Methodological approach
of antibacterial surfaces characterization

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The surface biocontamination is a very widespread phenomenon in the food industries, medical appliances... This biofouling can alter the products, leading to economic losses linked or not to health problems. Stainless steel is extensively used in the above mentioned markets because of its good cleanability, high corrosion resistance, stability, inertia under most circumstances.

Nevertheless, in material research, an important way of investigation is the elaboration of material able to kill microorganisms that are in contact with or limit their proliferation. The development of such surfaces requires robust and standardized methods to quantify the antimicrobial activity of those materials. Only few standards are available such as JIS Z2801 which is based on a culture method. This standard was assessed and compared to alternative methods. The study was carried out using stainless steel as the reference material, "antimicrobial" materials, and two bacterial species: E. coli and S. aureus.

Because viability is not easily defined with a single physiological or morphological parameter, the obtained results show the possible overestimation of the material antimicrobial activity with cultivation methods such as the JIS Z2801 standard. Thereby, to assess the antibacterial activity of materials, it is preferable to combine different methodologies, such as culture and in situ detection.

Keywords: Biocontamination, material, antimicrobial efficiency, standard, evaluation, alternative methods, stainless steel, copper, silver

INTRODUCTION

Bacteria can be found living into two states, either floating in a liquid medium as planktonic bacteria or embedded within a matrix which is called a biofilm. Bacteria more commonly exist in the sessile form than in free-floating form [1]. It is well known that, compared to planktonic bacteria, sessile bacteria or within biofilms exhibit a higher resistance to antimicrobial agents [2, 3, 4, 5] due to different metabolism activities such as, growth rates [6] or ATP[a] level [7] for example or due to the delayed penetration of the antimicrobial agent through the biofilm matrix [5]. Developing effective methods to reduce the surface biocontamination is a growing area of material research. Two approaches have been taken to prevent biofilm formation. One is to develop surfaces inhibiting bacterial adhesion on surfaces [8]. Another way to limit biocontamination is the elaboration of material able to kill microorganisms which are in contact, during the initial stage of biofilm formation [9]. The development of such "anti-

[a] Adenosine TriPhosphate (ATP) is the universal energy molecule for all living cells.

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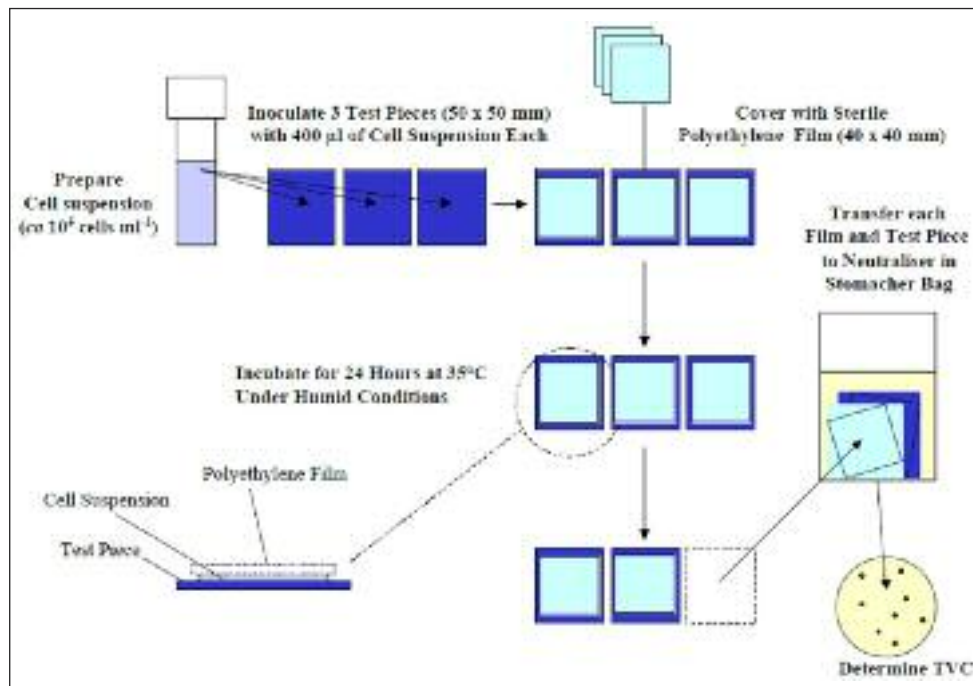Como, 21-23 September 2011, organized by AIM*

bacterial" surfaces requires robust and standardized methods to quantify the antimicrobial activity of that kind of material. This study deals with the description of the most used methods and the synthesis of their advantages and drawbacks.

First of all, it's necessary to define the term "antibacterial". It's an inappropriate term for microbiology even though it's often used [10]. It's necessary to well define following terms:
- **Bacteriostatic** means that the surface inhibits the microbial grow in favourable conditions for the cell multiplication [11]. A reduction of the contamination can take place but the suppression of the microorganism multiplication is enough.
- **Bactericide** is defined as *a product that kills vegetative bacteria in well defined conditions* [10 NF EN 14 885]. As a function of the tests, it requests a 4 or 5 log10 reduction of the bacterial contamination with defined strains. This efficiency level has to be reached for sanitizers on planktonic cells but is difficult to obtain for treated surfaces with adhering bacteria.
- A third intermediate level of activity exists and corresponds to **a microorganism reduction activity** in a 1 to 4 log10 range [12, 13, 14].

Numerous standards are available to assess the efficiency of antimicrobial agents such as sanitizers, antibiotics. They can be classified in 3 groups:
- methods based on micro-organism growth,
- direct measurement of the biocontamination,
- methods based on the detection of cellular components.

Concerning the methods to test the antimicrobial activity of surfaces, there are only few standards such as the JIS Z2801:2000 [12], the ASTM E 2149 [13] and the ISO 22196 [14] standards. Both are based on culture methods. So, in this report, the JIS Z2801:2000 standard was assessed and compared to alternative methods based on growth methods and on direct measurement of the biocontamination. The selected alternative methods were

FIG. 1

Schematic representation of the JIS Z 2801 standard [12].

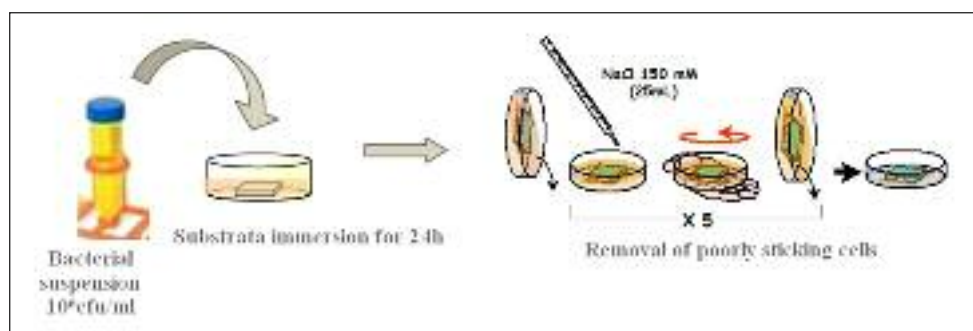Rappresentazione schematica della norma JIS Z 2801 [12].

FIG. 2

Schematic representation of the adhesion experiment.

Rappresentazione schematica dell'esperimento di adesione.

the modified dilution broth method, and the epifluorescence technique.

MATERIALS & METHODS
JIS Z 2801:2000
This standard specifies the testing method to evaluate antimicrobial activity and antimicrobial efficiency on bacteria on the surface of antimicrobial products. It also defines the main term used in this Standard: **antimicrobial means the condition inhibiting the growth of bacteria on the surface of products**.
As shown on the figure 1, each tested piece is put in a sterile Petri dish, is then inoculated with a bacterial suspension at 2,5 to 10×10^5 cfu/ml. The inoculum is covered with a film that is pressed to spread the inoculum. The Petri dish is incubated at a temperature of 35°C+/-1°C and a relative humidity of not less than 90% for 24h+/-1h.
At T0 and after the incubation time, the tested piece and the covering film are placed in a neutralizer broth in a stomacher bag and are massed to detach bacteria. Immediately the viable cells are counted by the serial dilution and the agar plate method after 40-48h of incubation at a temperature of 35°C+/-1°C. The number of viable bacteria (N) is calculated as follows:

$$N = C \times D \times V \tag{1}$$

where C is the number of colonies; D is the dilution ratio and V is the volume of neutralizer broth used to detach bacteria.
The following relationship gives the antimicrobial activity value:

$$R = [\log(B/A) - \log(C/A)] = [\log (B/C)] \tag{2}$$

where R is the value of the antimicrobial activity, A the average of the number of viable cells at T=0 of incubation on control piece, B is the average of the number of viable cells after 24h of incubation on control piece and C is the average of the number of viable cells after 24h of incubation on the antimicrobial piece.
As a stomacher is not available in the lab, bacteria were detached from the surfaces by ultra-sounds. Before the evaluation of the antimicrobial surfaces, preliminary tests were performed to validate the ultra-sounds step doesn't alter the bacterial cultivability, the neutralizer broth is not toxic for bacteria and is efficient to neutralize antimicrobial compounds.

The modified dilution broth method
Another procedure for antibacterial agent sensitivity testing involves a dilution assay. A series of two-fold dilutions of each antibacterial agent are made in the tubes and then all tubes are inoculated with the same test organism. After incubation, the inhibition of the growth by the various agents can be observed by measuring the turbidity. Sensitivity can be expressed as the highest dilution which completely inhibits growth [15].
In our experiments, the tested materials (1x1cm²) are put in 20 ml of nutrient broth (TSB - Bio-Rad, France). Then, the broth is inoculated with the test organism and incubated at 35°C. The microbial growth is monitored by turbidity measurements. At the end of the incubation time, cultivable cells are counted by the serial dilution and the agar plate method.

	Cr	Ni	Mn	Si	Mo	Nb	Ti	Co	V	C (ppm)	Cu
304 BA	18.17	8.03	1.220	0.42	0.25	0.008	≤0.001	0.14	0.100	490	0.27

TAB. I   *Chemical composition of the stainless steels used, expressed in weight percentage.*

Composizione chimica dell'acciaio inossidabile utilizzato, espresso come percentuale in peso.
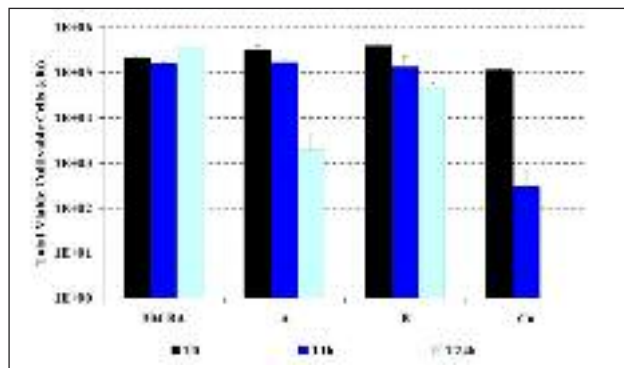

FIG. 3   *Total viable cells of S. aureus 53.154 strain at T0 and after 1 and 24h of contact with the tested surfaces.*

Cellule vitali totali di S. aureus 53.154 per le varie superfici in prova, con tempi di contatto di 0, 1 e 24h.
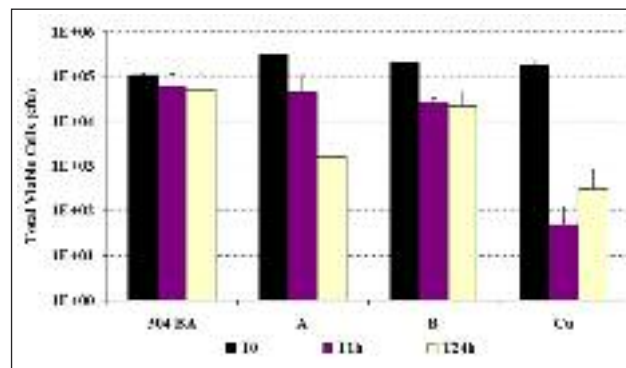

FIG. 4   *Total viable cells of strain at T0 and after 1 and 24h of contact with the tested surfaces.*

Cellule vitali totali di E. coli 58.55 per le varie superfici in prova, con tempi di contatto di 0, 1 e 24h.

The cell labelling and *in situ* detection

Coupons were immersed in the bacterial suspension (10^6 cells/mL) in static conditions at room temperature for 24h. Non-sticking cells were removed by five successive rinses with NaCl 0,15M.

Adherent cells were labelled with a solution of two fluorochromes (DAPI and Sytox Green®, Molecular Probes, USA). Blue-labelled (DAPI) cells represent total adherent bacteria whereas green (SYTOX GREEN®) labelled bacteria were considered as injured [16]. The fouled samples were observed with an epifluorescence microscope (obj x 100). For each field observed, numbers of total (N_{tot}) and injured ($N_{injured}$) cells were determined by image analysis (Analysis, USA) and expressed as cells/cm². Viability percentage can be calculated with equation 3.

$$\text{Mortality (\%)} = (N_{injured}/N_{tot}) \times 100 \qquad (3)$$

Bacterial strains

The strains used in this study, *Staphylococcus aureus* CIP 53.154 and *E. coli* CIP 58.55, were provided by the Pasteur Institute (France) and stored at –196°C in glycerol. Work stocks were kept at –20°C.

Cells were subcultured twice in the same medium as the work culture TSB (Bio-Rad, France). Microorganisms were grown at 35°C in static conditions until the early-stationary phase culture. Then, bacteria were harvested by centrifugation (7000g, 10 min.), washed twice and resuspended in NaCl 0,15M to obtain the necessary concentration for the experiments.

Coating	Composition	Thickness
A	Ag	100 nm
B	Ag in a matrix	60 nm

TAB. II   *Characterizations of the tested antimicrobial coatings.*

Caratterizzazione dei rivestimenti antimicrobici sottoposti a prove.

Tested materials

Stainless steel used in this study is an austenitic grade with a bright annealing finish. The detailed chemical composition of the industrial grade is presented in Table I.

The assessed antimicrobial surfaces were copper (purity 99.5%) and stainless steel with coating containing silver. The characteristics of the coatings are described in Table II.

Prior to any testing, bare stainless steel and copper were degreased first by an acetone/ethanol (50/50) blend, then soaked for 10 min at 50°C in a 2% (v/v) RBS 35 (Société des traitements chimiques de surface, Lambersart, France) and rinsed 5 times for 5 min in distilled water at 50°C and 5 times for 5 min in distilled water at room temperature. As for the coatings, sterilization was carried out under UV irradiation for 20min.

RESULTS – DISCUSSION
JIS Z 2801:2000

Figures 3 and 4 present the numbers of viable cultivable cells counted for *S. aureus* 53.154 (Fig. 3) and *E. coli* 58.55 (Fig.4) at T=0, 1h and 24h of contact with the tested materials.

Black bars represent the initial inoculum spread on the surfaces. The counts are similar for all the tested surfaces. After 1h of contact between this inoculum and the surfaces, no significant loss of cultivability is observed for all materials, excepted for copper which one presents a significant reduction (>2 $\log_{10}$).

After 24h of incubation, materials show different behaviours. The counts of viable cultivable cells on 304 BA and B are not reduced. As 304 BA is known to be an inert surface, this result is conformed to expectations. On the opposite, the deposit B which is supposed to be antimicrobial, is not efficient to inhibit the S. aureus growth. The A coating presents an intermediate behaviour with a cultivability reduction of 2 $\log_{10}$. On copper, cultivable cell counts fall from 5 $\log_{10}$ to less than 1 $\log_{10}$ cell can be counted.

As for *S. aureus* 53.154, similar results to this test are obtained for *E. coli* 58.55 (Fig.4). It means that 304 BA and the B coating don't inhibit the growth of *E. coli* 58.55; the A coating presents

	A	B	Cu
S. aureus 53.154	2,33	0,90	5,57
E. coli 58.55	1,50	0,36	2,22

TAB. III *Antimicrobial activity value (R) of each tested material against S. aureus and E. coli.*

Valore dell'attività antimicrobica (R) contro S. aureus e E. coli di ogni materiale sottoposto a prova.

		Blank	304 BA	Copper
S. aureus 53.154	Slope (µ)	0,51	0,59	0,55
	Tg (h)	1,36	1,17	1,26
E. coli 58.55	pente (µ)	1,2	1,1	0,96
	Tg (h)	0,58	0,63	0,72

TAB. IV *Slope and calculated generation time (Tg) for S. aureus 53.154 and E. coli 58.55 grown in TSB containing or not a tested material.*

Pendenza e tempo di generazione calcolato (Tg) per S. aureus 53.154 e E. coli 58.55 cresciuti in TSB contenente o meno uno dei materiali sottoposti a prova.

an intermediate behaviour with a cultivability reduction of approximately 2 $\log_{10}$; and the cultivability reduction with copper is higher than 2 $\log_{10}$. It is to note that copper is less efficient to inhibit the growth of this *E. coli* strain compared to *S. aureus* 53.154. Table III summarizes the antimicrobial activity values (R) for each assessed materials on the both bacterial strains.
As previously mentioned, the JISZ2801 standard results show that copper is the most efficient to inhibit the growth of both strains. The coating A presents an intermediate inhibition and B is inefficient on both bacteria. Under the tested experimental conditions, both strains have the same silver sensibility.
It is to note that the growth reduction efficiency of copper is not reproducible between the bacterial models. It can be supposed that in the experimental conditions, *E. coli* 58.55 seems to present a higher resistance to copper than *S. aureus* 53.154.
This method allows the classification of antimicrobial surfaces as a function of the efficiency on specific strains to inhibit the cell growth. However, depending on material and bacterial properties, it is known that cells can more or less strongly stick to surfaces. Ultra-sounds may not be sufficient to detach cells from materials leading to an overestimation of the antimicrobial efficiency. The removal from the surface of damaged or dead cells can be easier in comparison with survivor cells. In agreement with other study [17], this methodology can overestimate the efficiency of "antimicrobial' surface.
Furthermore, this method takes into account only viable cultivable cells: what about other cell physiological states such as viable but non cultivable cells or stressed cells?

The modified dilution broth method
Growth curves of *S. aureus* 53.154 and *E. coli* 58.55 in TSB alone and TSB with a coupon of copper or stainless steel are represented on figure 5. Results are expressed in Optical density measured at 600nm (OD) as a function of incubation time.
Growth curves are generally divided into 3 parts:
- the latency period: time to the strain to be adapted at the growth conditions (temperature, broth...),
- the exponential phase: the maximal reproduction speed is reach during this period. It allows to calculate the generation time,
- The stationary phase: the cell number is stable.
Curves have different kinetic for the assessed bacterial strains. *E. coli* presents a shorter latency period compared to *S. aureus* 53.154, respectively 2 and 4h. For *E. coli* and *S. aureus*, the exponential phase runs respectively for 3h and 6h. *E. coli* reaches the stationary phase after only 5h of incubation and 10h for *S. aureus*. It is to note that measured optical densities during the stationary phase are similar for both strains. Moreover, at the end of the growth curves, cultivable cells were counted. For both strains, results were similar (data not shown). All that descriptions confirm that *S. aureus* has a slowest multiplication speed than *E. coli* but reaches the same stationary phase level.
Differences can be observed between strains but for both strain, the addition of stainless steel or copper coupons in the nutrient broth doesn't impact the growth characteristics (Table VI).
The presence of a stainless steel or a copper piece in the nutrient broth doesn't alter the generation time, nor the time of each growth curve part. Those results confirm that stainless steel has no antimicrobial activity.
The previously observed antimicrobial activity of the copper is not confirmed. It means that copper antimicrobial efficiency needs a very close contact between bacteria and copper or a higher concentration of released copper ion into the nutrient medium. Moreover, this results show that the presence of organic matter in the solution affect the antibacterial efficiency of copper. The solid matter can decrease the copper leakage in the solution acting as a barrier by adsorption at the liquid-copper interface [18]. As one of the copper ion target is proteins [19], the soiling matter can act as a substitute leading to a decrease of its antimicrobial activity.
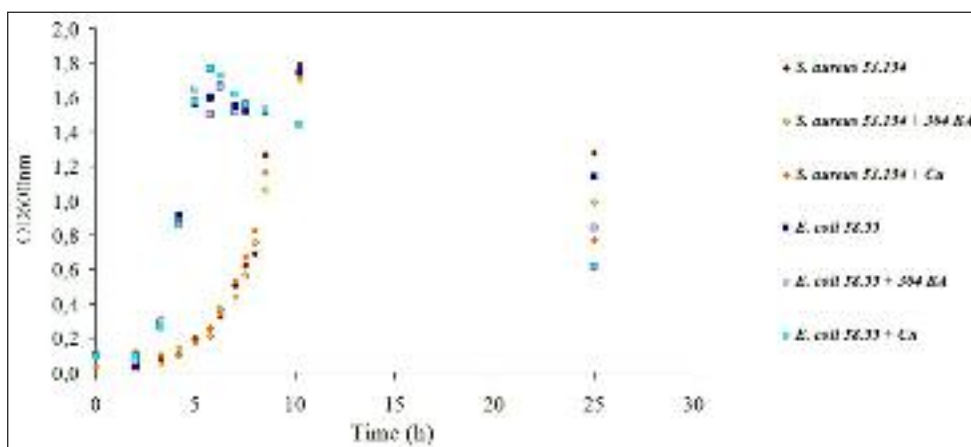

FIG. 5
Growth curves of S. aureus 53.154 and E. coli 58.55 in TSB w/wo 304 BA or copper.

Curve della crescita di S. aureus 53.154 e E. coli 58.55 in TSB solo o in presenza di 304 BA o rame.
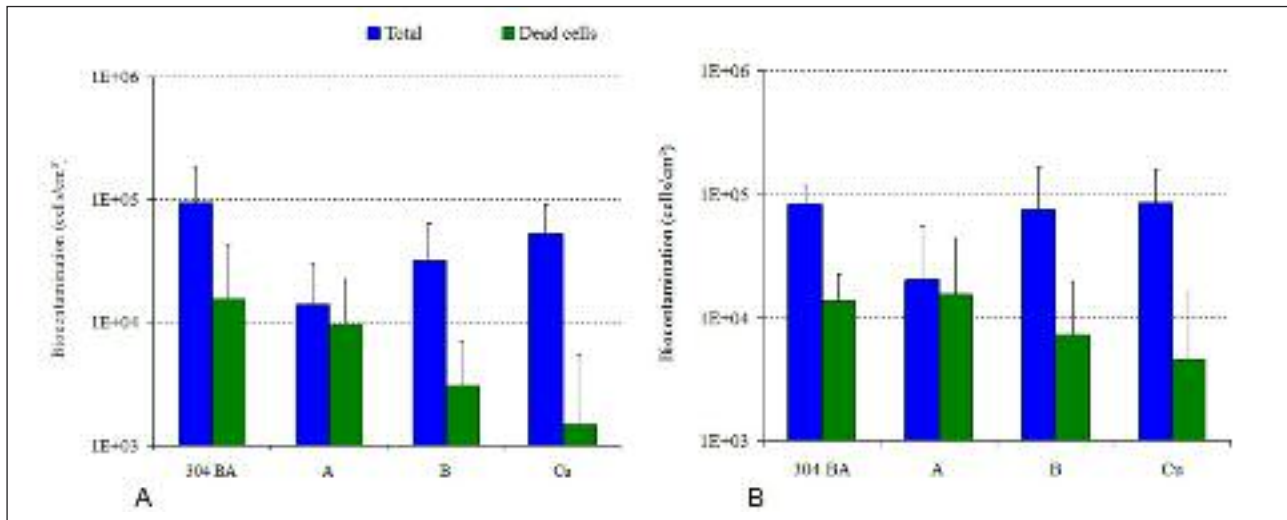
FIG. 6 *Biocontamination and dead levels (cells/cm²) of S. aureus 53.154 (A) and E. coli 58.55 (B) adhering cells on tested materials after 24h of contact.*

Biocontaminazione e livelli di mortalità (cellule/cm²) di cellule di S. aureus 53.154 (A) e E. coli 58.55 (B) aderenti ai materiali sottoposti a prova dopo 24h di contatto.

In situ detection

For each tested materials, the biocontamination (number of total cells) and dead levels are represented on the figure 6A for *S. aureus* 53.154 and on figure 6B for E. coli 58.55. They are expressed as a number of cells/cm².

For both strains, the most contaminated surfaces are 304 BA, copper and the coating B and the less contaminated material is B. This material ranking in terms of total biocontamination is due to the surface physico-chemical properties of each material leading to adhesion forces. The small differences between both bacterial strains are due to their own surface properties and analytical variability.

Concerning the number of dead adhering cells, we can observe that injuring level seems to be inversely proportional compared to the adhering cells number. The percentage of dead cells are summarised in table V.

The maximal injuring percentages are observed for the material A for both strains while B seems to be less efficient. For coatings containing silver, the ranking is in agreement with the one obtained for the JISZ 2801:2000 standard.

On the other hand, copper presents the lowest level of injured cells while copper has a great antimicrobial efficiency according to the JISZ standard. The difference of results for both methods between silver based coating and copper means that both methods are complementary.

Thus, in our experiments, copper has just an inhibiting activity on microbial growth while the silver ions alter the cell membranes. In the used experimental conditions, silver is more efficient on the tested strains, probably due to its targets or kinetics of reaction, than copper. The silver and copper solubilisation and leakage have to be considered in those experiments to explain their antimicrobial efficiency.

The *in situ* detection of biocontamination gives data about biocontamination level of materials, the distribution of cells *i.e.* single cells, cluster, size of the bacterial cluster (data not shown), injuring level of the adhering cells. As a function of the chosen fluorochrome, it's also possible to determine the metabolism activity and the cell division activity. Compared to the JISZ standard, this method takes into account the adhesion strengths involved in the biocontamination mechanism.

CONCLUSIONS

In agreement with previous studies [20], the results show a striking difference between the numbers of CFU (JISZ: 2801-2000) and the numbers of total and injured adhering cells (*in situ* detection) for some antimicrobial materials. Indeed, it is well known that changes in the bacterial surface arrangement and metabolism follow the adhesion to materials. [2,3,4].

Those differences generally lead to an overestimation of the number of dead cells by cultivation method and thereby to overestimate the antimicrobial efficiency of materials as observed in this study. Table VI summarizes the characteristics of the assessed methods in this work.

The growing methods usually used to assess the material antimicrobial efficiency, mean that the microbial flora is composed of viable and cultivable vegetative cells. Thus, the sample analysis depends on all the growth factors: bacterial strain, nutrient medium, time and temperature of incubation.

Because viability is not easily defined in terms of a single physiological or morphological parameters that kind of method doesn't allowed the detection of stressed cells or viable but non-cultivable cells (VBNC). Moreover, a previous study shows that some cells are able to divide but not enough to reach the stage of macro-colony on agar [20].

Furthermore, the results depend on the efficiency of detachment step, on the recovering medium. Thus, the antimicrobial activity of the surfaces may be overestimated with cultivation methods. So, to assess the antibacterial activity of materials, it is preferable to combine different measures, such as culture, enzymatic activity, membrane permeability, that complete the results with the biocontamination level, the cell physiological state...

	304 BA	A	B	Copper
S. aureus 53.154	16,46	43,89	9,97	5,38
E. coli 58.55	19,37	32,08	13,01	3,08

TAB. V *Percentage of dead cells for S. aureus 53.154 and E. coli 58.55 adhering cells on each tested material.*

Percentuale di cellule morte di S. aureus 53.154 (A) e E. coli 58.55 (B) aderenti ad ogni materiale sottoposto a prova.

	JIS Z 2801	Dilution Assay	In situ detection
Material	Classical lab material		Epifluorescence microscope
Technical difficulty	++	+	+++
Time to result (for 1 sample)	Adhesion: 24h Culture: 24-48h = 48-62h	Culture: 24-48h	Adhesion: 24h Labelling+observation: 2h = 26h
Number of step	1-adhesion 2-detachment + dilution 3-culture	1-OD measures	1-adhesion 2-labelling
Data	Growth inhibition of detached cells	Cell growth inhibition	Biocontamination level + Physiological state of adhering cells
	Qualitative & quantitative	Qualitative & quantitative	Qualitative & quantitative

TAB. VI *Synthesis of the assessed methods characteristics.*   *Sintesi delle caratteristiche dei metodi valutati*

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Abstract

Approccio metodologico per la caratterizzazione di superfici antibatteriche

Parole chiave: Caratterizzazione materiali - acciaio inossidabile

La biocontaminazione superficiale è un fenomeno molto diffuso nelle industrie alimentari, nei dispositivi medici, ecc... Questa forma di contaminazione può portare all' alterazione dei prodotti, e alla conseguente perdita economica più o meno legata a problemi sanitari.

L'acciaio inossidabile è ampiamente utilizzato in questo tipo di mercati per la sue caratteristiche di buona lavabilità, di elevata resistenza alla corrosione, di stabilità, di inerzia in quasi tutte le circostanze.

Tuttavia, nel campo della ricerca dei materiali, un importante filone di indagine riguarda l'elaborazione di un materiale che sia in grado di uccidere i microrganismi con cui esso viene in contatto o almeno di limitare la loro proliferazione. Lo sviluppo di superfici di questo tipo richiede metodi provati e standardizzati che permettano di quantificare l'attività antimicrobica di tali materiali. A tale proposito sono disponibili solo poche normative, come la JIS Z2801 che si basa su un metodo di coltura. In questa memoria tale norma è stata valutata e confrontata con metodi alternativi. Lo studio è stato effettuato con acciaio inox come materiale di riferimento, materiali "antimicrobici", e due specie di batteri: E. coli e S. aureus.

Poiché la vitalità non è facilmente definibile con un singolo parametro fisiologico o morfologico, i risultati ottenuti mostrano la possibile sovrastima dell'attività antimicrobica del materiale con metodi di coltivazione come quali quelli prescritti nella norma JIS Z2801. Quindi, per valutare l'attività antibatterica dei materiali, è preferibile utilizzare diverse metodologie abbinate, quali la coltura e la rilevazione in situ.