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**Interplay between surface properties of standard, vitamin E blended and oxidised ultra high molecular weight polyethylene used in total joint replacement and adhesion of Staphylococcus aureus and Escherichia coli [\*V.Allizond is the corresponding author]**

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## **Abstract**

This study was aimed at assessing the different adhesive strength of some of the most common bacteria associated with periprosthetic infection on various types of Ultra High Molecular Weight Polyethylene (UHMWPE) components. Quantitative *in vitro* analysis of the adhesion of biofilm producing strains of *Staphylococcus aureus* and *Escherichia coli* to physico-chemically characterized standard UHMWPE (PE), Vitamin E blended UHMWPE (VE-PE) and oxidized UHMWPE (OX-PE) was performed by using a sonication protocol. A significant decreased bacterial adhesion was registered for both strains on VE-PE, in comparison with that observed on PE, within 48h of observation (*S. aureus*  $p=0.0243$  and *E. coli*  $p=0.0081$ ). Biomaterial associate infections are still an issue in total joint replacement where often result in a series of implant-related *sequelae* that can lead to implant removal with clinical and economic consequences of significant importance. Since Vitamin E reduces bacterial adhesive ability, VE-stabilized UHMWPE could be a joint arthroplasty valid technology by presenting excellent quality on mechanical, wear, oxidation and adhesion properties.

**Key words:** *Staphylococcus aureus*, *Escherichia coli*, bacterial adhesion, UHMWPE, Vitamin E, oxidation.

## Introduction

Biomaterial associated infections (BAI) are still an issue in total joint replacement (TJR) surgery,<sup>1-4</sup> where often result in a series of implant-related *sequelae* that can lead to implant removal with clinical and economic consequences of significant importance.<sup>5</sup> The initial bacterial adhesion and subsequent growth of microorganisms to the biomaterials implant surface are complex processes related to physico-chemical interactions between substratum and microorganisms. The physical properties of the biomaterial surface, such as roughness, hydrophobicity, surface energy, electrostatic charge, and coating can strongly influence the feasibility and kinetics of microbial adhesion.<sup>3,6,7</sup> Ultra High Molecular Weight Polyethylene (UHMWPE) has a long successful clinical record as bearing material in TJR and remains the most commonly used material in modern joint replacement prostheses.<sup>7</sup> However, it has been demonstrated that oxidation has been the main cause of many dramatic implant failures in the last two decades.<sup>3,7-9</sup> Therefore many efforts have been made to improve the quality and the performance of UHMWPE *in vivo*, through reducing or eliminating the oxidation. Vitamin E (VE; alpha-tocopherol) was first introduced into conventional UHMWPE in an attempt to decrease the delamination caused by oxidative fatigue. Currently, VE is one of the antioxidants approved by the FDA for use in UHMWPE for medical use.<sup>10,11</sup>

In a previous study,<sup>3,12</sup> we have quantified the adhesion of *Staphylococcus epidermidis*, the leading etiologic agent of TJR infections, to standard UHMWPE, VE blended UHMWPE and oxidized UHMWPE. We have postulated a correlation among different degree of hydrophilicity related to different protein and other molecules absorption, direct effect of VE itself, even if present in very low concentration in the polyethylene, and *S. epidermidis* adhesion. The most interesting result was that VE blended UHMWPE, regardless of its concentration in the polyethylene matrix, reduced the adhesion of all three *S. epidermidis* strains tested in a manner strictly strain dependent.<sup>3,12</sup>

Taking into consideration that virtually all bacteria are able to cause BAI, and that any opportunity to reduce the microbial adhesion to the biomaterial could be an attractive in orthopedics, the objective of this current work was to quantify the adhesive strength of other pathogens to the same types of UHMWPE components. Strains of *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) were tested considering that various implant-related infections due to them have been described, including infections associated with prosthetic joints.<sup>13,16</sup> In fact, among the most frequently isolated organisms causing prosthetic joint infections, coagulase negative staphylococci (*S. epidermidis*) account for most of these (30%–43%), with *S. aureus* as the second most common (12%–23%); Gram-negative organisms, included *E. coli*, are less common than Gram-positive, causing around 6–8% of cases.<sup>14,15</sup>

## Materials and Methods

**Biomaterials.** Cylindrical specimens (height 14 mm, diameter 5 mm), were punched out from compression molded sheets of standard GUR 1020 UHMWPE (PE) and of UHMWPE blended with 0.1% w/w Vitamin E (VE-PE) (MediTECH/Quadrant, Fort Wayne, IN, USA). Half of the standard PE samples were exposed to accelerated ageing in a ventilated oven at 95°C for 210 h, to obtain a third group of samples: oxidized UHMWPE (OX-PE). To preserve the materials' chemical and physical properties, sterilization was achieved through 70% ethanol immersion followed by washing in sterile demineralized water. The same manufacturing steps were used for all specimens, to obtain comparable surface roughnesses.

**Surface roughness and water contact angle measurements.** The surface roughness  $R_a$  of samples was determined by using a roughness tester (Form Talysurf 50, Taylor-Hobson, Leicester, UK). Six measurements were made for each sample and then averaged.

Static contact angle (CA) measurements were conducted by assessing drop method using a DSA 100 (KRÜSS, Hamburg, Germany) apparatus and distilled water. The volume of the individual water droplet was 5  $\mu$ l. The water CA was measured at four locations on each sample to ensure homogeneity.

**Scanning Electron Microscopy.** The surface topographies of the UHMWPE samples were observed using a Stereoscan 420 SEM (Leica Cambridge Instruments, Cambridge, UK). Samples were sputter-coated with approximately 30 nm of gold and the microscope was operated at 15 kV, with magnifications from  $\times 200$  to  $\times 1,000$ . At least four independent images were taken from each sample.

**FTIR spectroscopy.** Attenuated Total Reflectance (ATR) - FTIR spectra of the sample surfaces before and after the adhesion assays were collected using a FTIR Microscope (Spectrum Spotlight, Perkin-Elmer, Waltham, MA, USA) equipped with an ATR objective (Germanium, incidence angle of the IR beam 45°, 100x100  $\text{mm}^2$  nominal surface area). The average beam penetration was on the order of 1  $\text{mm}$ . Each spectrum was collected using 32 scans at a 4  $\text{cm}^{-1}$  resolution and corrected for the wavelength dependence of the beam penetration by the Atrcorr algorithm of Grams AI/8.0 (Thermo Electron corporation, Bremen, Germany). One sample per group was kept in bacteria-free Tryptone Soya Broth (TSB; Basingstoke, Hampshire, UK) at 37°C for 48 h and was used as an FTIR control to evaluate possible modifications surface chemical properties, exerted by cultural medium. An average of 10 spectra was collected per sample; the most representative are reported.

**Bacterial cultures.** Well-characterized biofilm producing strains of *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were used for adhesion assays as representative pathogens of implant infection. Additionally biofilm producing *S. aureus* and *E. coli* recently isolated from orthopedic implant infections and previously characterized as *in vitro* biofilm producers were also tested. Each strain

was cultured on Tryptic Soy Agar (TSA; Merck KGaA, Darmstadt, Germany); young colonies (18-24h) were picked to approximately 364 McFarland standard and inoculated into cryovials containing both cryopreservative fluid and porous beads to allow bacteria to adhere (Microbank, Biomérieux, Rome, Italy). After inoculation, cryovials were kept at -80°C for extended storage.<sup>17,18</sup>

**Adhesion assays.** All strains were cultured over night at 37°C in TSB. After incubation, bacteria were re-suspended in 100 µl of TSB, harvested by 10 min centrifugation at 4,000 rpm and then diluted in TSB to 10<sup>7</sup> colony-forming-unit (CFU)/ml, as confirmed by colony counts on TSA. The sterile biomaterials were placed in 2 ml of bacterial suspension and incubated by shaking for incubation times of 3, 7, 24, 48h at 37°C to allow bacterial adhesion, biofilm formation and its maturation. Controls represented by bacteria incubated in TSB in the absence of the biomaterial were also performed. The number of strongly bound bacteria remaining on the cylinders after incubation, was quantified after sonication (40 kHz) for 7 min at 22°C in 1.5 ml of sterile NaCl 0.9% (Bieffe Medital S.p.A., Grosotto SO, Italy). The number of CFU in each sonication product was quantified by serial plate counts into TSA an ideal protocol for dislodging biofilm bacteria without affecting bacterial viability.<sup>3,19</sup> All the experiments were performed simultaneously for each biomaterial. The adherence experiments were assayed in triplicate and repeated a minimum of three times.

**Statistical analysis.** The adhesion assay results (CFU/ml) were analyzed by descriptive statistics (mean and standard deviation, SD) and tested by unpaired T-Student test, to highlight significant differences ( $p < 0.05$  significant and  $p < 0.01$  highly significant) between the biomaterials using the Graphpad Prism version 6 for Windows (Graphpad software, San Diego, CA, USA). As control, material PE was used.

## Results

Figure 1 shows some representative SEM micrographs taken from the surface of the three polyethylene biomaterials. The average surface roughness  $R_a$  of the UHMWPE samples was  $0.8 \pm 0.2$  µm and water contact angles measured on the three PE samples were  $93 \pm 2$ ,  $91 \pm 2$  and  $84 \pm 2$  degrees for PE, VE-PE and OX-PE, respectively.

The ATR-FTIR spectra of the three biomaterials before the adhesion assay (Figure 2A) showed no differences between PE and VE-PE, while a strong absorption centered at  $1718 \text{ cm}^{-1}$  was observed in the spectrum of OX-PE, confirming the presence of abundant oxidation products. After the suspension in TSB (Figure 2B), new, broad absorption bands centered in the  $3350$  and  $1500\text{-}1600 \text{ cm}^{-1}$  area were observed in the spectrum of OX-PE only, while no differences were found in the spectra of PE and VE-PE.

Quantitative analysis of bacterial adhesion on various polyethylenes were performed by using ATCC biofilm producing *S. aureus* (Figure 3A) and *E. coli* (Figure 3B). After 3h of incubation, the initial *S. aureus* adherence on the different biomaterials achieved adhesion rates similar on PE and VE-PE, ranging from  $4.12 \times 10^6$  to  $3.98 \times 10^6$  CFU/ml, but significantly higher on OX-PE ( $9.82 \times 10^6$  CFU/ml;  $p=0.0041$ ). A similar trend of staphylococcal adhesion was detected on PE, VE-PE and OX-PE even after 7h ( $1.31 \times 10^7$ ,  $1.34 \times 10^7$ ,  $3.7 \times 10^7$  respectively) and 24h ( $1.61 \times 10^7$ ,  $1.29 \times 10^7$ ,  $3.72 \times 10^7$  respectively). On the contrary, after 48h a lower staphylococcal adherence on VE-PE was observed compared with that seen on both PE ( $p=0.00243$ ) and OX-PE (Figure 3A).

No significant difference in the numbers of *E. coli* adhering to PE, VE-PE and OX-PE was noted after 3h of incubation ( $1.01 \times 10^7$ ,  $1.09 \times 10^7$ ,  $1.19 \times 10^7$  respectively), 7h ( $1.18 \times 10^7$ ,  $1.00 \times 10^7$ ,  $1.13 \times 10^7$  respectively), and 24h ( $1.65 \times 10^7$ ,  $1.66 \times 10^7$ ,  $1.85 \times 10^7$  respectively). On the other hand, at 48h the *E. coli* adherence to VE-PE was significantly lower than to PE ( $p=0.0081$ ) and OX-PE (Figure 3B).

The microbiological findings obtained with biofilm producing *S. aureus* and *E. coli* recently isolated from orthopedic implant infections were concordant with those registered with collection strains: again, after 48h of incubation, a significantly lower bacterial adhesion was detected on VE-PE for both *S. aureus* and *E. coli* compared with that registered on PE and OX-PE (data not shown).

## Discussion

It is widely known that BAI in TJR causes a decrease in the success rate of the implant with consequent enormous burden on the patients and high cost to the healthcare system and the role of UHMWPE wear in determining the failure of a TJR is well documented in literature.<sup>1,9,20</sup> In particular, it has been demonstrated that oxidative degradation of UHMWPE gamma-sterilized in air increased its wear while decreasing mechanical strength, constituting the main cause of the dramatic implant failures occurred in the last years.<sup>21-23</sup> As a consequence, many efforts have been made to improve UHMWPE quality and performance, for example through the reduction or elimination of the oxidation. The VE stabilization of UHMWPE has been recently proposed to improve oxidation resistance while maintaining wear resistance and fatigue strength.<sup>7,20,23,24</sup> VE, a free radical scavenger and well established biological antioxidant, prevented oxidation and delamination and had a favorable long-term effect on fatigue performance of the UHMWPE, increasing the resistance to fatigue cracks associated with oxidation without negatively altering the biocompatibility of the implant.<sup>20,24</sup> Moreover, it has been shown that VE when added to UHMWPE does not have any cytotoxic effects and acts as an effective anti-inflammatory.<sup>2</sup>

In this study we quantified the adhesion of well-characterized biofilm producing collection strains of *S. aureus* and *E. coli*, as pathogens mostly associated with periprosthetic infection, to standard UHMWPE, to VE blended UHMWPE and to oxidized UHMWPE taking into account that for a given material surface, different bacterial species and strains adhere differently since different species and strains have different physicochemical characteristics:<sup>14,15</sup> slime producer *S. epidermidis* and *S. aureus* have a comparatively thicker and more rigid peptidoglycan layer respect to a fimbriae producer *E. coli*, and extensive contact of their external cell-structure with the implant surface may be quite different. *S. aureus* produces many toxins and tissue damaging exoenzymes, compared to *S. epidermidis*.

The two laboratory collection *S. aureus* and *E. coli* strains were chosen for the reproducibility of data: our experimental protocol, in fact, required a well characterized model with number of uncontrolled parameters as small as possible to highlight the potential different effects exerted by three prosthetic polyethylenes. The two biofilm producer *S. aureus* and *E. coli* strains recently isolated from orthopedic implant infections were used for comparison.

Our earliest studies indicated no difference in the surface roughness among the three biomaterial samples;<sup>3</sup> the present SEM observations confirmed no significant differences in their surface topography (Figure 1). On the other hand, a lower static CA was observed for OX-PE, when compared to the other two groups, suggesting a lower hydrophobicity of the oxidized surface. Accordingly, the ATR-FTIR analyses indicates the adsorption of protein-like substances on the surface of OX-PE (Figure 2), while the same effect was not observed on the surface of the other biomaterials.

The results of the adhesion assays on PE, VE-PE and OX-PE samples led to the observation that the surface oxidation and, possibly, the protein absorption facilitates the microorganism adhesion, confirming what previously reported with *S. epidermidis*.<sup>3</sup> In fact, a significant highest adherence of collection biofilm producing *S. aureus* (Figure 3A) was detected on OX-PE. In contrast, a significant decreased bacterial adhesion was registered for both bacteria on VE-PE, in comparison with that observed on PE, within 48h of observation (Figure 3 A and B), underlying the role of VE, probably related to its well established antioxidant properties.<sup>9,20</sup> The mechanism by which VE may affect the bacterial adherence to UHMWPE it is currently unknown, even if potential effects of VE on infection are currently investigated.<sup>25</sup> The CA measurements showed no significant variations in hydrophilicity between PE and VE-PE suggesting that other, concurrent factors must be involved in the different bacterial adhesion. A direct effect of VE itself, even if present in very low concentration in the polyethylene matrix, on the bacterial adhesive ability cannot be ruled out.



A similar trend was even observed, within 48h of observation, for clinical biofilm-producing *S. aureus* and *E. coli* strains, recently isolated from orthopedic infections, with a bacterial adhesion to VE-PE significantly lower than to PE and OX-PE (data not shown). The results obtained by testing both collection and clinical strains of *S. aureus* and *E. coli* highlighted similar adhesion bacterial capacity although indicating some variability in the initial adherence to inert surfaces. These results are consistent to the trends previously reported by us with *S. epidermidis*,<sup>3</sup> but in contrast with some literature data. Molina-Manso *et al.*<sup>25</sup> reported that collection *S. aureus* strains showed significantly less adherence to VE blended- UHMWPE while some of the clinical strains tested, not all, failed to confirm this effect.<sup>25,26</sup> The current available literature data are hard to compare owing to either different experimental adopted procedures or different observation periods of time. Anyway the conclusion that can be drawn is that the very early stage of colonization process is strongly affected by intrinsic intra and inter-species variability among different bacterial genera.<sup>3,25-27</sup> At the moment clinical data about bacterial colonization of different biomaterials are still scanty.<sup>28</sup>

The clinical significance of the data collected in this study must be emphasized if one considers that orthopedic implants that become infected are normally exposed to relatively small numbers of bacteria much lower than the bacterial inoculums size used in our *in vitro* assay. Moreover our *in vitro* experimental conditions do not take in account the role of the host and none of his defense factors that *in vivo* make a major contribution to the outcome of an infectious process.<sup>16,29</sup> To transfer into clinical practice experimentally obtained results, an *ex vivo* failed prosthetic component analysis will be necessary to correlate biomaterials surface with infection.

Taking together, these data may have important clinical implications concerning one aspect of the multi-factorial septic loosening in TJR. We highlighted that the adhesion of both *S. aureus* and *E. coli* with different physicochemical characteristics on inert surfaces is closely influenced by biomaterial surface chemistry and that only VE blended - UHMWPE reduces both *S. aureus* and *E. coli* adhesive ability probably related to the addition of VE with its antioxidant properties. This fact introduces an added value for VE-stabilized UHMWPE that could be a joint arthroplasty valid technology by presenting excellent quality on mechanical, wear, oxidation and adhesion properties. Further studies are required to evaluate the adhesion of other microorganisms involved in TJR (fungi included) on other well characterized innovative biomaterials, such as cross-linked UHMWPE.

## Acknowledgement

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### **Conflict of interest**

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

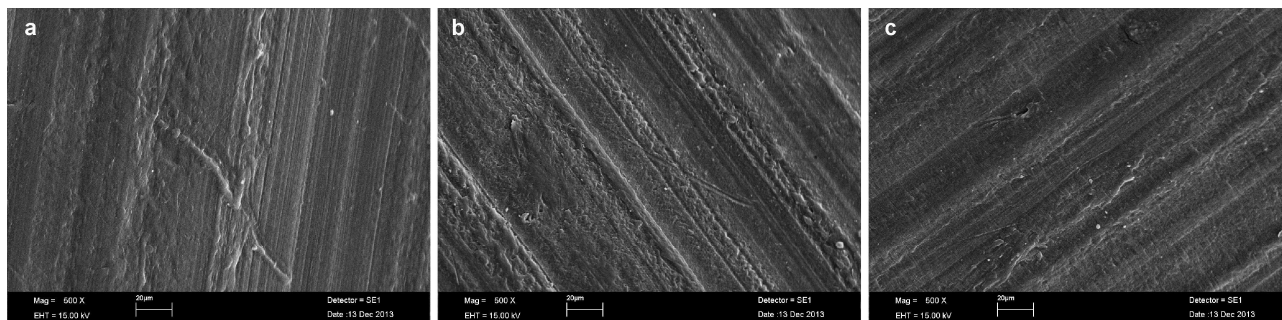
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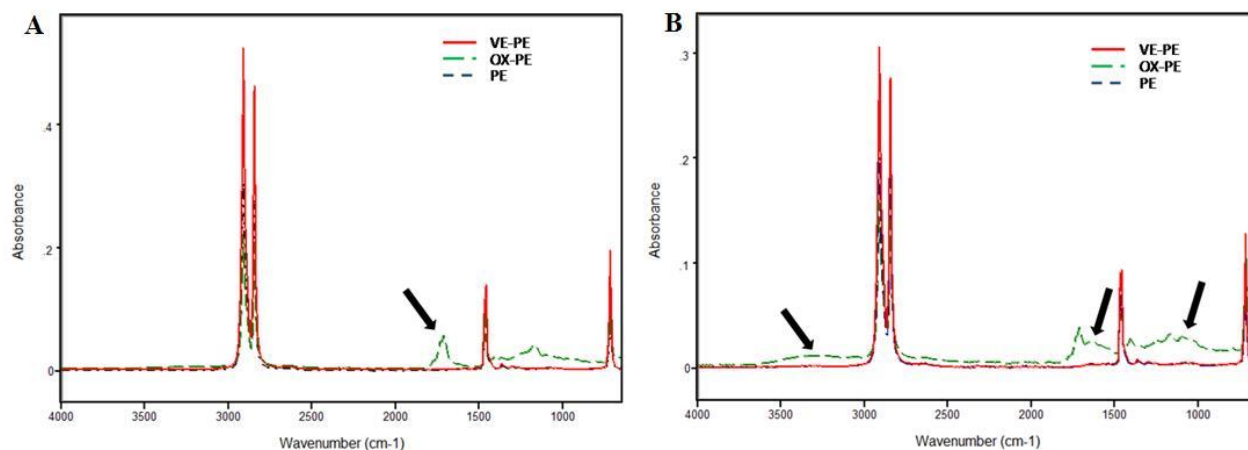
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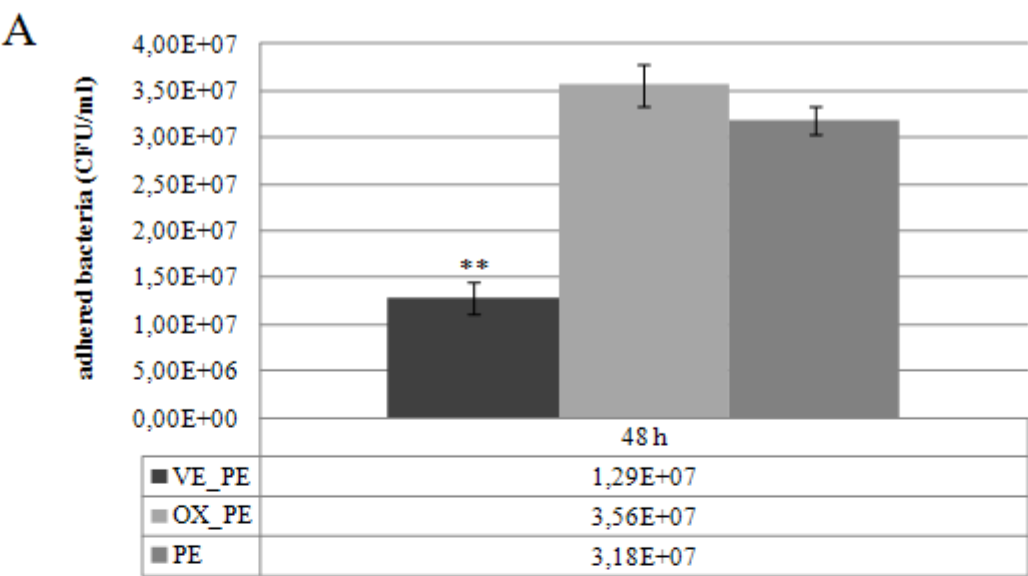
**Figure 1.** Representative SEM micrographs taken from the surface of the three polyethylene biomaterials: standard UHMWPE (PE; **a**), oxidized UHMWPE (OX-PE; **b**) and VE blended UHMWPE (VE-PE; **c**).



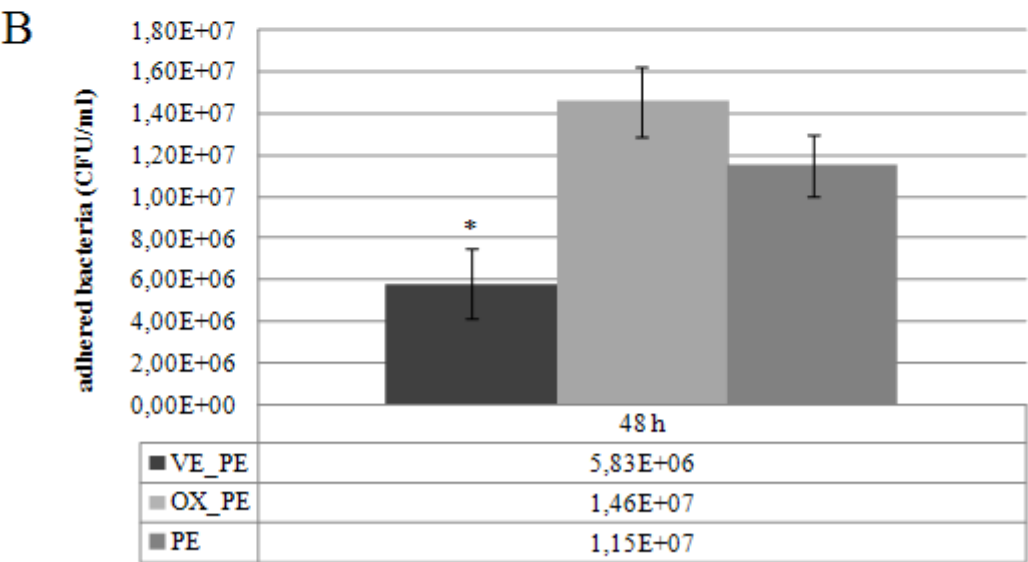
**Figure 2.** The ATR-FTIR spectra of VE blended UHMWPE (VE-PE), oxidized UHMWPE (OX-PE) and standard UHMWPE (PE) before the adhesion assay (A) and after the suspension in TSB (B).



**Figure 3.** Comparison of adherence for the well characterized biofilm producing *Staphylococcus aureus* ATCC 29213 (A) and *Escherichia coli* ATCC 25922 (B) on VE blended UHMWPE (VE-PE) and oxidized UHMWPE (OX-PE) versus standard UHMWPE (PE) at 48 hours of incubation.



\*\*= significantly different from standard UHMWPE (p< 0.05)



\* = significantly different from standard UHMWPE (p< 0.01)