcome.

1.2.6 Uses of synthetic bone subtitutes

HA has many clinical uses, which include augmentation of atrophic ridges, repair of long bone defects, repair of ununited bone fractures, middle ear prostheses, spinal/vertebral fusions, cranioplasty and craniofacial repair. It has also been used extensively in dentistry as well as for biomolecular and drug delivery (Murugan and Ramakrishna, 2005).

TCP and biphasic HA/TCP bone substitute materials can be manufactured with porosity such that its replacement produces architecture similar to that of real bone. In addition the porosity is a fundamental feature, which allows the bone graft to be used as a potential local drug release agent (Palazzo et al., 2005). Drugs and agents that will preferentially bind to bone mineral include bisphosphonates and tetracyclines. As bone is continually turning over, there is an opportunity to deliver bioactive molecules or therapeutic agents to these sites and this has the potential to improve efficacy and reduce undesired side effects (Shea and Miller, 2005).

1.2.7 Dental applications of hydroxyapatite and tricalcium phosphate graft materials

The use of porous HA to treat intra-bony defects in patients with periodontal disease has been shown to decrease pocket depth (Kenny *et al*., 1988). Cararopoli *et al*. (2006) investigated the orthodontic movement of migrated teeth through infrabony defects that have been previously augmented with a biomaterial. The biomaterial was a collagen bovine bone mineral (not HA) and the study reported no detrimental effects to the implant material from orthodontic tooth movement. However, only three patients were studied making it difficult to draw meaningful conclusions. There were no controls and it is impossible to know whether the final bone heights recorded were a result of orthodontic tooth movement alone. However, I have included this as there are few studies on this topic and the concept of tooth movement through a bone graft site is important in terms of the developing patient and particularly for cleft palate patients where the

permanent canine teeth erupt into a graft site and where orthodontic tooth movement is frequently carried out (Mitchell, 2007).

Wolford et al. (2002) advised against the use of PBHA when grafting in the alveolar cleft, specifically when eruptive or orthodontic tooth movement was anticipated in this region. This was due to the risk of infection and loss of the graft. A distinction was however made regarding the much more favourable situation when PBHA was used in maxillary osteotomy sites, even when this was associated with the maxillary sinus.

Secondary alveolar cleft augmentation involves the placement of a graft in a difficult area. The aim is not only to replace the bone, but also to stabilise the pre-maxilla, support the alar base, aid closure of residual oronasal fistulae and facilitate eruption of the permanent canine (Mitchell, 2007).

Linton *et al*. (2001) investigated the use of calcium phosphate ceramic as an alternative to secondary alveolar bone grafting in cleft lip and palate patients by placing HA implants with high biodegradation rates in beagle dogs. No differences were found for tooth eruption for the beagles through the four graft materials compared with controls. The authors did suggest further investigation would be useful, including increasing the graft site and subsequent follow up to proceed beyond eruption as well as investigating orthodontic tooth movement of the teeth which had erupted through the graft site.

A case report by Proff *et al*. (2006) put forward the potential use of bone substitute materials prior to orthodontic space closure after tooth extraction, as well as for use in residual defects in cleft lip and palate patients. The objective in both these

scenarios is structural preservation of the alveolar ridge. A case was presented where immediate insertion of bone graft substitute, Nanobone®, into a fresh extraction site appeared to preserve structure of the alveolus prior to beginning orthodontic space closure. However, this is an isolated case and although it indicates the possibility for such use of this material, it is not possible to draw any other conclusions.

HA powder has been investigated for use in dental composite materials as it is deemed an appropriate reinforcement for organic polymers by virtue of its mechanical and biological properties. According to Santos et al. (2001) the main reasons for the potential use of HA powder in dental composites are that it is the 'structural prototype for the principal inorganic crystalline constituent of tooth', as well as being radio-opaque, highly resistant to moisture and of ideal hardness. This led them to research the mechanical properties and *in vitro* bioactivity of composites in which HA was used as a filler. Results of this study revealed composites of higher stiffness and that these materials could form a compact and continuous calcium phosphate layer on their surface after 4 weeks of immersion in simulated body fluid. However this study did not test these composite materials within the oral cavity.

HA has also been used to improve the bond between other implant materials. The technique of plasma spraying is used to deposit a thin, dense layer of HA onto a titanium substrate. Implants with a surface of HA are referred to as bioactive due to the strong bond, which is produced between the bone and implants made of sintered HA. Thin coatings are recommended rather than the use of implants made solely of sintered HA,

due to the poor biomechanical properties of this material. De Groot *et al*. (1987) demonstrated the difference between the inert material titanium by means of which only a close contact with bone can be achieved (osseointegration) and the bioactive apatite, which not only shows osseointegration but also bone bonding. The latter is characterised by continuity between the crystals of HA in the graft material and those in bone (Bonfield and Luklinska, 1991).

1.2.8 Stability and dissolution of hydroxyapatite

According to Ducheyne et al. (1993) the variation of the crystal structure of calcium phosphate ceramics produces a wide variation in dissolution behaviour. The results of this study showed HA to have the slowest rate of dissolution of the monophasic calcium phosphate ceramics tested and tetracalcium phosphate to have the highest rate of dissolution. This was based on measurements of calcium and phosphate concentration in solution over defined time periods at a constant pH of 7.3 and a temperature of 37°C.

Degradation of calcium phosphate ceramics was investigated by Koerten and Van der Meulen (1998) and this research determined that the rate of degradation depended on the type of ceramic - beta-tricalcium phosphate > HA > fluorapatite. It was shown that the dissolution rate of the ceramics is pH dependent, with the higher pH resulting in less dissolution. The samples used were in the form of spheres (average diameter 11.3 $\pm$ 6.3μm) and degradation was characterised by dissolution occurring between the necks and formation of cracks and irregularities between the grains. As a consequence grains were released.

It is well documented that a pH of less than 5.3 is required before dental HA starts to dissolve, above this pH, the enamel remains in tact (Stephan and Miller, 1943).

Oonishi *et al.* (2000) compared bone growth behaviour in granules of Bioglass®, A-W glass ceramic and HA and concluded that the rate of bone formation correlates with the rate of dissolution of the particles.

A similar study investigating bony ingrowth of bone graft substitutes compared a ceramic graft material (ProOsteon), demineralised bone matrix and a composite material (Collagraft) following implantation in the femoral condyle of a rabbit. The study demonstrated that there were differences in the biodegradation and bony ingrowth of these three materials. The ceramic graft averaged 43% bony ingrowth but with most of the graft remaining. The composite graft averaged the greatest bony ingrowth at 56%, again with most of the graft remaining. The demineralised bone matrix showed the least bony ingrowth at 35% but this was associated with nearly complete resorption of the graft. This may demonstrate that the timing of dissolution is important in the success of the graft material in terms of enabling bony ingrowth.

In a study by Maxian *et al*. (1993) the theory that dissolution of various apatite coatings on a metal orthopaedic prostheses promoted enhanced bone bonding led to an investigation into coating dissolution rate. Results showed that the amount of calcium dissolved from calcium phosphate coated implants was strongly dependent on the chemistry of the coating, with the poorly crystallised HA undergoing the most

degradation and the amorphous coating being more stable. Dissolution was less dependent on either pH or incubation time.

1.2.9 Bacterial influence on ceramic degradation

A study by Kurkcuoglu (2001), investigated 'the effect of staphylococci on dissolution of synthetic calcium phosphate biomaterials'. In Kurkcuoglu's study three calcium phosphate materials were tested in the form of powder or fired discs. The results showed that dissolution of the commercially available calcium phosphate materials being tested were dependent on the individual material. Biotal HA (even that with 15% TCP impurity) did not dissolve when exposed to strains of Staphylococcus *epidermidis* (*S. epidermidis)*. Bioland HA and TCP were shown to dissolve more in the presence of *S. epidermidis* and Staphylococcus *aureus (S. aureus)* than without. When powder forms of these materials were incubated in the presence of *S. epidermidis*, Bioland HA and TCP showed more dissolution, as indicated by calcium release into the medium when they were exposed to the bacteria than when there were no bacteria. Biotal HA powder did not dissolve in the presence of bacteria and even in a further experiment performed with an impure Biotal HA powder with 15% TCP impurity, no increase in calcium concentration in the medium after 24hr was shown.

Although there is little known about bacterial degradation of bone graft materials, there has been investigation into degradation of other ceramic material. Concrete, natural stone and glass are ceramic materials, which are commonly used in construction of buildings. Sand and Bock (1991) carried out a study into the biodeterioration of ceramic

materials by biogenic acids. They found that microorganisms contribute to the degradation of concrete and that this is caused by excretion of mineral or organic acids. They were able to demonstrate differences in the resistance of various concretes and reported that chemical and/or physical testing of materials only is not sufficient to determine how resistant they are to biological attack.

Most microorganisms can degrade materials and this is generally caused by excretion of metabolic intermediates and/or end products, deterioration of a material is generally caused by a combination of factors. Determining these factors is often difficult because some compounds may undergo metabolic turnover, for example organic acids (Sand, 1997).

1.3 Infections associated with biomaterials

1.3.1 Infection of synthetic bone grafts

There are conflicting reports regarding hydroxyapatite and its interaction with bacteria. Opalchenova *et al*. (1996) tested the influence of biphasic calcium phosphates on laboratory-isolated Gram-negative bacteria, including *S. aureus* and *S. epidermidis.* and demonstrated a reduction of the bacterial cell population within 24hrs, suggesting an antibacterial effect. It was noted however, that it was difficult to directly compare this to any clinical situation because of the complex surface reactions which could occur in the presence of the various chemical mediators, particularly during the inflammatory process, which is inevitable following implant placement

A prospective study to evaluate the long-term clinical and radiographic results following placement of coralline porous block HA (PBHA) grafts in orthognathic surgery and craniofacial augmentation recorded a high percentage of success and efficacy (Cottrell and Wolford, 1998). At follow up of five years, clinical evaluations included signs and symptoms of infection, wound dehiscence, implant exposure, displacement and changes in the overlying mucosa as well as development of oronasal or oroantral fistulae. Only 4.9% of implants were removed during the evaluation period and lateral maxillary wall grafting had 95.7% success. 14% of midpalatal implants used for midpalate expansion were lost and this was attributed to exposure of the implant to the oral or nasal cavity at the time of surgery. There was 100% failure of alveolar cleft grafts when PBHA was used. This comprised 5 implants, four of which were removed within 3 months of placement due to infection and the fifth was removed at 20 months, also due to infection. It was suggested that lack of rigidity of the implant and contamination with oral/nasal flora led to failure of the grafts in the alveolar cleft site. (Cottrell and Wolford, 1998).

HA orbital implants are used in cases of eye enucleation or evisceration and in secondary implant cases. Orbital implants are significant due to the fact that complications have gradually become apparent since their initial clinical use in 1985. Although few implant infections have been reported, many of these have resulted in actual removal of the implant to correct the symptoms (Oestreicher *et al*., 1999).

Oestreicher *et al.* (1999) reported a case involving a patient who had undergone routine secondary HA orbital implant surgery and subsequently developed signs and symptoms of infection. Removal of the implant settled the problems and resolution was

rapid. Although culture results were negative, histopathological examination of the implant revealed intertrabecular spaces with clusters of organisms resembling Aspergillus (Oestreicher *et al*., 1999).

Bacterial infection in six cases of HA orbital implants were reported by You *et al.* (2003). The patients were treated in the same hospital for the signs and symptoms of infection, but not all had their initial surgery in that hospital. All were different ages, mixed gender, had different post - operative times and surgical histories and medical histories were not detailed. Microbiological testing of cultures revealed bacterial colonisation in all six cases and symptoms resolved following removal of the HA implant. The recommendations from this study were that at the first signs of bacterial infection systemic antibiotics and eye drops should be administered immediately. If this does not eradicate the infection, the implant should be considered the focus of infection and be removed immediately.

A Cochrane review article on 'horizontal and vertical bone augmentation techniques for dental implant treatment' reviews only 3 articles on the comparison of autogenous bone with bone substitutes (Meijndert 2007; Felice 2008; Fontana 2008) and in these studies only two types of bone substitute were used; BioOss and Regenaform. The results of these studies did not highlight a significant problem regarding infection of the bone substitute materials and although autogenous bone is thought of as being the 'gold standard' when it comes to grafting, this was not evident in the findings of these studies (Esposito *et al*., 2010). This is a very limited review on what is a large subject matter and further study would be required to draw meaningful conclusions.

A study by Verheyen *et al*. (1993) studied the integrity of a HA coating in the presence of staphylococci in an attempt to reveal a possible mechanism for loosening of ceramic coated implants. This was an in vitro experiment in which a plasma sprayed HA coating showed dissolution during a 24hr incubation period with S. aureus and S. epidermidis. Dissolution was measured via an increase in pH and a release of calcium in the buffer solution. There was initial calcium release in all media containing HA coated specimens, including the controls without bacteria, but after 8 hours the specimens with bacteria exhibited an increased calcium release compared with the controls. There was significantly more *S. aureus* than *S. epidermidis* when counted on the scanning micrographs, but bacteria could only be counted for up to 4 hours with *S. epidermidis* and 8 hours with *S. aureus* due to calcium phosphate precipitate forming on the surface. The scanning micrographs provided evidence that the integrity of the HA coating was damaged by the bacteria. However, the study could not confirm that this was the mechanism for loosening of ceramic coated implants as only marginal damage to the coating could be observed due to the early precipitation of calcium phosphate.

The performance of calcium phosphate ceramics in an infected site was investigated by Van Blitterswijk *et al*. (1986). Macroporous and dense HA were evaluated histologically after implantation in the middle ear of a rat with an induced *S. aureus* infection. Degradation of the HA was found to occur to a similar extent as previously implanted material in the non-infected middle ear. The author concluded that HA was 'highly suitable for middle ear implantation'. A follow on study was conducted,

again using the middle ear of rats, but this time evaluating HA during short-term infection with *S. aureus*. The histological results corresponded well to those reported for the infected middle ear cavity without an implant, indicating the bacteria did not have any influence on the degradation rate. (Van Blitterswijk *et al*., 1986)

Kinnari *et al*. (2009) carried out a study into the surface porosity and pH on the adherence of bacteria to HA and biphasic calcium phosphate (BCP) materials. The reasoning behind this study was that the porosity of these materials and the pH reduction as a result of surgical trauma predispose to bacterial infections. The author reasoned that the pH decrease could influence the surface charge of HA and BCP and that these reactive changes in the bone implant environment would influence susceptibility to implant related infections. Total porosity was measured at 20% for HA and 50% for BCP. Results showed that when pH decreased from 7.4 to 6.8, the adherence of *S. aureus* and *S. epidermidis* to both materials was significantly reduced. At both pH values the number of *S. aureus* adhered to the HA surface was lower than for BCP. Theories put forward by the author to explain the unexpected reduction in adherence of bacteria included lowering of pH damaging staphylococci or alternatively the relative difference in solubility of the ceramics but this was speculation.

This study also provided evidence that the HA and BCP under investigation have insufficient pore size to allow Staphylococci within the material structure. The pores present in these materials were measured using mercury intrusion porosimetry and they ranged from 50nm in diameter to 300nm, with a mean diameter of 200nm. As discussed

below, staphylococci range in size from 0.5-1.5µm meaning they are too large to enter the pores of the HA or BCP used in this experiment.

1.3.2 Staphylococci

Staphylococci are Gram-positive cocci. They have a diameter ranging from 0.5-1.5µm and grow in pairs, tetrads and small clusters. They usually produce the enzyme catalase and are non-spore forming. In anaerobic conditions, almost all Staphylococci produce acid from glucose, lowering the pH of the surrounding environment. The ability to clot plasma separates them into coagulase-positive or coagulase-negative staphylococci. (Pace *et al*., 2006)

Coagulase-negative Staphylococci (CNS) such as *S. epidermidis* are opportunistic pathogens and as such they have become more of an issue with the increase in the use of transient or permanent implanted medical devices. CNS are a major component of the normal flora of the cutaneous ecosystem, including the skin and mucous membranes. (Kloos and Bannerman, 1994)

The importance of staphylococci in biomaterial-associated infections is well documented. Coagulase-negative staphylococci, especially *S. epidermidis* are recognised as common pathogens associated with infections of medical implant devices in hospitals. (O'Gara and Humphreys, 2001). Pfaller and Herwaldt (1988) believed the most important factor contributing to the increasing number of nosocomial coagulase-negative staphylococcal infections was the presence of indwelling prosthetic devices in both compromised and non-compromised hosts.

With respect to CNS, the generally benign relationship with their host where they function as commensal or saprophytic organisms can change upon direct implantation of foreign bodies as this can allow these organisms to gain entry to the host. Depending upon their ability to adhere to host or foreign body surfaces, breach or avoid the host immune system, multiply, and produce products that damage the host, they may develop the lifestyle of a pathogen. A main focus on mechanisms of pathogenesis has been with foreign body infections and the role of specific adhesins and slime produced by *S. epidermidis*. There is now some understanding of the sequence of events leading to the establishment of biofilm on polymers, though the story is not complete and may be somewhat different for the establishment of infection in native tissue. It is now clear that biofilm can act as a barrier to antibiotics and limit the effectiveness of antibiotic therapy. (Kloos and Bannerman, 1994). One or more of the following have been proposed as the possible mechanisms of biofilm resistance to antimicrobial agents:
(i) delayed penetration of the antimicrobial agent through the biofilm matrix, (ii) altered growth rate of biofilm organisms, and (iii) other physiological changes due to the biofilm mode of growth (Donlan and Costerton, 2002).

Implant materials and devitalized tissues are foreign bodies and as such the boundaries with the normal host tissues are a thousand fold more vulnerable to sepsis. The inflammation generated is therefore likely to be chronic and may result in implant loosening and bacteraemia may occur due to the release of the bacteria from the biofilm into surrounding tissues. (Gristina, 1994)

1.4 The aim of this study

One of the complications of bone graft materials is infection (Felice *et al*., 2008). However it is not known whether different materials are more or less susceptible to bacterial adhesion and whether bacteria can influence the dissolution properties of the materials. In the presence of organic and inorganic compounds most microorganisms excrete organic acids while metabolising and many are capable of metabolising organic compounds by fermentation. The resulting organic solvents may react with materials, such as the bone substitute materials, causing dissolution and eventually deterioration (Sand, 1997). The aim of this study is to investigate the effects of contamination of synthetic bone substitutes with respect to potential dissolution. There are many synthetic bone substitutes available for use in the human body, however the scope of this research is focused on materials with a potential use within the oral cavity.

HA and β tri calcium phosphate are frequently used in non-load bearing regions such as the oral cavity. Another popular choice in this region is BioOss®, which is comprised of the mineral portion of bovine bone. Samples of five test materials, including HA, β tri calcium phosphate and bovine derived bone mineral will be incubated in the presence of *S. epidermidis*, an opportunistic pathogen that is known to be associated with the infection of biomaterials (Gristina, 1994). The effect of *S. epidermidis* on pH and dissolution of the test materials will be tested and scanning electron microscopy will be used to assess the adherence of bacteria to the material and any visual evidence of dissolution.

2. MATERIALS AND METHODS

2.1 Acquisition and preparation of bone substitute samples

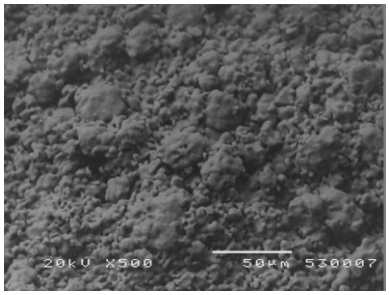
Five bone substitutes were investigated in this study. These materials were provided, for experimental use, by the companies detailed in table 2.1 following provision of a protocol based on this study. All of these samples are routinely used in the medical and dental field, except for Ossbone (Curasan), which is still in the development stage.

Table 2.1: Details of bone substitutes investigated in this experiment

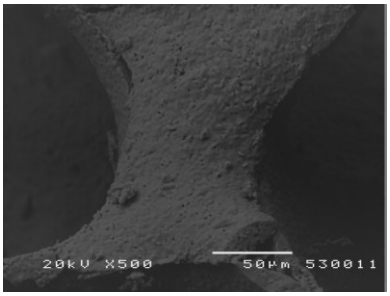
Material	Company	Composition	Structure
Cerasorb® (H1) 1000-2000μm	Curasan	Pure phase β Tri calcium phosphate	35% porosity (micropores)
Ossbone (H2) 1000-2000μm	Curasan	Hydroxyapatite	Openecell
BioOss® (H3) 0.25-1mm	Osteohealth®	Bovine bone mineral	Trabecular architecture and fine crystalline structure
Cerasorb® M (H4) 1000-2000μm	Curasan	Pure phase β Tri calcium phosphate	65% porosity (micro-, meso- and macropores)
Bonesave(H5)	Stryker®	80% Tri calcium	50% porosity

2-6mm		phoshate, 20% hydroxyapatite	

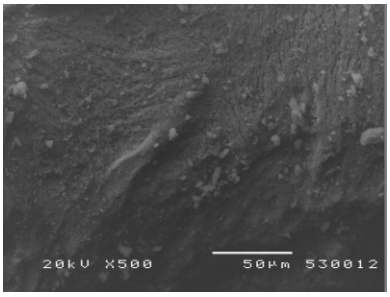
As described, the five bone substitutes varied in composition and structure and this was reflected in the widely varied morphology of the granules. This can be observed in the following SEM images (See micrograph 2.1).



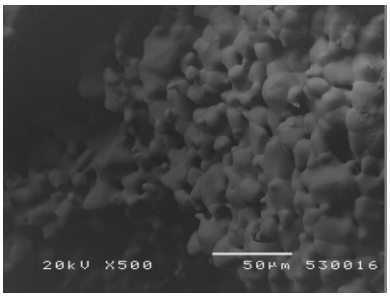
Cerasorb® (H1) - Pure phase β Tri calcium phosphate



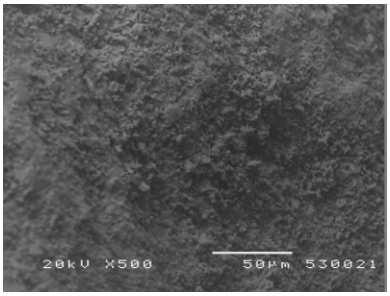
Ossbone (H2) - Hydroxyapatite



BioOss® (H3) – Bovine bone mineral



Cerasorb® M (H4) - Pure phase β Tri calcium phosphate



Bonesave (H5) – 80% tri calcium phosphate, 20% hydroxyapatite

Micrograph 2.1: H1-H5: Each showing representative granule (direct from packet) shown at X500 magnification to show surface characteristics

The synthetic bone samples ranged in size from 0.25 to 6mm granules. 0.1g +/- 0.05g of each material were weighed directly into the lid of the 50ml Corning bottles in which the testing was to proceed. The bottle was then screwed on to the lid before being inverted, thus improving the accuracy of the measurement by ruling out an intermediate vessel.

2.2 Part 1 (Preliminary work): Investigation of the influence of autoclaving on pH, calcium and phosphate concentration of the culture medium with and without the substitute bone test material

2.2.1 Culture media

Tryptone Soya Broth (TSB) was used as a liquid culture media and was prepared according to manufacturers instructions:

30g TSB powder (Oxoid, UK) was dissolved in 1000ml of distilled water. The dissolved mixture was autoclaved in a glass bottle at 120°C for 15minutes at 1.1 bar pressure.

2.2.2 pH measurement

The pH of the TSB with and without the substitute bone test material was measured using a Mettler Delta 320 pH meter, (model 3310) (Mettler Toleto Ltd, UK). The meter was calibrated before each set of samples measured, using a standard buffer solution of pH 4.0 and 7.0. The bulb of the pH meter was rinsed with distilled water between measurements.

2.2.3 Preparation of the samples

The TSB was then aliquotted into 20ml volumes in 50ml autoclavable Corning centrifuge bottles. The bone substitute materials (0.1g +/- 0.05g) were then added to these and the start pH recorded, as described above. A 5ml sample of TSB was aliquotted from each test specimen into a bijou bottle to be frozen, to allow later measurement of calcium and phosphate concentration. The table below (table 2) lists the samples prepared for this part of the testing.

Table 2.2: Part 1 (Preliminary work) – test samples

Sample name	Contents	No. of samples
B	20ml TSB only	3
B + H1	20ml TSB + 0.1g Cerasorb®	3
B + H2	20ml TSB + 0.1g Ossbone	3
B + H3	20ml TSB + 0.1g BioOss®	3
B + H4	20ml TSB + 0.1g Cerasorb® M	3
B + H5	20ml TSB + 0.1g Bonesave	3

2.2.4 Autoclaving the test samples

The samples in the above table were autoclaved at 120°C for 15minutes at 1.1 bar pressure. The lids of the 50ml autoclavable Corning bottles were loosened to allow steam to penetrate and to prevent distortion of the plastic. Samples were left to cool before completing the experimental measurements. The samples were centrifuged for 5minutes

at 5000 RPM at room temperature before a further 5ml of TSB was removed from each sample for freezing prior to calcium and phosphate measurement. A second pH measurement was then taken.

2.2.5 Measurement of calcium concentration

Calcium measurements were carried out using the Quantichrom^{TM} calcium assay kit (BioAssay systems, USA), which provided a quantitative colorometric calcium determination at 612nm. The procedure was carried out using a 96-well plate. The standards provided were diluted (see table 3 below) and 5µl of the diluted standards and samples transferred into wells of a clear bottom 96-well plate, 200µl working reagent was then added and the plate tapped lightly to mix the contents.

Table 2.3: Calcium assay – diluted standards

No	STD + H_2O	Vol (µl)	Ca (mg/dl)
1	100µl + 0µl	100	20
2	80µl + 20µl	100	16
3	60µl + 40µl	100	12
4	40µl + 60µl	100	8
5	30µl + 70µl	100	6
6	20µl + 80µl	100	4
7	10µl + 90µl	100	2
8	0µl + 100µl	100	0

The samples were then incubated for 3minutes at room temperature and the optical density (OD) was read at 570-650nm (peak absorbance at 612). The amount of calcium in the sample could then be calculated. The blank OD was subtracted from the standard OD values and these were plotted against the calcium standard concentrations. Linear regression analysis was then used to determine the slope of the graph, enabling the calcium concentration of the sample to be calculated using the following equation:

$$= \frac{OD^{\text{sample}} - OD^{\text{blank}}}{\text{Slope}} \quad \text{(mg/dL)}$$

2.2.6 **Measurement of phosphate concentration**

Phosphate measurements were carried out using the Quantichrom^{TM} phosphate assay kit (BioAssay Systems, USA), which provided a quantitative colorimetric phosphate determination at 620nm. The procedure was carried out using 96-well plate. The transfer of 50µl Blank (distilled water), standard (0.28mg/dL) and samples were carried out into duplicate wells of a clear bottom 96-well plate. This was followed by the addition of 100µl of reagent (supplied in the assay kit) and light tapping to mix. The plate was then incubated for 30minutes at room temperature and the OD read at 620nm (600-660nm)

The phosphate concentration was then calculated from the OD using the following formula:

$$\frac{\text{OD sample} - \text{OD blank}}{\text{OD standard} - \text{OD blank}} \times 0.28 \text{ (mg/dL)}$$

If the sample OD was higher than the OD for the standard, the samples had to be diluted in distilled water and the assay repeated.

2.3 Part 2 (Test results): Investigation into the influence of bacteria, with and without the substitute bone test material, on pH, calcium concentration and phosphate concentration

2.3.1 Bacteria

As discussed previously, *S. epidermidis* is one of the bacteria known to be frequently associated with infection of biomaterials. There are many strains of *S. epidermidis*, but for the purposes of this investigation a single strain, NCTC 11047, was selected and tested throughout. NCTC 11047 cells were used because this is a standard bacterial strain, which is non-pathogenic and easy to culture. It was also used to ensure continuity between these experiments and previous studies undertaken within the same laboratory.

2.3.1.1 Bacterial culture

Stocks of bacterial isolates were stored on beads (Pro-lab, UK) in a medium containing 10% glycerol, which was immediately frozen and maintained at -20°C for subsequent use. Commercial blood agar plates were used as the solid media and these were inoculated with *S. epidermidis* 11047. Inoculation was carried out using a wire bacteriological loop, which was flame sterilised prior to each application. The bacteria were then incubated for 24hrs at 37ºC.

2.3.1.2 Preparation of bacterial suspension

Bacterial suspensions were prepared in sterile 30ml universal bottles (Sterilin; Bibby Sterilin Ltd., UK). Sterile TSB was used as the liquid media. In aseptic conditions 10ml of sterile TSB was pipetted into the universal bottle. A disposable sterile bacteriological loop was used to remove one single colony from the agar plate and this was then agitated in the TSB to transfer the colony to produce the suspension. The universal bottle was placed on a rotary shaker and incubated overnight at 37ºC. Subsequent bacterial growth could be confirmed by a cloudy appearance of the TSB.

2.3.1.3 Diluting the bacterial suspension

The overnight bacterial culture was diluted 1 in 10 in TSB and the absorbance at 500nm wavelength was determined using a spectrophotometer. This was then adjusted to 0.5 by dilution in TSB. The diluted bacterial suspension was then immediately added to the prepared experimental samples.

2.3.2 Preparation of the samples

The preparation of samples for this part of the experiment was similar to that of the initial experiment on the effects of autoclaving. The difference in the second set of samples was the addition of 0.1ml of the diluted bacterial suspension of *S. epidermidis* following autoclaving (See table 4).

Table 2.4: Part 2 (Test results) – test samples

Sample name	Contents	No. of samples
B	20ml TSB only	3
B + H1	20ml TSB + 0.1g Cerasorb®	3
B + H2	20ml TSB + 0.1g Ossbone	3
B + H3	20ml TSB + 0.1g BioOss®	3
B + H4	20ml TSB + 0.1g Cerasorb® M	3
B + H5	20ml TSB + 0.1g Bonesave	3
SB	20ml TSB + 01ml bacterial suspension	3
SB + H1	20ml TSB + 0.1g Cerasorb® + 0.1ml bacterial suspension	3
SB + H2	20ml TSB + 0.1g Ossbone + 0.1ml bacterial suspension	3
SB + H3	20ml TSB + 0.1g BioOss® + 0.1ml bacterial suspension	3
SB + H4	20ml TSB + 0.1g Cerasorb® M + 0.1ml bacterial suspension	3
SB + H5	20ml TSB + 0.1g Bonesave + 0.1ml bacterial suspension	3

2.3.3 Experimental procedure

Once the test samples were prepared, the pH was measured prior to autoclaving, as previously described. The bacterial suspension (0.1ml) was added to the appropriate sample, as outlined in table 4 and all samples were then incubated for 24hrs at 37ºC.

Following incubation all samples were centrifuged for 5 minutes at 5000rpm and

Room temperature and pressure, to ensure bacteria and synthetic bone granules settled to the bottom of the bottle. At this point 5ml of each sample was pipetted from the top of the bottle for immediate freezing and storage until calcium and phosphate assays could be carried out .The pH of each sample was then measured.

This experiment was repeated on three separate occasions, more than 24hrs apart.

2.4 Part 3: Scanning electron microscopy of the bone substitute before and after testing

2.4.1 Preparation of bone substitute samples for the scanning electron microscope (SEM)

The samples selected for inspection under the SEM included an average sized granule/s of each of the synthetic bone materials being tested, taken straight from the packet to represent the structure of the material before testing. A sample of the materials after testing was selected from experiment-3. Both of these sample types had to be prepared for viewing under the SEM.

2.4.1.1 Pre-test bone substitute samples

The preparation of the pre-testing granules first required mounting of the granule onto an aluminium stub. The widest part of the sample had to be in contact with the flat aluminium stub for stability and to reduce ‘charging’ during viewing with the SEM. This was more difficult where the granule was spherical and in such cases silver DAG (Agar Scientific, Stansted, UK) was used to stick the samples to the stub. Once stable, the

samples were coated in a thin layer of gold by the technique of sputtering, using an Emitech K550X sputter coater; the gold conducts away the electrons, reducing charging.

2.4.1.2 **Post-test bone substitute samples**

The samples that had undergone testing potentially had live bacteria on the surface and careful preparation was required to preserve their morphology. The liquid medium was drained from the samples and the remaining bone substitute material was transferred to a sterile 24 well plate. Samples were first fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, then dehydrated in ethanol solutions in the following order: 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and 100%. Each sample was soaked in each solution for 10minutes with rapid transfer to avoid drying in air between each step.

The samples then had to be dried, without destroying the morphology of any bacteria present. This was carried out using the technique of critical point drying. Following dehydration the 100% ethanol was replaced by liquid CO2, which sublimes at approximately 32°C temperature and 83 bars pressure (the critical point) to gas, which is then released slowly, leaving the samples dry. This was carried out in a Polaron critical point drier.

The samples could then be prepared in the same way as the pre-test materials with regard to mounting and coating prior to viewing with the SEM.

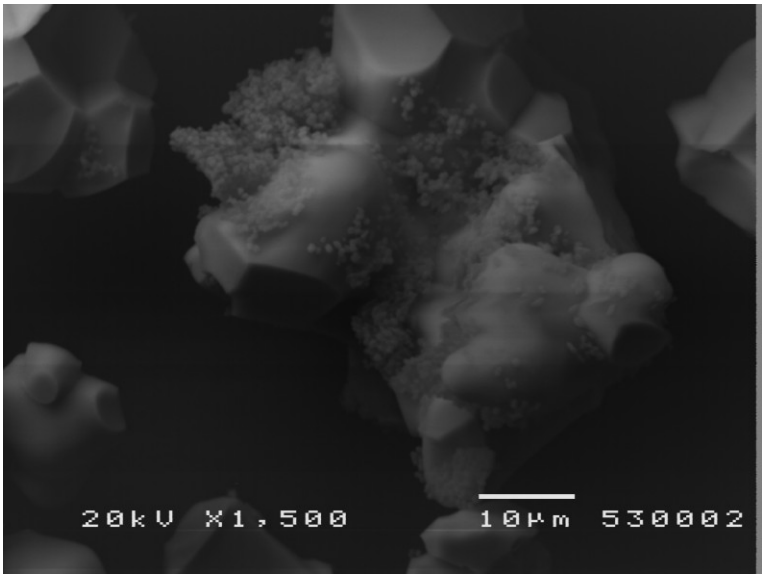
2.4.2 Viewing bone substitute samples with the SEM

Samples were viewed using the Jeol 5300 LV SEM (Jeol Ltd., UK) operating in high and low vacuum mode at 20 or 30 Kv. The Joel 840A SEM (Joel Ltd., UK) was then used to

produce clearer images at high vacuum. Representative images for each sample were recorded using Semaphore software at a number of magnifications ranging from x50 to x5000.

2.4.3 Assessing SEM images of bone substitute granules

Each sample was assessed as being either positive or negative for visibility of *S. epidermidis* based on identification of typical staphylococcal 'grape like' clusters of bacteria (Micrograph 2.2). Images were then matched for magnification, allowing comparison of granule size to be measured in the original state as well as pre and post testing.



Micrograph 2.2 Sample H4 (a) at 20KV, X 1,500 magnification showing 'grape like' form of *S. epidermidis* on granule of Cerasorb® M. These clusters were not present on the original samples

2.5 Statistical analysis

2.5.1 **Part 1 (Preliminary work)**

For pH, calcium and phosphate measurements each sample was tested before and after autoclaving and this was repeated to provide 3 sets of results. The mean and standard deviation of the data was calculated for each sample and a t-test was carried out to determine if there was a statistical difference between samples before and after autoclaving. In the pH group an analysis of variance was also carried out to test the difference between groups. (See appendix I)

2.5.2 Part 2 (Test results)

In part 2 the same experiment was repeated on three separate occasions and each experiment underwent the same statistical analysis (See appendix II-IV):

The pHs of the samples were measured 24 hrs apart for two different groups – with and without bacteria. The mean and standard deviation of the data was calculated for each set of results, then a t-test was applied to determine if there was a statistical difference between the two groups.

The calcium and phosphate measurements were taken at one time only and the comparisons were made between the control group (without bacteria) and the test group (with bacteria). The mean and spread of the data was calculated and a t-test was carried out to demonstrate any statistical difference.

3 RESULTS

3.1 Part 1 (Preliminary work): The influence of autoclaving on pH, calcium and phosphate concentration of the culture medium with and without the substitute bone test material

Preliminary tests were carried out because the TSB and samples had to be sterilised in order to subsequently test the effect of bacteria on the substrates. It was possible that autoclaving would affect the pH and this could mask any subsequent changes due to the bacteria. The pH of the culture medium with added biomaterials was therefore measured before and after autoclaving. For the same reasons, calcium and phosphate concentrations of TSB (plus biomaterials) were also measured before and after autoclaving.

3.1.1 pH changes of the liquid medium before and after autoclaving

The average starting pH of the suspension was 7.33. The results show that after autoclaving there was a small but significant difference between the two sets of data with a consistent reduction in pH of less than 0.1 (average 0.09) and the lowest recorded pH being 7.2.

The change in pH was statistically significant at the $P = 0.01$ level in all cases except H5 ($P = 0.05$). There was no statistically significant difference between the pH of

the TSB in the presence of the 5 synthetic bone substitute test materials either before or after autoclaving (P = 0.03).

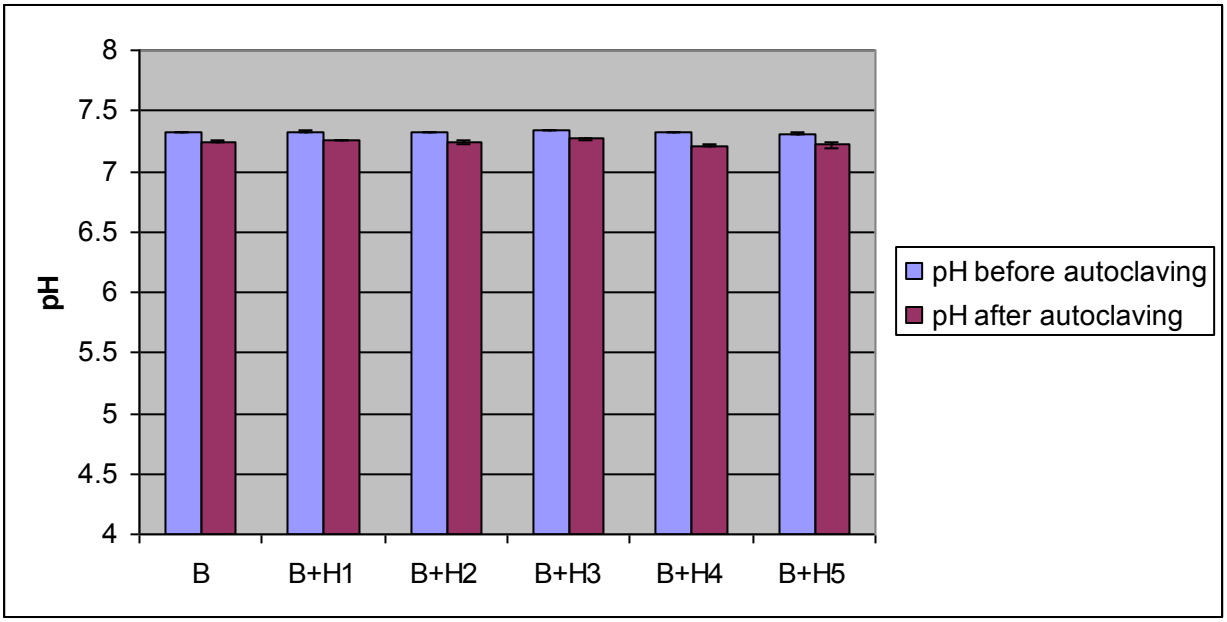


Figure 3.1: Influence of autoclaving on pH of TSB in the presence and absence of synthetic bone substitute materials

Although there was a statistically significant reduction in pH on autoclaving the reduction was very small. The greatest change in pH following autoclaving was an average reduction of 0.107 in the sample containing Cerasorb® M. The small pH changes experienced are more clearly demonstrated in the graph below (figure 3.2).
The knowledge of the effect of autoclaving on pH enabled the second part of the study to proceed.

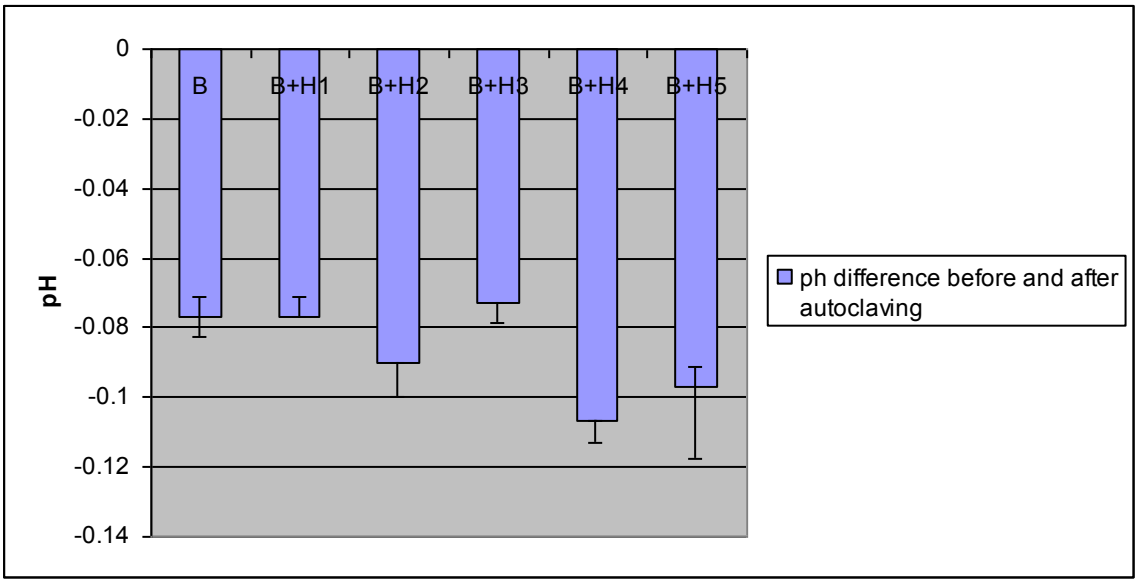


Figure 3.2: The pH difference recorded following autoclaving of the samples

3.1.2 Calcium concentration of the liquid medium before and after autoclaving

Calcium concentration was measured and the difference in the average concentration before and after autoclaving was calculated. In Figure 3.3 below these changes are shown as positive if the concentration increased after autoclaving or negative if the concentration reduced. The relative difference in concentration is measured in mg/dl and these differences were not statistically significant, except in the case of H5 (P=0.01). This is consistent with the relative maintenance of pH post autoclaving as dissolution of the synthetic bone substitutes would not be expected. Again this enabled the results of the second part of this study when bacteria were present to be interpreted independently of autoclaving.

It should be noted when interpreting the results for H4 (Cerasorb® M), that a single outlying measurement prior to autoclaving could help explain this relative difference in average calcium concentration (see figure 3.3). However, even with this

peak in calcium concentration the maximum difference experienced following autoclaving is only 0.206mg/dl.

The statistically significant pH difference affecting the H5 sample suggests that the process of autoclaving had an effect on the calcium concentration that could not occur by chance but this difference is clinically small and still allows the second part of the experiment to continue without influence of autoclaving.

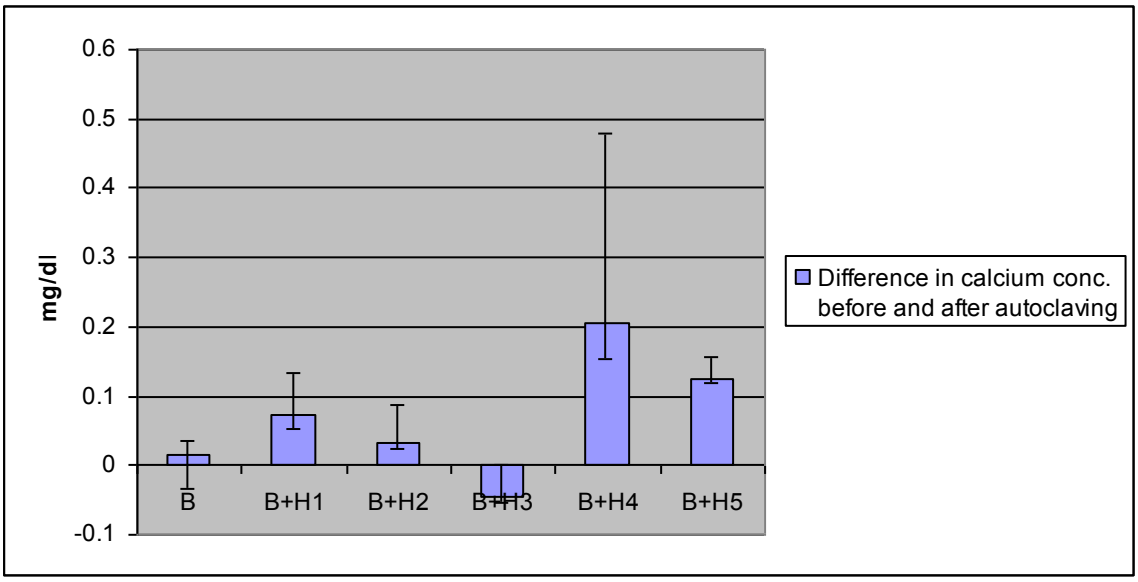


Figure 3.3: The difference in calcium concentration (mg/dl) before and after autoclaving in the presence and absence of the synthetic bone test materials

3.1.3 Phosphate concentration of the liquid medium before and after autoclaving

The phosphate concentration of the liquid medium was measured before and after autoclaving and the difference in these measurements can be seen in figure 3.4. The greatest difference in phosphate measurement was less than 0.08mg/dl and there was no statistically significant difference in phosphate concentration before and after autoclaving, therefore it was demonstrated that the process of autoclaving did not

significantly affect the levels of phosphate in the liquid medium. The measurement of phosphate levels prior to autoclaving was omitted in the second part of the study.

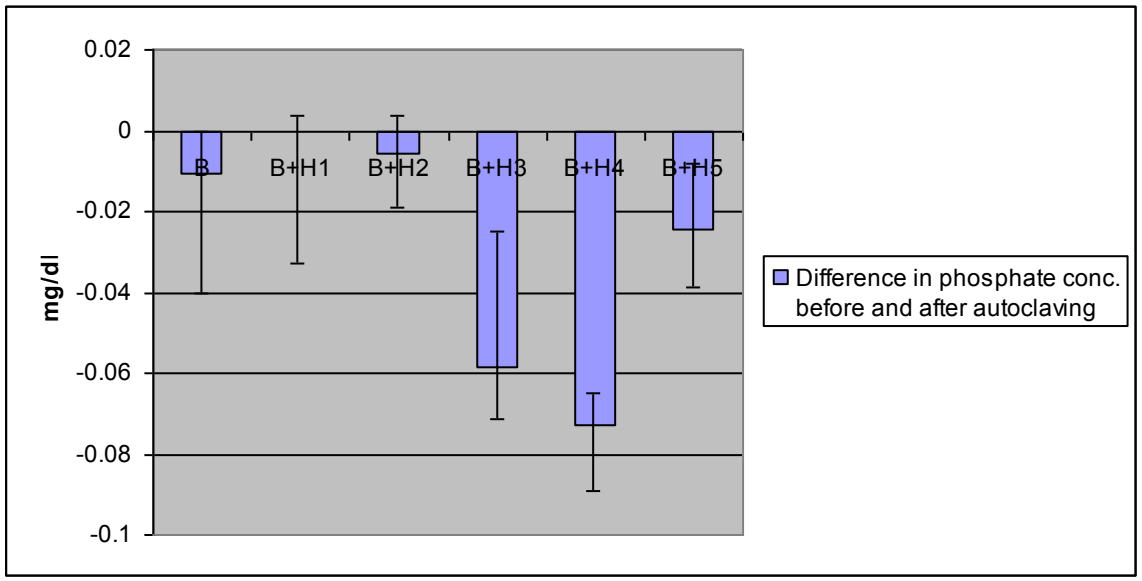


Figure 3.4: The difference in phosphate concentration (mg/dl) before and after autoclaving in the presence and absence of the synthetic bone test materials

3.2 Part 2 (Test results): Influence of bacteria, with and without the substitute bone test material, on pH calcium concentration and phosphate concentration

3.2.1 pH

The pH of the liquid medium was measured prior to autoclaving and then after 24 hrs of incubation with or without the addition of S. *epidermidis*. This was repeated on three separate occasions at least 24 hrs apart. The results of the three repeated experiments

have been collated and are represented in the graphs below (Figure 3.5 and figure 3.6). In both graphs the starting pH has been corrected by a reduction of 0.1 based on findings from part 1 of this study, which showed that the process of autoclaving reduced the pH by an average of 0.1.

It is interesting to note that in both graphs below, the average starting pH is fairly consistent across all groups with an average of 6.7. However in part 1 of this study the average start ph was higher at 7.3. The average start pH would be expected to be similar in all groups of both experiments. A possible explanation for this difference could be the use of a different batch of TSB, as experiment 1 was completed first and a new batch of TSB was used for all of experiment 2.

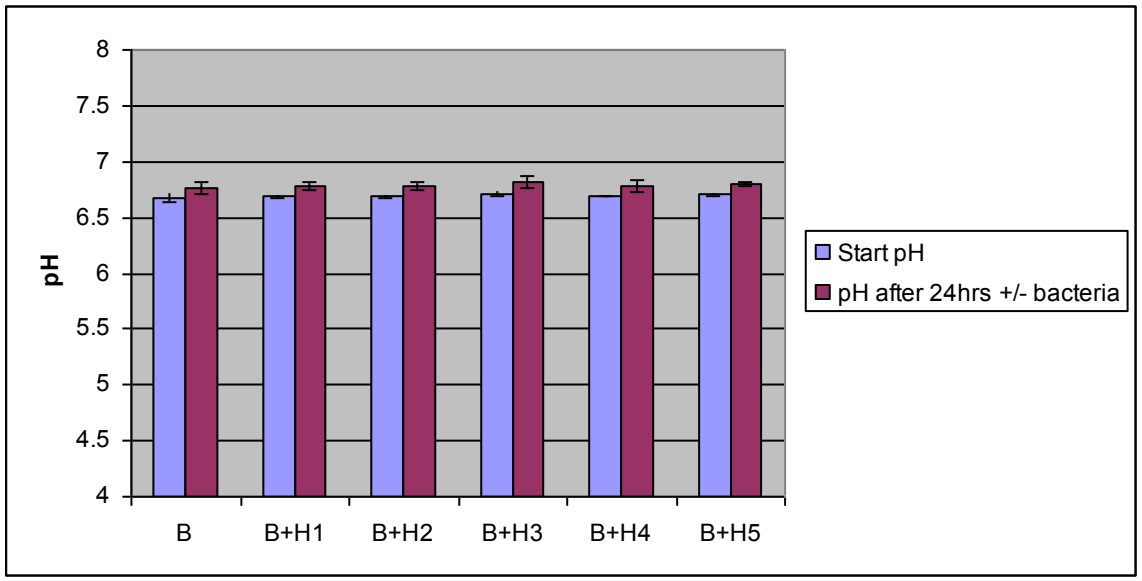


Figure 3.5: pH of the liquid medium before autoclaving and after 24hrs, without addition of bacteria. Start pH corrected by 0.1 as per findings of part 1

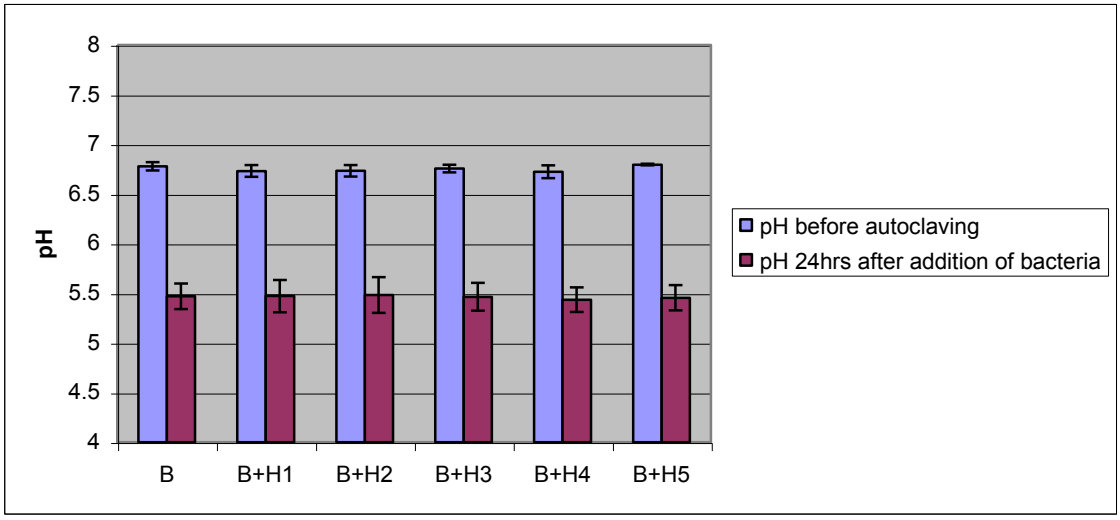


Figure 3.6: pH of the liquid medium before autoclaving and 24hrs after addition of Bacteria. Start pH corrected by 0.1 as per findings of part 1

The results clearly show that 24hrs after the addition of bacteria (*S. epidermidis*) the pH drops more in the test groups than in the controls. The average drop in pH falls below 5.5 in all test groups containing bacteria, but the upper limit of the error bars shows that not all pH values dropped below this level. No pH measurement fell below the previously mentioned critical pH of 5.3, which suggests that dissolution should not occur in test materials, which behave in the same way as dental HA. This applies to samples containing HA (H2 and H3) or tricalcium phosphate (H1 and H4).

The reduction in pH in the test group after 24 hrs was statistically significant at the 99% confidence interval ($P =< 0.01$) in all cases of all three experiments. The change in pH of the samples with materials but no bacteria in comparison with the control broth was statistically significant at the 95% confidence level ($P =< 0.05$) in the case of H3 in experiment 2 and in comparison to the test groups the change in pH was small (see

appendix IIii). The difference in the pH of the individual samples within both the control and the test groups can be more easily visualised in the graph below (figure 3.7). The average pH change in the presence of bacteria was a reduction of 1.2 and in the absence of bacteria there was an increase of 0.1.

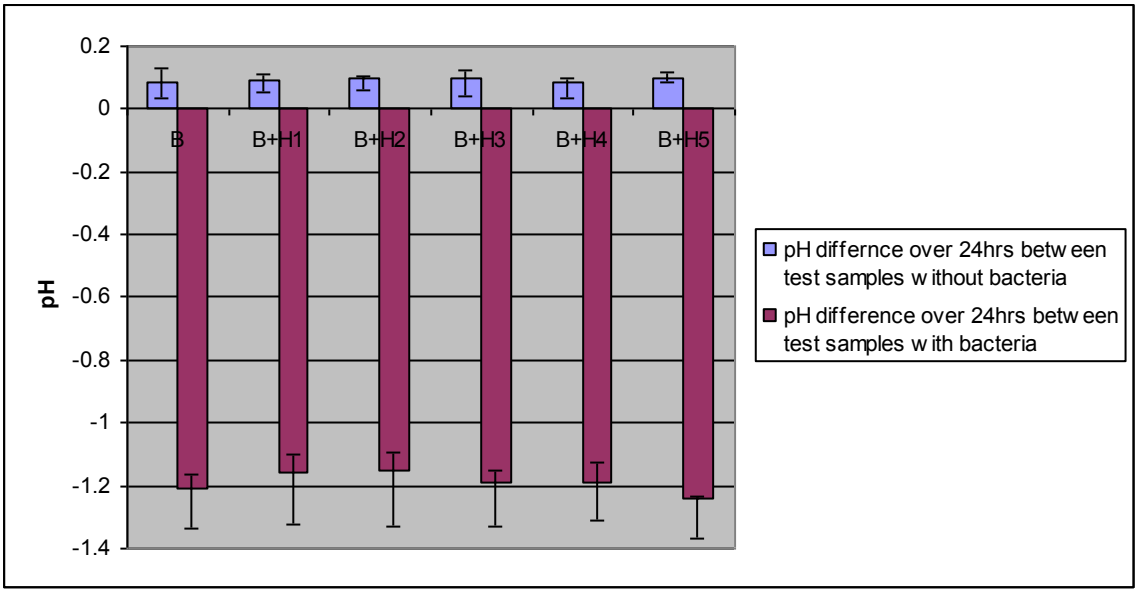


Figure 3.7: pH difference of the liquid medium before autoclaving and 24hrs after addition of bacteria

3.2.2 **Calcium**

Calcium concentration was measured in the test samples and control samples after the 24hr test period. The results have been collated and are presented in figure 3.8 below. The negative values represent a drop below that of the calibrated standard. Although the average calcium concentration is higher in each test sample compared with the paired control, the large error bars and the small differences in calcium concentration (max = 0.0

64) make it difficult to draw conclusions. When the results were analysed within each group there were some statistically significant results, but these were different in each experiment. When the results were collated so that the means from each experimental group were compared there were no statistically significant results (see appendix IV).

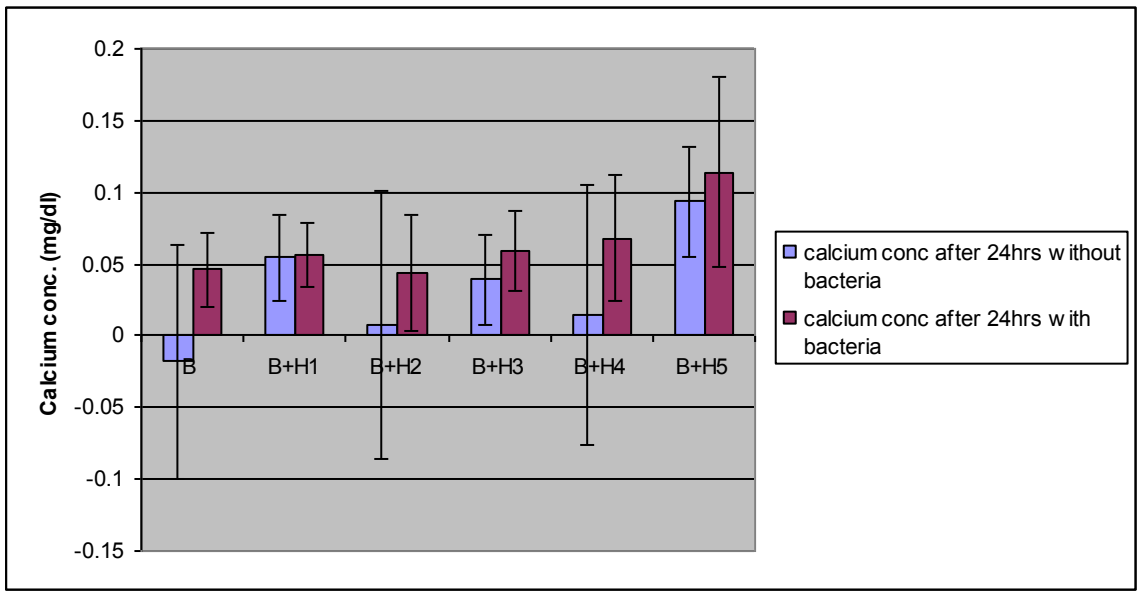


Figure 3.8: Calcium concentration after the 24hr test period

When the results are presented as the difference in average calcium concentration between the test and control samples they look more meaningful, but there are large margins of error and small difference in calcium concentration. In addition, the calcium concentration increases in sample B (broth only), which cannot signify dissolution, as there is no test material. The increase in calcium may be attributed to the bacteria themselves.

B+H5 shows the highest calcium concentration of the control samples, with the recorded mean being similar to that of matched test group. This may indicate that another factor may be affecting the dissolution, other than the presence of bacteria.

The measurement of calcium concentration is dependent on a process, which is itself subject to error. The optical density of the treated culture medium is calculated using a constant, which is derived from linear regression of the calcium standard curve for each set of results. The linear regression model should form a straight-line plot in each case and although variation from a straight line allows use of a line of best fit the less linear the points the less accurate the final measurements. It is acknowledged that this demonstrates the difficulty in obtaining accurate, reproducible measurements at such low concentrations of calcium and phosphate.

3.2.3 **Phosphate**

Phosphate concentration was also measured in the test and control samples after the 24hr test period. The combined averages are shown below in figure 3.10. The error bars are large and the differences in the averages between tests and controls are small (max < 0.05mg/dl). For ‘B’ and ‘H1’ the phosphate level was higher on average in the test group compared to the controls, however all other test groups showed on average lower phosphate levels than controls (table 3.11). The higher phosphate concentration in the case of test sample ‘B’ may affect the significance of the rest of the phosphate concentration results, as there is no biomaterial source of phosphate in this sample. A decrease in phosphate concentration may signify bacterial metabolism, but with such small changes it is impossible to draw any conclusions.

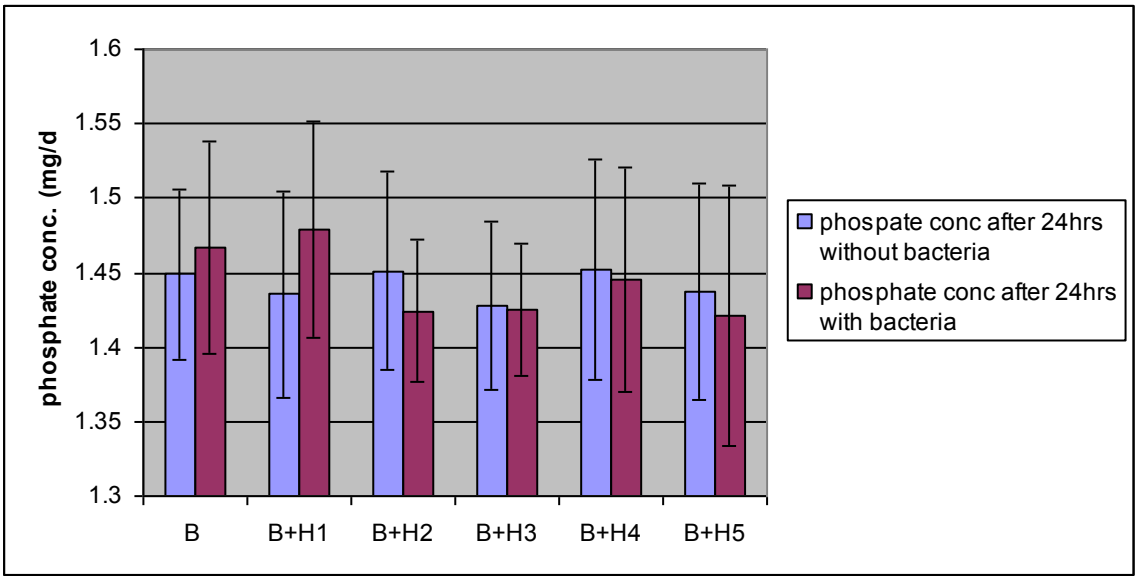


Figure 3.9 Phosphate concentration after the 24hr test period

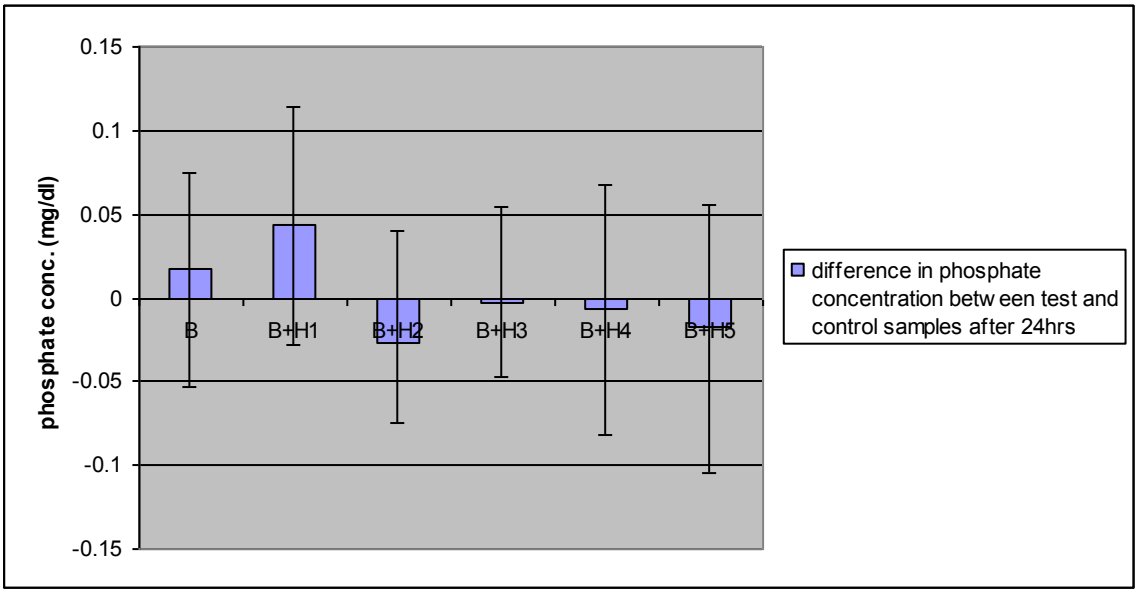


Figure 3.10 Difference in phosphate concentration between test samples (with bacteria) and control samples (without bacteria) after 24hrs

Experiment 1 showed no significant statistical difference in phosphate measurements. Experiment 2 and 3 showed a statistical difference in H2, but this represented a reduction in phosphate levels, which would not have been expected if there had been dissolution of the bone substitute material.

3.3 Part 3 (SEM imaging)

Following experimental use, the samples were prepared (as previously discussed) to assess presence/absence of bacteria. Prior to testing, the average granule size ranged from 250 to 6000μm, as described in the supporting literature but this was not measured. For the majority of samples the structure of the synthetic bone material appeared similar to the naked eye pre-testing, post-autoclaving as well as after the test period (24hrs - with and without bacteria). However, H4 (Cerasorb® M), which is a pure phase β Tri calcium phosphate, with 65% porosity (micro-, meso- and macropores) appeared to breakdown into smaller particles. SEM imaging enabled direct measurement of the average granule size at a defined magnification.

3.3.1 Presence/absence of bacteria

A representative sample was mounted and prepared to allow viewing at both low and high vacuum with the SEM. The mounted samples were assessed for presence or absence of bacteria (figure 3.12). Staphylococci were not detected on any of the control samples under high or low vacuum SEM. This was expected as no bacteria were added to these

samples and the experiment was carried out using aseptic techniques to avoid contamination.

Staphylococci were identified in at least one of the three samples for each bone substitute material. For two of the bone substitute materials (H2 and H4), Staphylococci were seen on all three samples.

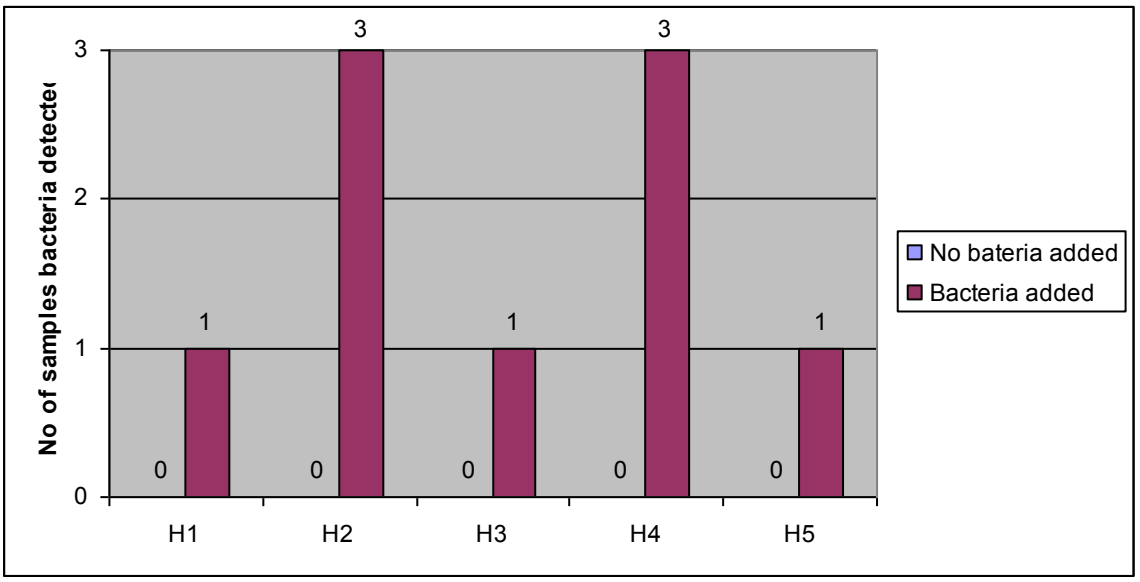
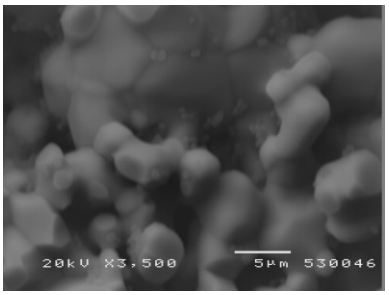
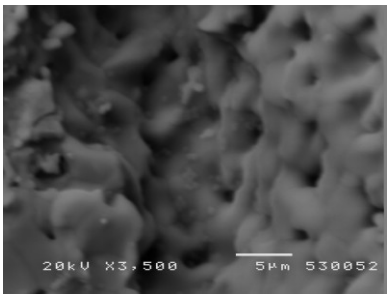


Figure 3.11: Number of samples in which bacteria was detected using the SEM in both the control and test samples

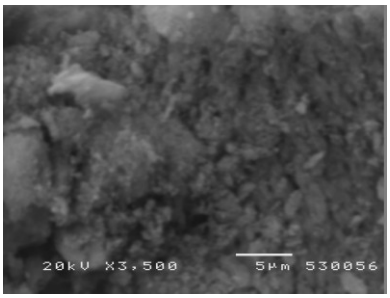
The positive identification of staphylococci may have been affected by a number of factors: the surface of the bone substitute material (see micrograph 3.1), the preparation of the sample and the representative view of the material may not have been indicative of the entire sample. As a result it is hard to quantify or qualify this information regarding the bacteria and their relationship with each bone substitute material. However, it could be demonstrated that all test samples had potential to be contaminated with *S. epidermidis*.



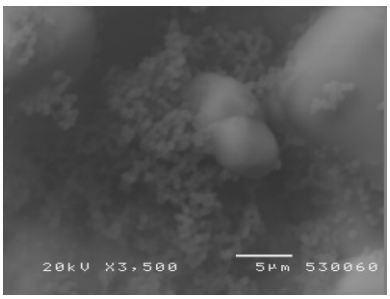
Cerasorb® (H1) with bacteria



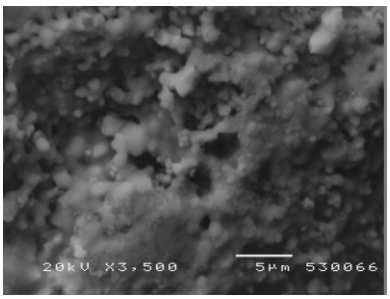
Ossbone (H2) with bacteria



BioOss® (H3) with bacteria



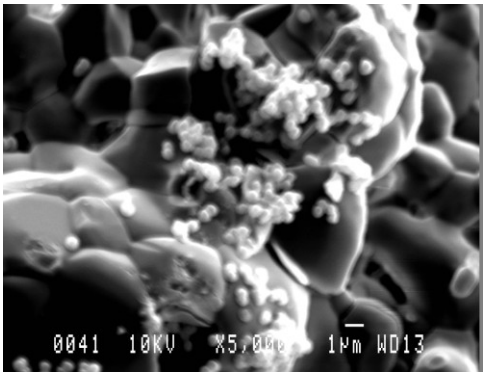
Cerasorb® M (H4) with bacteria



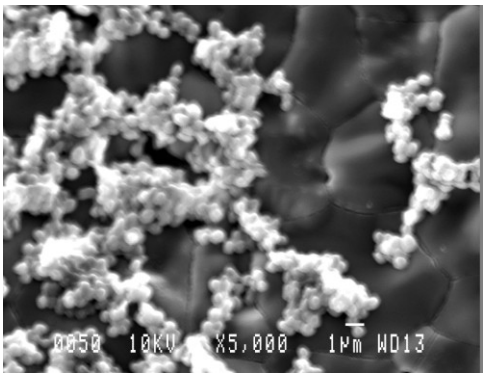
Bonesave (H5) with bacteria

Micrograph 3.1: H1-H5: From Experiment iii, each showing representative granule and presence of Staphylococci shown at X3500

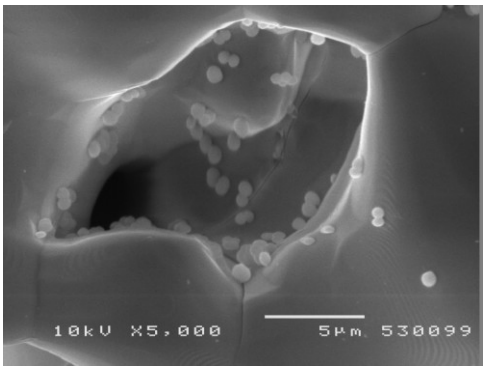
The bacteria seemed to be more abundant on the H4 sample than the other test materials, but still inconsistent in distribution. The relative abundance of bacteria may have been in part due to the ease of identifying the bacteria on this sample. In the few cases where high magnification micrographs were achieved and captured, the bacterial adherence was observed as being greater within pores and along crack and grain boundaries:



Cerasorb® (H1) with the majority of bacteria adhering in crevices and grain boundaries, but some adhering to what looks like smooth surfaces



Ossbone (H2) with bacteria adhering in 'grape like' clusters along cracks and grain boundaries

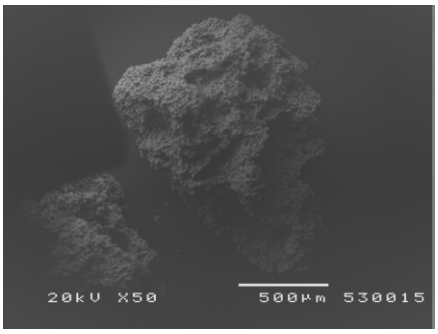


Cerasorb® M (H4) with bacteria adhering within a pore and along grain boundaries

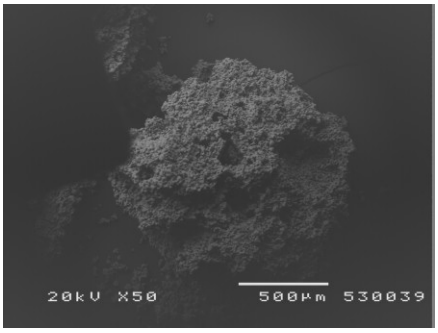
Micrograph 3.2: H1, H2 and H4: each showing presence of bacteria at X5000 magnification

3.3.2 Synthetic bone substitute degradation

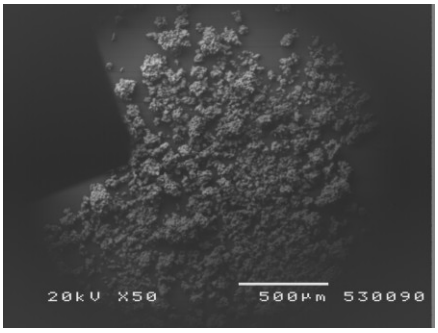
Only the synthetic bone substitute sample H4 exhibited significant degradation in the presence of bacteria over the course of the experiment, as shown in the following SEM images:



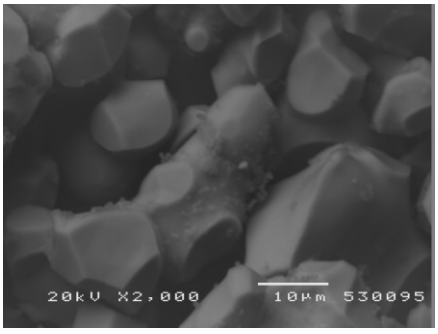
A: Cerasorb® M (H4) – granule of sample direct from packet



B: Cerasorb® M (H4) – granule of control sample following exp iii



C: Cerasorb® M (H4) – representative test sample following exp iii



D: Cerasorb® M (H4) – magnified sample of above test sample following exp iii

Micrograph 3.3: Demonstrates the relative degradation of the H4 sample – images from experiment iii

The above images show that the test group (with bacteria) underwent more physical degradation than the control group in the case of H4. The test sample was broken down to only small fragments with the largest observed fragment being approximately 200µm, whereas the largest fragment of the control sample was approximately 1500µm, similar to the original sample. The amount of degradation was similar in all 3 experiments for the H4 test material.

4. DISCUSSION

4.1 Background

This study was intended primarily to ascertain if bacteria affected the pH in the environment of a synthetic bone graft and if this pH change resulted in break down of the synthetic bone graft as measured by the calcium and/or phosphate content of the surrounding medium. It was hypothesised that reduction in pH may play a part in failure of the graft due to early dissolution of the synthetic bone material. It is acknowledged that dissolution is required for true integration of the implant over time but that if this occurred to quickly the graft could fail. The five different test samples had different compositions and as such a different dissolution rate would have been expected in each case. β-TCP is known to be more soluble than HA and is utilised for this property (Murugan and Ramakrishna, 2005). However, the behaviour of calcium phosphate materials may be less predictable when implanted in human or animal models.

4.2 Part 1 (Preliminary results)

4.2.1 The effect of autoclaving on pH and dissolution

The initial part of the experiment was to eliminate the process of autoclaving as a contributing factor to reduction in pH of the test medium. Autoclaving was required to provide a sterile environment prior to the addition of the test bacteria. Testing of the pH at this point in part 2 of the study would have introduced potential for contamination of the sample, as pH measurement could not be carried out aseptically. A statistically significant reduction in the pH of the culture medium occurred after autoclaving, but the

decrease in pH was very small and not associated with a corresponding change in calcium and phosphate levels. The effect of autoclaving could therefore be corrected for and the experiment could proceed to part 2 (test results), testing the effect of the bacteria.

The changes in calcium and phosphate measurements on autoclaving were not statistically significant except for the H5 sample calcium measurement. This increase in calcium concentration of the culture medium would occur if there was dissolution of the sample. H5 is composed of 80% β-tri-calcium phosphate, 20% HA, and the tri-calcium phosphate portion of this material is known for its increased solubility as discussed previously. The pH of the H5 culture medium did not fall below 7.2 and as such dissolution was not expected. It is possible that the 'critical pH' for dissolution of this material may be higher than expected. Alternatively, it is possible that this material is thermally unstable, a variable which was not measured in this study

4.3 Part 2 (Test results)

4.3.1 The effects of addition of bacteria on pH

The addition of bacteria to the TSB had the effect of lowering the pH whether or not there was synthetic bone material present. The reduction in pH was consistent and statistically significant in all test groups. This would suggest that the *S. epidermidis* 11047 produced an acid when incubated in the TSB for 24hrs. TSB acts as the energy source for bacterial growth as it consists of a mixture of amino acids and dextrose. As the bacteria grow and metabolise, sugar acids of the carboxylic or tricarboxylic acid cycle are produced as metabolites and their release into the culture medium lowers the pH. When the TSB, with or without the bone substitute samples were incubated over 24 hrs, there

was no clinically significant effect on the pH. Unexpectedly, the results (see figure 3.5) did not show the same statistically significant reduction in pH as in the pre and post autoclave group from experiment 1, which may mean that there is a mild buffering effect from the incubation of the samples for 24 hrs.

4.3.2 **The effect of addition of bacteria on dissolution**

This experiment attempted to quantify potential dissolution as a measure of the relative change in calcium and phosphate in the culture medium. This was measured using a calcium and phosphate assay.

The results of calcium and phosphate measurements would suggest that there was no detectable dissolution of any of the bone substitute materials, although the experiment should be repeated with larger samples and a longer incubation time. The inconsistencies in these results support the need to repeat these measurements. With respect to these results, it should be noted that the pH of the culture medium did not drop below 5.3, the assumed 'critical pH' at which HA dissolves (as previously discussed), in any of the test or control samples and as such dissolution would not have been expected. B-TCP is likely to dissolve to a greater extent at low pH but reprecipitation of calcium phosphate may occur, masking any changes (Kohri *et al*, 1993). This should be investigated further by incubating for longer periods and SEM could be used more extensively to look for any evidence of crystal formation due to reprecipitation on the surface, as described in this study. The bone substitute materials used in this experiment are varied in both composition and structure and as such may show wide variation in their dissolution properties.

4.3.2.1 Calcium concentration

The measurement of calcium levels were dependent on first producing a calcium standard for each separate part of the experiment and from this, linear regression was used to calculate the calcium concentration from the optical density of the sample. This was a potential source of error, as the linear regression models (see 3.10) did not have a perfect straight line and best fit was used. The results were statistically significant in some cases but did not show a pattern that could be interpreted. The pH change did not fall below the previously described 'critical pH' of 5.3 and as such dissolution would not have been expected. It is acknowledged that this theory does not apply to the test materials composed of β-tricalcium phosphate (β-TCP), but there is no 'critical pH' described in the literature for this material. Bohner *et al*., (1997) investigated the kinetics of dissolution of β-TCP and concluded that the rate of dissolution decreases very sharply with time and that this effect increases at higher pH, but no exact explanation of dissolution was established.

The changes in calcium concentration were small and samples containing 'broth only' showed similar changes even in the absence of a calcium source. From this it could be concluded that the results may not be representative of the true calcium concentration change, as none would be expected in the case of the 'broth only' sample. Ideally this part of the experiment should be repeated and the calcium standard curve should be a straight-line for each set of data before measurements could be calculated and compared

accurately. Alternatively or in addition, there could be a second method of testing for calcium concentration to rule out inaccuracies.

4.3.2.2 Phosphate concentration

The phosphate concentration was also measured using an assay, which required multiple steps to calculate the final phosphate level. The results did not show an expected pattern, being on average higher in test samples 'B' and 'H1' and lower in all remaining test samples. There is wide variation in the repeat measurements and this makes interpretation of results less reliable.

The results were not statistically significant, except for H2 (experiment ii and iii) and in these results there was a relative decrease in the phosphate concentration, which would not have been expected if dissolution had occurred. This may have occurred due to bacterial metabolism, as bacteria can take up calcium and phosphate from the medium, but this conclusion cannot be drawn from these results.

The lack of statistically and clinically significant changes in the phosphate concentration correlate with the failure of the pH to drop below the presumed 'critical pH' of 5.3 but large error in repeat results makes this conclusion less reliable. This experiment would be more robust if these measurements were repeated using the same assay or if an additional method of phosphate measurement was included. The incubation time may also be a variable worth investigating in this experiment, as 24hrs may not be a long enough time period for the bacteria to have an effect on the phosphate levels.

4.4 Part 3: SEM imaging

The SEM images confirmed the presence of staphylococci in at least one of the three test samples from experiment iii, demonstrating that each sample had the potential to be contaminated. No staphylococci could be demonstrated in any of the control samples which is in keeping with the aseptic techniques used through out. When interpreting SEM images, it is acknowledged that the mounted material is merely a representative of the overall sample and as such could be open to false negative readings.

The structures of the bone substitute materials varied widely and this was demonstrated using the SEM (see micrograph 3.1). The surface structure may have played a part in the ease of adherence and colonisation of *S. epidermidis*, for example, staphylococci were more abundant and easier to identify in some bone substitute materials such as H4.

The degradation of the bone substitute material in this experiment was only measured in terms of dissolution, using calcium and phosphate concentration as an indicator. However it was observed that the bone substitute H4 was breaking down significantly, especially in the test material. It was difficult to work with, as the fragments were very brittle making it challenging to mount in preparation for the SEM. The degradation was more easily visualised using the SEM images to compare a representative sample at x50 magnification and it showed that the largest fragments of the test material were around 7 times smaller than those of the control and the untreated

specimen. A greater amount of degradation would have been expected in this material based on its composition, as it is a pure phase β tri-calcium phosphate, which as discussed previously is known to have a higher rate of degradation (Koerten and Van der Meulen, 1998). H4 also has the greatest porosity (65%) out of all the test samples used in this study, which would again increase the rate of degradation.

5. CONCLUSION

5.1 Part 1 (Preliminary work)

The results of this experiment indicate that if a TSB culture medium is autoclaved at 120°C for 15minutes at 1.1 bar pressure on its own or in the presence of any one of the five bone substitute test materials, there is:

- Statistically significant reduction in the pH of the culture medium across all samples, but only a small change in pH, to a minimum value of 7.2.
- No statistically significant change in the phosphate concentration of the culture medium
- No statistically significant changes in the calcium concentration of the culture medium, except in the presence of H5.

5.2 Part 2 (Test results)

5.2.1 pH

In the main part of this study, the pH of the culture medium was tested before autoclaving and 24 hrs later, following addition of *S. epidermidis* 11047 to the test samples:

- There was no statistically significant difference in pH in the majority of control samples (without bacteria) after 24 hrs, despite these having been autoclaved as part of the experiment. This was unexpected, as a statistically significant reduction of the pH would have been expected, similar to that of the control group in Part 1 (Preliminary work) of this study.

- The test samples (with bacteria) after 24 hrs showed a statistically significant reduction in pH of on average 1.2. The reduction approached the critical pH at which hydroxyapatite dissolves (pH of 5.3) in all cases.

The pH reduction of the culture medium after addition of *S. epidemidis* has the potential to affect the immediate environment of a bone graft. The potential for this pH change to affect a synthetic bone graft is dependent on the critical pH for dissolution of a graft material. This is likely to be affected by many factors including the composition and structure of the bone graft material and the surrounding medium. This experiment was '*in vitro'* and cannot be directly related to how a graft would behave in an infected site within the body. As such the results of this experiment are limited with respect to clinical application.

5.2.2 Dissolution

The calcium and phosphate levels of the culture medium were measured 24 hrs after autoclaving, following addition of *S. epidermidis* (11047) to the test samples only. The results of this experiment indicate:

- No significant reproducible differences in calcium or phosphate concentrations of the TSB were detected after incubation of any of the samples with or without bacteria.
- The phosphate measurement for 'B' and 'H1' was higher on average in the test group compared to the controls. All other test groups showed on average lower phosphate levels than controls.

- The higher phosphate concentration in the case of test sample 'B' would not be expected, as there is no additional source of phosphate in this sample and this could affect the validity of these results.
- There was no statistical significance between the test and control phosphate measurements, except in the case of H2 (experiments ii and iii) and this was associated with a drop in phosphate concentration.

5.3 Part 3 (SEM imaging)

- Surface images of the bone substitute materials tested showed the wide variation in their structure.
- Staphylococci were identified adhering to at least one of each of the test samples and no staphylococci were identified in the control samples.
- The bone substitute material H4 underwent physical degradation, which was obvious to the naked eye and could be further demonstrated using SEM images. The degradation resulted in fragments up to 7 times smaller in the test sample as compared with the control and untreated sample. This behaviour is in keeping with this materials composition and structure as it is pure phase β Tri calcium phosphate with high percentage porosity (65% micro-, meso- and macro).

5.3 Further work

Part 2 (Test results) of this study should be repeated to gain more robust information on the possible dissolution of the materials at different pH's. Further information could also be gained from:

- Leaving the test samples to incubate for longer than 24 hours as this may result in changes to the effects on pH or dissolution. It may give a greater insight into the mode of failure of an infected bone substitute graft within the clinical setting.
- High resolution SEM would provide more information about the distribution of the bacteria and their behaviour regarding dissolution on the surface of the test material.
- Weighing the samples before and after incubation with bacteria may provide more accurate results as to the extent of the dissolution affecting synthetic bone graft materials.
- Testing other kinds of bacteria, such as S*treptococcus mutans* or *Lactobacilli* – common oral microorganisms that favour an acidic environment, may provide greater insight into the use of synthetic bone graft materials within the oral cavity.

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APPENDICES

I Statistical analysis: Part 1 (Preliminary work)

Difference in pH before and after autoclaving

t-TEST: PAIRED TWO SAMPLE FOR MEANS
Part 1 (Preliminary work): difference in pH before and after autoclaving

SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	7.323333	7.246667
	t Statistic	23	
	P value	0.001885	
H1	Mean	7.326667	7.25
	t Statistic	23	
	P value	0.001885	
H2	Mean	7.33	7.24
	t Statistic	15.58846	
	P value	0.00409	
H3	Mean	7.34	7.266667
	t Statistic	22	
	P value	0.00206	
H4	Mean	7.32	7.213333
	t Statistic	32	
	P value	0.000975	
H5	Mean	7.313333	7.216667
	t Statistic	6.653056	
	P value	0.021854	

Difference in pH between test groups – before and after autoclaving

Samples	B	H1	H2	H3	H4	H5
A	0.08	0.08	0.08	0.08	0.11	0.12
B	0.07	0.07	0.1	0.07	0.11	0.1
C	0.08	0.08	0.09	0.07	0.1	0.07

Anova: Single Factor

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002667	5	0.000533	3.692308	0.0296	3.105875
Within Groups	0.001733	12	0.000144			
Total	0.0044	17				

Difference in calcium concentration - before and after autoclaving

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 1 (Preliminary work): difference in calcium concentration before and after autoclaving			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	1.126333	1.142333
	t Statistic	-0.44704	
	P value	0.698596	
H1	Mean	1.090667	1.163
	t Statistic	-2.71952	
	P value	0.112792	
H2	Mean	1.138	1.170333
	t Statistic	15.58846	
	P value	0.442722	
H3	Mean	1.138	1.170333
	t Statistic	1.742574	
	P value	0.223531	
H4	Mean	0.961333	1.167333
	t Statistic	-1.31931	
	P value	0.317853	
H5	Mean	1.068333	1.193667
	t Statistic	-8.98042	
	P value	0.012174	

Difference in phosphate concentration - before and after autoclaving

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 1 (Preliminary work): difference in phosphate concentration before and after autoclaving			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	1.147667	1.137
	t Statistic	0.936329	
	P value	0.447948	
H1	Mean	1.142	1.142
	t Statistic	0	
	P value	1	
H2	Mean	1.13	1.124333
	t Statistic	0.94299	
	P value	0.445226	
H3	Mean	1.187333	1.129
	t Statistic	2.516228	
	P value	0.128252	
H4	Mean	1.13	1.124333

	t Statistic	0.94299	
	P value	0.445226	
H5	Mean	1.187333	1.129
	t Statistic	2.516228	
	P value	0.128252	

II Statistical analysis: Part 2 (Test results)

i. Part 2i

2i: Difference in pH after 24 hrs without bacteria

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in pH after 24 hrs without bacteria			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	6.79	6.8
	t Statistic	-1.73205	
	P value	0.225403	
H1	Mean	6.796667	6.8
	t Statistic	-0.5	
	P value	0.666667	
H2	Mean	6.823333	6.843333
	t Statistic	-2	
	P value	0.183503	
H3	Mean	6.823333	6.843333
	t Statistic	-2	
	P value	0.183503	
H4	Mean	6.806667	6.806667
	t Statistic	0	
	P value	1	
H5	Mean	6.803333	6.823333
	t Statistic	-3.4641	
	P value	0.07418	

2i: Difference in pH after 24 hrs with bacteria

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in pH after 24 hrs with bacteria			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	6.73	5.416667
	t Statistic	38.82197	
	P value	0.000663	
H1	Mean	6.706667	5.376667
	t Statistic	133	
	P value	5.65E-05	
H2	Mean	6.703333	5.396667
	t Statistic	64.4444	
	P value	0.000241	

H3	Mean	6.733333	5.38
	t Statistic	76.72679	
	P value	0.00017	
H4	Mean	6.68	5.36
	t Statistic	114.3154	
	P value	7.65E-05	
H5	Mean	6.786667	5.373333
	t Statistic	424	
	P value	5.56E-05	

2i: Difference in calcium concentration after 24 hrs

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in calcium concentration after 24 hrs			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	0.018667	0.051667
	t Statistic	-0.87256	
	P value	0.47491	
H1	Mean	0.073667	0.063333
	t Statistic	0.80015	
	P value	0.507564	
H2	Mean	0.058	0.048667
	t Statistic	0.381314	
	P value	0.739667	
H3	Mean	0.077667	0.07
	t Statistic	0.347489	
	P value	0.761386	
H4	Mean	0.080667	0.111
	t Statistic	-3.4618	
	P value	0.074268	
H5	Mean	0.132333	0.2
	t Statistic	-4.41304	
	P value	0.047704	

2i: Difference in phosphate concentration after 24 hrs

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in phosphate concentration after 24 hrs			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	1.409	1.434
	t Statistic	-1.51492	
	P value	0.269014	

H1	Mean	1.377667	1.426667
	t Statistic	-4.2277	
	P value	0.051652	
H2	Mean	1.376333	1.4
	t Statistic	-1.51476	
	P value	0.26905	
H3	Mean	1.393333	1.391667
	t Statistic	0.112338	
	P value	0.920814	
H4	Mean	1.398667	1.468
	t Statistic	-1.90107	
	P value	0.197658	
H5	Mean	1.415333	1.397
	t Statistic	0.817353	
	P value	0.499607	

ii. Part 2ii

2ii: Difference in pH after 24 hrs without bacteria

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in pH after 24 hrs without bacteria			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	6.813333	6.79
	t Statistic	7	
	P value	0.019804	
H1	Mean	6.803333	6.806667
	t Statistic	-1	
	P value	0.42265	
H2	Mean	6.8	6.796667
	t Statistic	0.229416	
	P value	0.839872	
H3	Mean	6.836667	6.853333
	t Statistic	-5	
	P value	0.03775	
H4	Mean	6.803333	6.81
	t Statistic	-1	
	P value	0.42265	
H5	Mean	6.813333	6.803333
	t Statistic	1.732051	
	P value	0.225403	

2ii: Difference in pH after 24 hrs with bacteria

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in pH after 24 hrs with bacteria			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	6.806667	5.376667
	t Statistic	247.6833	
	P value	1.63E-05	
H1	Mean	6.686667	5.376667
	t Statistic	226.8987	
	P value	1.94E-05	
H2	Mean	6.696667	5.36
	t Statistic	401	
	P value	6.22E-06	
H3	Mean	6.733333	5.386667
	t Statistic	404	
	P value	6.13E-06	
H4	Mean	6.693333	5.366667
	t Statistic	150.4299	
	P value	4.42E-05	
H5	Mean	6.8	5.393333
	t Statistic	159.501	
	P value	3.93E-05	

2ii: Difference in calcium concentration after 24 hrs

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in calcium concentration after 24 hrs			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	0.045	0.035
	t Statistic	0.50358	
	P value	0.664548	
H1	Mean	0.071	0.072
	t Statistic	-0.10521	
	P value	0.925807	
H2	Mean	0.075333	0.073
	t Statistic	0.152499	
	P value	0.892789	
H3	Mean	0.009333	0.067667
	t Statistic	-9.13493	
	P value	0.011772	
H4	Mean	0.062	0.036
	t Statistic	0.912983	

	P value	0.457627	
H5	Mean	0.083667	0.079333
	t Statistic	0.991241	
	P value	0.426036	

2ii: Difference in phosphate concentration after 24 hrs

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in phosphate concentration after 24 hrs			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	1.496	1.553
	t Statistic	-2.18746	
	P value	0.16022	
H1	Mean	1.500667	1.555667
	t Statistic	-2.3883	
	P value	0.139539	
H2	Mean	1.519	1.481667
	t Statistic	8.53992	
	P value	0.013436	
H3	Mean	1.497333	1.470667
	t Statistic	1.871121	
	P value	0.202231	
H4	Mean	1.524	1.514
	t Statistic	0.487757	
	P value	0.673952	
H5	Mean	1.516	1.511333
	t Statistic	0.116108	
	P value	0.918175	

iii. Part 2iii

2iii: Difference in pH after 24 hrs without bacteria

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in pH after 24 hrs without bacteria			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	6.73	6.706667
	t Statistic	32.27273	
	P value	0.681489	
H1	Mean	6.77	6.74
	t Statistic	1.192079	

	P value	0.355497	
H2	Mean	6.78	6.743333
	t Statistic	2.2	
	P value	0.158809	
H3	Mean	6.786667	6.75
	t Statistic	3.050851	
	P value	0.092735	
H4	Mean	6.783333	6.723333
	t Statistic	2.267787	
	P value	0.151472	
H5	Mean	6.79	6.786667
	t Statistic	1	
	P value	0.42265	

2iii: Difference in pH after 24 hrs with bacteria

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in pH after 24 hrs with bacteria			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	6.8	5.616667
	t Statistic	32.27273	
	P value	0.000959	
H1	Mean	6.8	5.66
	t Statistic	19	
	P value	0.002759	
H2	Mean	6.8	5.69
	t Statistic	34.53049	
	P value	0.000838	
H3	Mean	6.8	5.626667
	t Statistic	352	
	P value	8.07E-06	
H4	Mean	6.796667	5.58
	t Statistic	365	
	P value	7.51E-06	
H5	Mean	6.803333	5.6
	t Statistic	136.4452	
	P value	5.37E-05	

2iii: Difference in calcium concentration after 24 hrs

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in calcium concentration after 24 hrs			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	-0.11833	0.051333
	t Statistic	-9.62435	
	P value	0.010624	
H1	Mean	0.019333	0.032333
	t Statistic	-1.07466	
	P value	0.394967	
H2	Mean	-0.11167	0.01
	t Statistic	-6.12085	
	P value	0.025668	
H3	Mean	0.031	0.038667
	t Statistic	-0.9477	
	P value	0.443313	
H4	Mean	-0.09967	0.056667
	t Statistic	-3.51797	
	P value	0.072164	
H5	Mean	0.064	0.063
	t Statistic	0.094351	
	P value	0.933432	

2iii: Difference in phosphate concentration after 24 hrs

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in phosphate concentration after 24 hrs			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	1.441667	1.412667
	t Statistic	2.740242	
	P value	0.111365	
H1	Mean	1.427333	1.456
	t Statistic	-0.73834	
	P value	0.537193	
H2	Mean	1.458333	1.39
	t Statistic	4.22613	
	P value	0.051688	
H3	Mean	1.392333	1.413333
	t Statistic	-0.52593	
	P value	0.651433	
H4	Mean	1.433	1.355

	t Statistic	1.984191	
	P value	0.185672	
H5	Mean	1.380667	1.353333
	t Statistic	0.422428	
	P value	0.713794	

iv. Difference in calcium concentration after 24 hrs, 2i-2iii

t-TEST: PAIRED TWO SAMPLE FOR MEANS Part 2 (Test results): difference in calcium concentration after 24 hrs, 2i-2iii			
SAMPLE	STATISTIC	VARIABLE 1	VARIABLE 2
B	Mean	-0.01822	0.046
	t Statistic	-2.12733	
	P value	0.066067	
H1	Mean	0.054667	0.055889
	t Statistic	-0.18227	
	P value	0.859907	
H2	Mean	0.007222	0.043889
	t Statistic	-1.55649	
	P value	0.158204	
H3	Mean	0.039333	0.058778
	t Statistic	-1.59383	
	P value	0.149641	
H4	Mean	0.014333	0.067889
	t Statistic	-1.72414	
	P value	0.122972	
H5	Mean	0.109556	0.114111
	t Statistic	-0.23208	
	P value	0.822303	