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Correlation between microbiological contamination of the environment and the care-associated infections (ICAs) in public (hospital care) and private (nursing homes and/or home care) facilities.

Scientific Disciplinary Sector ING –IND/10

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To my family: Claudio, Dino, Serena, Argentina and Felice

Contents

Definitions

Antibiotic: an active substance of synthetic or natural origin which is used to eradicate bacterial infections in humans or animals [1].

Bacteria: generally divided into two groups: Gram-negative bacteria do not retain the crystal violet stain (Gram's stain), while Gram-positive do.

Bioburden: the microbiological load (i.e., number of viable organisms in or on the object or surface) also known as "bioload" or "microbial load."

Biocide (According to the EU Biocides Regulation 528/2012 definition): a product able to destroy, deter or damage organisms, by any means, with action other than simple physical or mechanical action. It applies to all active substances of the products that contain or produce one or more active substances [I].

Biofilm: a set of bacterial colonies within a mucous matrix, able to adhere tensely to an even inanimate surface[11].

Care-associated infection (ICA) is a **nosocomial infection, also called "hospital-acquired infection" or "**healthcare-associated infection" **(HAI) and is defined as:** a microbial infection occurring in a patient in a hospital or other healthcare facility in which the infection was not present or incubating at the time of admission. This includes infections acquired in the hospital but appearing after discharge, and also occupational infections among staff of the facility. The term "nosocomial" comes from two Greek words: "nosus" meaning "disease" + "komeion" meaning "to take care of."
Hence, "nosocomial" should apply to any disease contracted by a patient while under medical care [2]. These infections are also caused by microorganisms that are resistant to antibiotics [3;4;5;6].

Cleaning: the removal of visible soil, organic and inorganic contamination from a device or surface, using either the physical action of scrubbing with a surfactant or water with appropriate chemical agents, defined as detergents [7].

Colony-Forming Unit (CFU): the minimum number of separable cells on the surface of or in semi-solid agar medium which gives rise to a visible colony of progeny in the tens of millions. CFUs may consist of pairs, chains, and clusters as well as single cells, and are often expressed as colony-forming units per meter (CFU/m^2).

Detergents: compounds that possess a cleaning action and have hydrophilic and lipophilic parts. Although products used for hand washing or antiseptic hand wash in a healthcare setting represent various types of detergents, the term "soap" is used to refer to such detergents in this guideline. Detergents make no antimicrobial claims on the label. [7].

Disinfectant: a chemical that destroys vegetative forms of harmful microorganisms (such as bacteria and fungi) especially on inanimate objects (surfaces), but may be less effective in destroying spores. Disinfectants are antimicrobial agents that are applied to the surface of non-living objects to destroy microorganisms that are living on the objects. [7;8]
Disinfectants are different from other antimicrobial agents such as antibiotics, which destroy microorganisms within the body, and antiseptics, which destroy microorganisms on living tissue. Disinfectants are also different from biocides — the latter are intended to destroy all forms of life, not just microorganisms. Disinfectants work by destroying the cell wall of microbes or interfering with their metabolism [8;9].

Microbiological contamination: Microbiological contamination refers to the non-intended or accidental introduction of infectious material such as bacteria, yeast, mould, fungi (dermatophytes), viruses, prions, protozoa or their toxins and by-products [10].
The existence of viable microorganisms in, on, or around materials constitutes microbiological contamination.

Microbiota: the gut flora and symbiotic microorganisms living in the human body without damaging it. [11].

Opportunistic infection: an infection caused by a microorganism that does not ordinarily cause disease but is capable of doing so under certain host conditions (impaired immune response) [1].

Pathogen: a disease-causing microorganism or microbe [1].

Probiotics: (meaning: for life), all strains of microorganisms that are ingested to improve the (eubiotic) balance of human microbiotics, which benefit and contribute to the well-being of the organism. Probiotics are microorganisms such as non-pathogenic and non-toxic bacteria or fungi that contribute to the balance of the human microbiata [12].

Sanitizer: substance that simultaneously cleans and disinfects [5].

Total Microbial Viable Count (TVC): a method of estimating the viable number of microorganisms (bacteria, yeasts and moulds) per unit sample. The term also refers to the estimated number of bacteria per unit sample, usually expressed as colony-forming units (CFUs) per square meter (m^2).

Abbreviations

ACC: aerobic colony count.

AST: Antimicrobial Susceptibility Testing.

ATCC: American Type Culture Collection.

CDC: European Center for Disease Control.

CFU: Colony-Forming Units.

CRE: Carbapenem Resistant Enterobacteriaceae

EPA: Environmental Protection Agency.

EUCAST: European Committee on Antimicrobial Susceptibility Testing.

FDA: Food and Drug Administration.

HAIs: Care-Associated Infections (ICA) / nosocomial infections (NI) / Hospital-Acquired Infections / Healthcare-Related Infections.

HPC: heterotrophic plate count.

LTCF: long-term care facility for geriatric patients.

MDR: Multi-Drug Resistant.

MDRO: Multi-Drug Resistant Organism.

MRSA: Methicillin-Resistant *Staphylococcus aureus.*

PVC: polyvinyl chloride.

RODAC: Replicate Organism Detection And Counting.

RSA: Health Care Residence for geriatric patients.

SOP: standard operating procedures.

TMC: Total Microbial Count.

TVC: Total Microbial Viable Count.

WHO: World Health Organization.

1. Introduction:

The microbial hazard derived from environmental contamination is a major problem that affects the safety of public health in every field (food, pharmaceutical, cosmetic and especially medical) [II].
In the medical field, healthcare plays an important role in the quality and safety of both public (hospitals) and private (nursing homes, home-care and long-term care) facilities.
Although health facilities are structurally different (hospitals, day hospitals, day surgeries and healthcare residence (RSA) or long-term care facility (LTCF) for geriatric patients), they have the common and fundamental requirement to ensure a hygienic environment, especially at the microbiological level.

Disease prevention is directly related to health promotion.
Currently, care-associated infections (HAIs) are on the rise. They will become an even more significant public health problem with an increasing economic and human impact because of the rising numbers and crowding of people with impaired immunity (age, illness and treatments); new microorganisms, defined as opportunistic, do not ordinarily cause disease but are capable of doing so in people with an impaired immune response [11;13;14] and increased bacterial resistance to antibiotics [15].
Long-term care services are crucial to the welfare of older people as their numbers rise throughout Europe. Endemic bacterial infections at LTCFs are a major health problem with high individual, social and economic costs, especially if these infections are caused by pathogens resistant to the major antibiotics used in treating them.
Presently, the spread of infection complications among patients with chronic conditions and the elderly is high. The incidence of health-related infections in LTCFs is comparable to that observed in hospitals. In this context, it is more appropriate to define hospital infections as "Healthcare-Related Infections"[16;17].
Healthcare facilities are confined and organized environments, where the risk of cross-contamination is high and consequently also the risk of infection.
Microbes are ubiquitous and present in all environments, including abiotic ones, and are colonized by a set of bacterial and mycotic cells that have rapidly producing means of adhesion on surfaces, one such being biofilm, a matrix of extracellular polymeric substances which protects the bacteria from chemical agents and allows their survival by living together.

Bacteria are distinguished in “commensal” and “pathogenic” bacteria.
Commensal bacteria belong to the normal flora of healthy humans. They are usually harmless to healthy people or even have a significant protective role by preventing colonization by pathogenic microorganisms. Some commensal bacteria may however cause infection if the host is compromised or if they are brought into the host’s tissue [11].
Pathogenic bacteria have greater virulence and cause infections regardless of the host’s status [1]. They are able to survive adverse conditions while remaining virulent for long periods of time.

There is a broad range of microbiological pathogens which can cause contamination and thus infection. The infection can originate from the outside environment, another infected patient, healthcare staff that may be infected, or in some cases, the source of the infection cannot be determined. In some cases the microorganism originates from the patient's own skin microbiota, becoming opportunistic after surgery or other procedures that compromise the protective skin barrier. Though the patient may have contracted the infection from their own skin, the infection is still considered nosocomial since it develops in a healthcare setting. [III]

Although the main source of nosocomial pathogens is likely the patient's endogenous flora, an estimated 20 to 40% of HAIs have been attributed to cross infection via the hands of healthcare personnel who have become contaminated from direct contact with the patient or indirectly by touching contaminated environmental surfaces. Contaminated environmental surfaces provide a significant and potential source for transmission of HAIs and increase cross-transmission [18;19;20].

The correlation between the microbiological contamination of the environment and the **HAIs** must be controlled and reduced by means of a valid sanitization process in order to decrease potential pathogens [21].

In compliance with the guidelines of the World Health Organization (WHO) and European Center for Disease Control (CDC), a well-functioning healthcare system requires an important procedure for preventing infections [22-23], and considerable evidence exists concerning the benefits of hospital cleanliness in reducing HAIs [IV].

Prevention of biological hazards in the hospital environment is essential in limiting the spread of potential pathogens at the environmental level and ensuring adequate and effective protection against infections of all people present and/or attending a healthcare facility (patients, healthcare workers, caregivers and visitors).

The sanitation procedures have the precise aim of reducing and containing the proliferation of microorganisms, especially in hospital environments [24].

The effectiveness of cleaning operations is vital, because it has a direct influence on the health of patients [25;26;27]. Disinfection of the surfaces and the environment becomes an absolutely critical component in preventing bacterial contamination and the spread of infections [28;29]. This represents the potential risk of cross-contamination for the patient, caused by direct and indirect contact of patients with potentially contaminated surfaces [30].

An innovative approach is the use of non-pathogenic microorganisms, called probiotics [12] and defined as live microorganisms which confer a health benefit on the host and colonize hard surfaces in order to counteract the proliferation of other bacterial species, according to the competitive exclusion principle [31; 32]. Traditional chemical disinfectants used in cleaning procedures in hospitals display several disadvantages such as time-limited biocidal activity and rapid bacterial re-contamination of treated surfaces. Recent experimental studies report the validity of a technique based on microbial bio-stabilization by using probiotic cleaners [33;34;35;36;37;38].

1.1 Healthcare-associated infections (HAIs)

According to the WHO and CDC, hundreds of millions of patients are affected worldwide by HAIs yearly [22; 23]. The burden of disease is much higher in low income and emerging economies countries [IV].

The data for the frequency of HAIs comes from surveillance systems, such as the US National Nosocomial Infections Surveillance System and the European Center for Disease Control.

The prevalence of patients with at least one HAI in acute care hospitals was 6% (country range 2.3%–10.8%).

The microorganisms most frequently contributing to HAIs are, in decreasing order, Escherichia coli (15.9%), Staphylococcus aureus (12.3%), Enterococcus spp. (9.6%), Pseudomonas aeruginosa (8.9%) Klebsiella spp. (8.7%), Coagulase-negative staphylococci (7.5%), Candida spp. (6.1%), Clostridium difficile (5.4%), Enterobacter spp. (4.2%), Proteus spp. (3.8%) and Acinetobacter spp. (3.6%) [23].

In Italy, the rate of health-related infections is between 5 and 8% for hospitalized patients and 5% for geriatric patients in RSAs [39; 40].

Care-associated infections are a very significant problem and outnumber the cases of influenza, HIV and tuberculosis aggregates. Primary blood infections account for about 6% of HAIs and are associated with the highest mortality rate (15%) [41]. In Italy, catheter-related blood infections (CR-BSI) account for between 8 and 12% of assistance-related infections [42; 43].

Furthermore, the most frequent type of infection hospital-wide is catheter-related urinary tract infections (UTI), often caused by *Candida* yeasts or *E. Coli* bacteria, which are able to attach to the bladder wall and form a biofilm that resists the body's immune response [44; 45].

Pathogens may be transmitted via the hands of patients or healthcare workers to environmental surfaces, where they can persist or proliferate if cleaning and disinfection are not performed.

Depending on the organism, microbes can persist in the environment for hours (some enveloped viruses), days or weeks (most vegetative bacteria and fungi), or months (bacterial spores and fungal spores) [21; 46; 47; 48]. Common surfaces in the rooms of patients colonized or infected with the methicillin-resistant bacteria S. aureus or vancomycin-resistant Enterococci (VRE) may become contaminated and touched by healthcare workers [49].

Prior room occupants colonized or infected with VRE, methicillin-resistant S. aureus (MRSA) or Clostridium difficile, also increase the risk of colonization or infection for the next occupant [50].

Infectious agents can also be transferred to patients and healthcare workers after contact with a contaminated surface, as demonstrated by healthcare worker hand-imprint cultures after contact with environmental surfaces in patient rooms [51]. One study found that environmental surface contamination is a determinant of transmission of Multi-Drug Resistance Organisms (MDROs), that are resistant to several antimicrobial agents (drugs or chemicals) [52]. Infections caused by resistant organisms pose significant challenges, especially among immune-compromised critical-care patients

e.g. the elderly, people with multiple pathologies or intensive care and oncology patients [53]. Antimicrobial resistance is an urgent and global threat. It leads to high morbidity and mortality, as well as economic burden [54]. The impact of antibiotic resistance on lives, health systems and economies is considerable and will continue to grow.

The yearly cost to the U.S. health system alone has been estimated at 21 to 34 billion dollars, as well as more than 8 million extra days in the hospital. In the EU, resistance to antibiotics costs an estimated €1.5 billion per year [55; 56].

There is a correlation between high antibiotic consumption and antibiotic resistance. Antibiotic use in human medicine, veterinary medicine, and agriculture has been linked to the rise of antibiotic resistance globally [57].

Antibiotics are not only essential to treat infections, but also help prevent them in a wide number of clinical conditions (e.g. during cancer treatment or surgery). Over-use and misuse of antibiotics drives the emergence of resistance in pathogenic microorganisms, which in turn raises mortality and morbidity rates associated with infectious diseases.

Antibiotic drugs have always been very useful in combating infections and have greatly reduced the rate/numbers of illness and death in humans and animals, but due to the misuse and over-prescription of antibiotics, many infectious microorganisms have adapted and become resistant to many first-line (older and less expensive) and even second-line (new and expensive) antibiotics [58; 59; 60] [IV; V].

The black list of the most important MDROs or Resistant 'Superbugs' in healthcare facilities includes: methicillin-resistant *Staphylococcus aureus* (MRSA); vancomycin-resistant *Enterococcus species* (VRE); Extended Spectrum β-Lactamase (ESBLs) producing Gram-negative bacteria such as *Escherichia coli* [VI]; carbapenem-resistant *Enterobacteriaceae* (CRE), such as *Klebsiella pneumoniae carbapenemase* (KPC); MDR *Acinetobacter baumannii*; MDR *Pseudomonas aerginosa* and MDR *Candida strains* [61].

On the 10th annual European Antibiotic Awareness Day, the European Centre for Disease Prevention and Control (ECDC) released its latest EU-wide data on antibiotic resistance and antibiotic consumption [VII]. In 2015, antibiotic resistance continued to increase for most bacteria and antibiotics under surveillance. In particular, the EU average percentage of carbapenem resistance in *Klebsiella pneumoniae* increased from 6.2% in 2012 to 8.1% in 2015 [VII]. The WHO, alongside the Food and Agriculture Organization of the United Nations and the World Organization for Animal Health, is developing a campaign on antibiotic resistance that includes interventions to reduce antibiotic use in food-producing animals [62; 63].

In this context, the experimental evaluation in the reduction of the microbiological load on hospital surfaces through probiotic-based cleaning procedures, in comparison with traditional chlorine-based chemical disinfectants, gives evidence that it is a new strategy and, therefore, a valid intervention in the control of nosocomial infections.

1.2 Procedures and protocols of cleaning and disinfection

Compliance with all major International Guidelines for sanitizing procedures recommended in hospital environments is fundamental for prevention and human health. In fact, the disinfection and antibiotic prophylaxis where patients are present are carried out in order to kill microorganisms present on surfaces and reduce their proliferation (biocide action) [11].

Cleaning and disinfection (or sanitation) consists of a set of procedures designed to make the environment hygienically healthy or safe.

Chemical biocides usually used in the hospital sector must meet certain conditions to be effective, such as [64; 65]:

• Sufficient contact time with the surface to be sanitized;

• Sufficient concentration, which may decrease if there is organic material;

• Low level of corrosive action.

• Broad-spectrum microbiocidal.

Traditionally, sanitizing procedures are carried out using chemical disinfectants, but they have several disadvantages:

• Limited biocidal efficacy over time, which normally ends within 20-30 minutes after application, with subsequent exponential growth of microbiological agents;

• The capacity of the microorganisms to develop continuous genetic mutations and defenses of different genes, making the biocidal action ineffective, with the resulting biocidal resistance phenomena;

• Environmental impact of chemical contamination of the natural environment.

Disinfectants are required to be registered at the SSN as Biocides (EU BPR 528/2012).

With accordance to European Standards (ENs), disinfectants are subjected to standard test methods to determine efficacy of the disinfection process (simulated-use test) in clean and dirty conditions [I; IX].

ENs include methods for evaluating bactericidal, fungicidal, sporicidal, micro-bactericidal, and virucidal activity as well as specific methods for disinfectant hand-washing in medical areas [X].

A biocide or disinfectant has the kinetics of microbial destruction, but each microbial species has its particular tolerance to chemical activity [65].

During a biocidal process the rate of bacterial killing is logarithmic (as is their rate of growth). The bacteria are killed at a rate proportional to the number of organisms present and to contact time. A linear curve is obtained *'in vitro'* (exponential kinetics) (Figure 1).

The killing rate decreases in proportion to the number of survivors (residual bioburden).

The reduction in decimals (LOG base x10) of treatment efficiency directly corresponds to the disinfectant used.

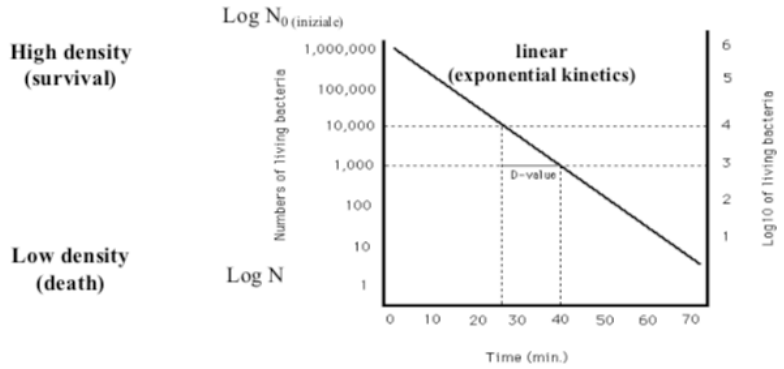


Figure 1: Linear kinetics of microbial destruction (Kinetics of the Inactivation of microorganisms) [66].

In field, however, we have different trends represented by the typical configurations of survival curves instead of linear (exponential kinetics):

1. concave upward (Figure 2);
2. sigmoidal or concave downward (Figure 3).

The sigmoidal (concave downward) type is obtained by:

- the presence of interfering substances or particulate material, the presence of spores and bacteria clusters (clump or bacterial aggregates), which slow the disinfectant activity;
- the presence of a few clumps, but with different degrees of resistance of the same species or presence of different species [67].

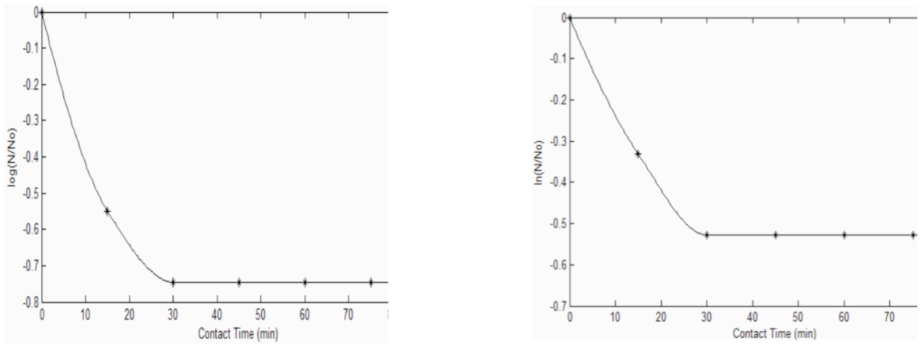


Figure 2: The kinetic of microbial killing: two different concave upward curves

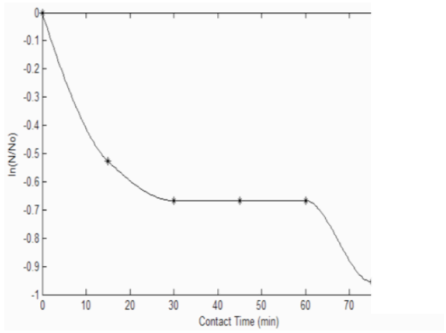


Figure 3: The kinetic of microbial killing: sigmoidal curve

(Source: Sunil B. Somani *et al.*)

An important factor in the effectiveness of disinfectants is the persistent activity, also called “residual activity”: the prolonged or extended activity that prevents or inhibits the proliferation or survival of microorganisms after application of the product.

Chemicals end their activity within about 30 minutes of application; other strategies are needed to achieve a prolonged effectiveness.

A second factor is the presence of biofilm. Most bacteria are able to promptly produce a glycocalyx, a gelatinous polysaccharide and/or polypeptide outer covering [68].

The glycocalyx is referred to as a capsule if it is firmly attached to the cell wall, or as a slime layer if loosely attached. This material produced by bacteria forms the structural matrix of biofilm (Figure 4_a and 4_b).

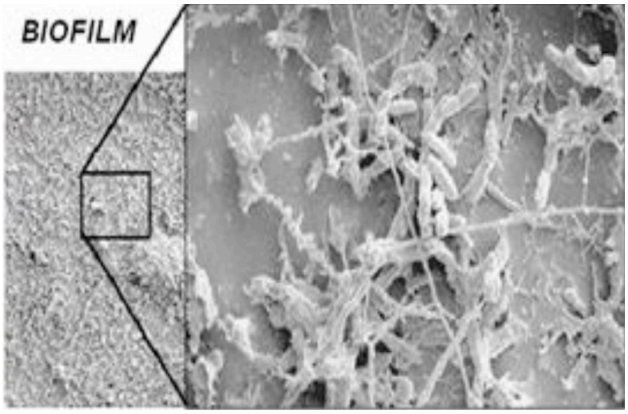


Figure 4_a: Biofilm: Gram negative bacteria *Pseudomonas* attach to surface and develop biofilm (Source: images https://it.wikipedia.org/wiki/Biofilm)

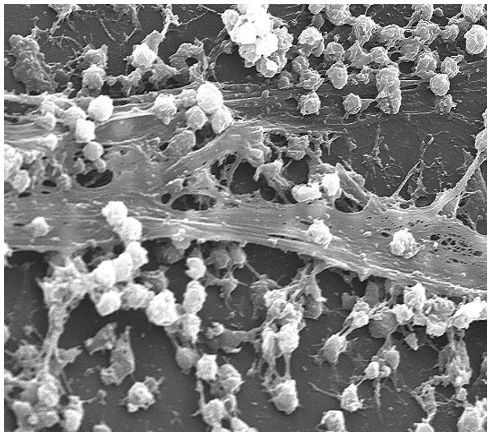


Figure 4_b: Scanning electron micrograph of Gram positive bacteria *Staphylococcus* that attach to medical device catheter and develop biofilm. (Source: Lippincott Williams & Wilkins. Images obtained from the CDC Public Health Image Library)

Rodney M. Donlan’s paper published in Emerging Infectious Diseases (Vol.8, No. 9, 2002) regards how the microorganisms attach to surfaces and develop biofilms [69].

The initial phase of microbial adhesion to surfaces is the most important.

Attachment is a complex process regulated by diverse characteristics of the growth medium, substratum, and cell surface. An established biofilm structure comprises microbial cells and EPS, has a defined architecture, and provides an optimal environment for the exchange of genetic material between bacteria. Bacteria may also communicate by quorum sensing, which may in turn affect biofilm processes such as detachment, resulting in the spread of contamination in the surrounding environment.

Biofilms are significant to public health because of their role in certain infectious diseases and variety of device-related infections. A greater understanding of biofilm processes should lead to new and effective control strategies for biofilm control.

Solid surfaces may have several characteristics that are important in the attachment process. Characklis et al. [70] noted that the extent of microbial colonization appears to increase as the surface roughness increases. This is because shear forces are diminished, and surface area is higher on rougher surfaces. The physicochemical properties of the surface may also exert a strong influence on the rate and extent of attachment.

It is generally thought that a surface can be kept as clean as it is smooth. However, a study presented in 2009 (N.W. Buijs, Innomet, May 2009) showed that a too-smooth surface can be more exposed to the risk of biofilm growth.

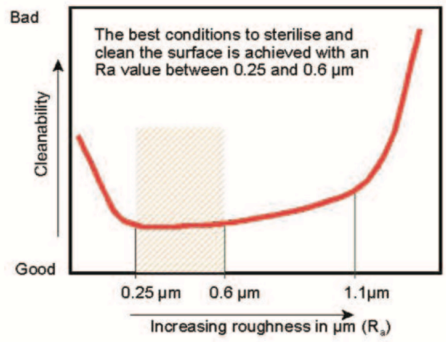


Figure 5: Stainless steel can best be cleaned if it has a roughness between 0.25 and 0.6 µm (source Leser Hamburg).

It is very important for a surface to be smooth, however not too smooth, as dangerous biofilms can develop with roughness levels under Ra = 0.2 µm as the adhesive forces between the surface and the bacteria then become substantially large. This is shown in the diagram in Figure 5, where the degree of cleaning is shown as a function of the roughness. In other words, the rule 'the smoother the surface the easier it is to clean' is not always true in practice.

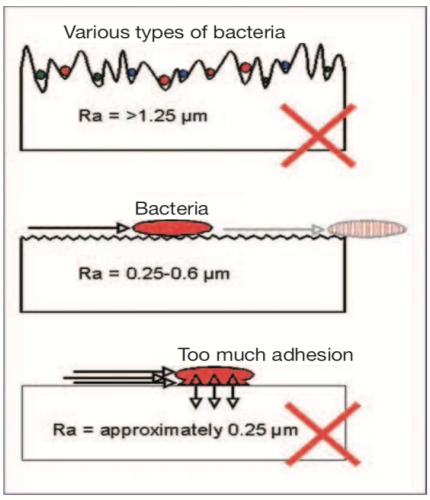


Figure 6: It is difficult to remove bacteria from a surface that is too smooth (source: Leser Hamburg).

The Figure 6 illustrates what happens in reality (in field).

While high roughness values provide bacteria and contamination high settlement opportunities, too smooth surfaces allow for high adhesion between bacteria and surface (e.g. metal).

Roughness values between 0.25 and 0.6 µm of surfaces provide the best conditions for limiting the formation of biofilms and to facilitate removal by cleaning [25].

Many studies currently include experimentation of antimicrobial surfaces.

Several experimental studies include techniques for creating "self-disinfecting" surfaces by coating medical equipment with metals such as copper, silver or zinc, and other inhibitors of microbial growth which can prevent biofilm formation [71; 72; 73; 74; 75; 76].

A different strategy is to evaluate the effect of a new method of biocontrol on the presence and survival of several microorganisms responsible for HAIs (*Staphyloccus aureus*, *Enterobacteriacea*, *Pseudomonas species*, *Acinetobacter species*, *Candida albicans* and *Clostridium difficile*) on surfaces in hospital settings. This technique of biocontrol of hospital surfaces can act as a sustainable alternative to chemical disinfectants.

Finally, the effectiveness of the disinfectant is important for reducing the bio-burden deposited on the surface: the surface becomes microbiologically safe; the microbial diffusion and the related cross-contamination cycle are stopped.

Equally important is the system of procedures, cleaning and disinfection protocols, training of operators, the provision of EC ergonomic equipment, and apposite cleaning tools and cleaning utilities.

Recently, sector associations such as AMNDO (National Association of Doctors of Hospital Departments), MANAGEMENT AND TRAINING (Scientific Association and School for Hospital and Territorial Health Services), FIASO (Italian Federation of Sanitary and Hospitals) FARE (Federation of Regional Health Associates and Providers) and FISE (Federation of Business Services of Confindustria) have developed a guideline which recommends the establishment of strict and well-defined cleaning and disinfection protocols [XII].

The protocols of cleaning and disinfection are required to be written in detail, and must give instructions on how to perform each step, as well as give a description of specifications.

These requirements are: standard operating procedures (SOP); protocols (for validation); and technical and safety sheets of each detergent and disinfectant [64].

The cleaning and disinfection system includes a protocol, sanitizers, suitable and specific equipment and cloths for each surface to be sanitized.

In general, the protocol provides both the use of mops and cleaning cloths, previously absorbed with sanitizing solution (Figure 7), for the floor line and the hand line respectively (Figure 8).

The mops and cleaning cloths are made of microfiber as it avoids the phenomenon of lifting dust from movement. In this way, we avoid re-contamination of surfaces determined by their re-sedimentation. Microfiber cloths are suitable for cleaning all surfaces [77; 78].

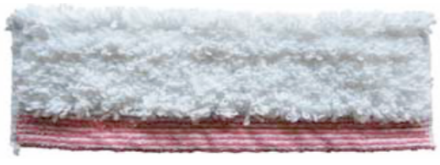


Figure 7: Microfiber mop with velcro back for fastening on handle.

Figure 8: Microfiber cloth for equipment and washbasin in different colors.

Microfiber is important because it is constructed from split conjugated fibers of polyester and polyamide that are able to trap dirt and remove it from the surface during cleaning, as shown in the Figure 9.

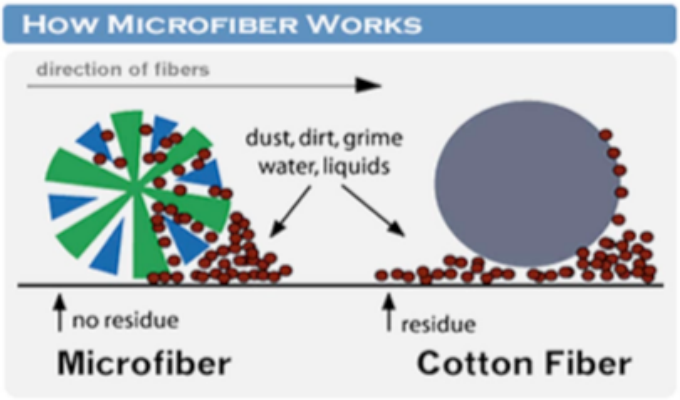


Figure 9: Cross sections: microfiber thread at left, cotton thread at right (Source: https://en.wikipedia.org/wiki/Microfiber).

Microfiber cleaning cloths used in hospitals and their ability to reduce microbial loads from a surface.

Both mechanical strength, especially the impingement, i.e. the pressure on the mop (floor line) or on the cloth (hand line), and the direction of the cleaning movement is very important for the mechanical removal of dirt and microbes from surfaces (Figure 10).

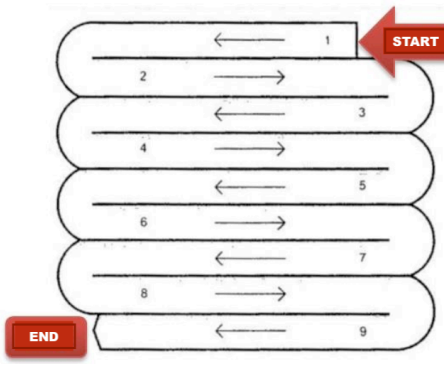


Figure 10: Illustration of the direction and correct movement (overlaid S-shaped motion), for better disinfectant performance.

1.3 Study of characteristics of materials (surfaces)

Hospitals are the basic sanitary institutes of health services. Hospital buildings are human habitats and characterized by their own potentially pathogenic microbiome environment. In hospital buildings, human comfort levels are measured by factors such as humidity, temperature and airflow [79]. These factors influence the diversity and distribution of microorganisms indoors. There exists a significant relationship between indoor environmental conditions—including relative humidity and temperature—and airborne bacterial community types [80].

The indoor climate can influence human health through direct effects on microbial populations [81; 82].

The experimental study includes the validation of the sanitizing procedures of the surfaces and furnishings of patient wards in several inpatient divisions such as medicine, geriatrics, long-term care, gynecology and general medicine, etc., in relation to the kind of hospital materials and environmental factors (room temperature, unit number of beds, type of disease determined by the department).

Noncritical environmental surfaces can be porous or nonporous and include bed rails, bedside tables, patient room furniture, and floors. Noncritical environmental surfaces are reservoirs for microbial contamination. Surfaces are frequently touched by hands (e.g. bedside tables, bed rails) and are a challenge for cross-contamination. Transfer of microbial contamination from hand contact with environmental surfaces or equipment surfaces to the patient or other surfaces represents an indirect mode of transmission [83]. The degree of microbial contamination of the floor could be an indicator of the general hygienic quality of a confined environment (hospital rooms, operating rooms, intensive care units) [83]. Microclimate of hospital premises is determined by the thermal state of the environment and depends on the temperature, humidity and air velocity. Comfortable microclimate conditions are provided with heating and ventilation and air conditioning units in separate rooms or central air conditioning. Indoor climates in hospitals are determined by a combination of temperature, humidity, air mobility, the temperature of the surrounding surfaces and thermal radiation [84].

In accordance with ISPESL 2006 "Microclimate, Ventilation and Lighting at Workplace". Microclimate of the patient ward is defined by the following parameters: air temperature, relative humidity, air purity, air pollution level and air velocity (Table 1).

Table 1: Characteristics of the patient room:

Patient ward	
Patients room	
Temperature	between 20°C and 25°C
Relative Humidity (U.R.)	between 40% and 60%
Ventilation: Air changes per hour	2 v/h
Illumination	300 lx
Sanitary facilities	
Temperature	between 20°C and 25°C
Relative Humidity (U.R.)	between 40% and 60%
Ventilation: Air changes per hour	3 v/h
Illumination	80/200 lx

The study is carried out in occupied rooms of an inpatient division of 13 public hospitals (11 North Hospitals and 2 South Hospitals) and 7 RSAs (6 North Hospitals and 1 South Hospital). Nine kinds of material (surfaces) per room are analyzed (Table 2) by plate contact before and after a probiotic-based cleaning procedure in comparison with traditional chlorine-based chemical disinfectants.

Table 2: Features of the different kinds of materials are shown in the following table:

Surface	Material
Grès porcelain stoneware (floor)	Compact ceramic
Marble (floor)	Marble
Rubber (vinyl or rubber flooring)	Rubber
Linoleum (floor)	Linoleum
Metal / steel (hospital corridor handrail /bedside railing	Metal
Synthetic leather	Vinyl resin
ABS / epoxy (headboard and footboard)	Plastic (Polymeric Material)
PVC (bedside table)	PVC (Polymeric Material)
Vitreous china (washbasin)	Glass based enamel coating

All the test surfaces have the following features:

• Easy cleaning (Cleanability)

• Water repellency

• Lightweight

• Wear resistance / Low level of corrosive action.

Cleanability: the ability to be easily cleaned without damage caused by the corrosive action of chemical treatments and permits easy removal of dirt deposited on the surface.

The type of flooring includes:

Grès: a tile flooring, type of material hardness that has been in use for many years.

The Porcelain Enamel Institute standards are used to rate tile on a 1-5 scale with 5 being the hardest and most durable. It is used for high-traffic flooring like corridors and especially in patient rooms and hospital bathrooms, because it is very durable and has a high cleanability suitable for heavy sanitization (acidic or alkaline cleaning solutions).

Other hospital flooring is mainly PVC, linoleum, and rubber, which have characteristics of robustness, resistance and low cost.

They are resilient, returning to their original position after being bent and compressed from high-traffic.

Epoxy flooring comes without joints that limit the uniformity of the surface. It's waterproof and easily cleaned compared to other materials. It has inhibitory properties of microbial adhesion.

Rubber flooring consists of natural rubbers that are also accompanied by filling compounds. The rubber, as a raw material in itself, is highly expensive.

The features of PVC, as with all plastics, are determined by its chemical composition and type of molecular structure (molecular formation: crystalline/amorphous structure) (Figure 11).

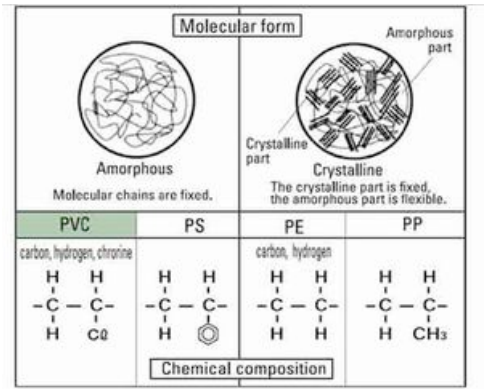


Figure 11: Molecular form and chemical composition of PVC and other plastics.
(Source: http://www.pvc.org/en/p/pvcs-physical-properties)

PVC resin has an amorphous structure with polar chlorine atoms in the molecular structure making it very chemically stable. This applies to PVC resins, which furthermore possess fire-retarding properties, durability, and oil/chemical resistance.
It has machinability and printability, which easily lends to many products with different colors (such as home improvement materials, agricultural films, items of furniture, bedside tables, etc.).
The required physical properties of PVC end-products, in addition to the flexibility, elasticity, impact resistance, etc., are also the ones that prevent microbial growth by mixing or coating of antimicrobial inhibitors [XII].

Vitreous china (sanitary porcelain): a type of ceramic used for sanitary wares of remarkable mechanical strength (shocks and tensions) and for the glossy enamel coating that is very resistant to abrasions and acids. It is a compact material with water absorption not exceeding 0.50 percent.

Vinyl resin types include:
Synthetic leather: leather-like material, defined vinyl artificial leather (faux leather) used for dialysis department armchairs.
Vinyl leather is a vinyl resin, mainly polyvinylchloride, used for ambulatory beds.

With regard to layout of both public hospitals and RSAs, the structure were subjected to microbiological monitoring as well.
Hospital design (in healthcare sectors, departments, inpatients divisions and areas of each division) largely depends on recommendations from the WHO [XIV]:

1. District Hospitals: Guidelines for Development, WHO 1992;
2. Building for Health Care: a guide for planners and architects of first and second level facilities, World Bank 1996 [85].

The total area of the department depends on the number of beds.

Inpatient division:

Parts and components of the inpatient division are:

Patient room 11.5 m^2/ bed – 8 m^2/bed with window

Patient room with bathroom

Area around beds (European dimension) must have sufficient space:

Area dimension of: 205÷215 (L) cm x 95 cm (La) as shown with dashed lines in Figure 9.

Features of the Articulated hospital bed:

Headboard, Footboard in Epoxy /ABS

Side railing: tube manufactured in steel with ABS lining or in metal (steel)

Measurements: overall bed sizes: 208÷210 (L) x 90÷96 (La) x 52÷60 (h) cm [86].

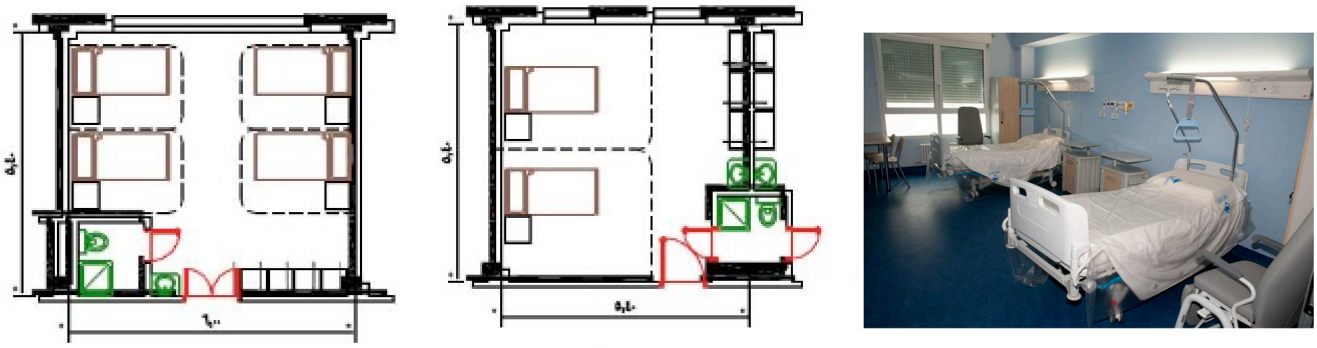
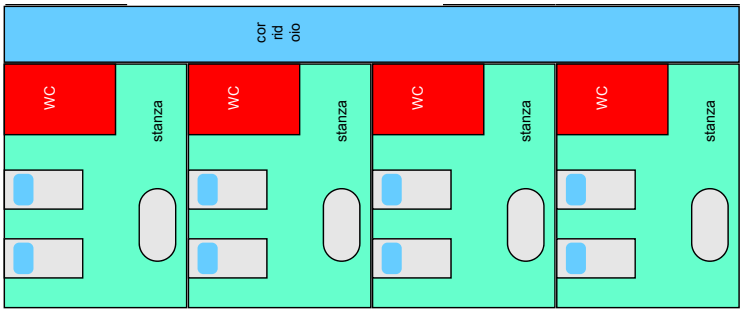


Figure 12: Two patient room schemes and image Unit bed.

The hygienic building requirements of patient rooms depends on critical issues (biological risks). Patient wards are divided into 4 areas, which are colored in the following Figure 13:



Legend: (explanation of the colors)

area low critical issues (hallway)
area high critical issues (hospital bathroom)
area medium critical issues (patient room)
area very high critical issues (bad Unit)

Figure 13: Patient wards scheme.

For the departments covered by this experimental study, see the following table:

Table 3:

Patient wards
Outpatient Clinic
Day hospital
Medicine
Oncology-Hematology
Otorhinolaryngology/ Laryngologist
Intensive care
Long-term care
Respiratory Diseases
Surgery / Urology
Traumatic Brain Injury Unit (UGC)
Rehabilitative Medicine Unit (UMR)
Nephrology
Post Acute Care
Gynecology and General Medicine
General surgery
Orthopedics and Rehabilitation
Geriatric Nephrology

Three hospital layouts representative of the experimental study are reported:

1. Hospital (2010): divided into three sectors, five buildings and four floors resting on a surface of 185 thousand square meters; new construction; advanced Hospital.
   The staff who work there consists of 380 medical directors, 66 managers, 142 university employees, 1408 people in the health sector, 442 technicians and 175 administrative employees. It includes 744 ordinary hospital beds and 116 day hospital beds.

2. North Hospital (2004): on a surface of 500 square meters with 9 patient rooms for 17 beds on each floor (8 rooms with two beds and one to a bed), 2 large, 40 square meter gyms and all with air-conditioning.
   Rehabilitation Center includes two units:
   UGC: Traumatic Brain Injury Unit with 40 beds
   UMR: Rehabilitation Medicine Unit with 30 beds

Figure 14: Corridor (stoneware / grès flooring)

3. North Hospital (1959): on a surface of 500 square meters with 22 departments, 64-bed Day Hospital and 545 beds (10 rooms with two beds on each side of the corridor) and all with air-conditioning.

1.Hospital

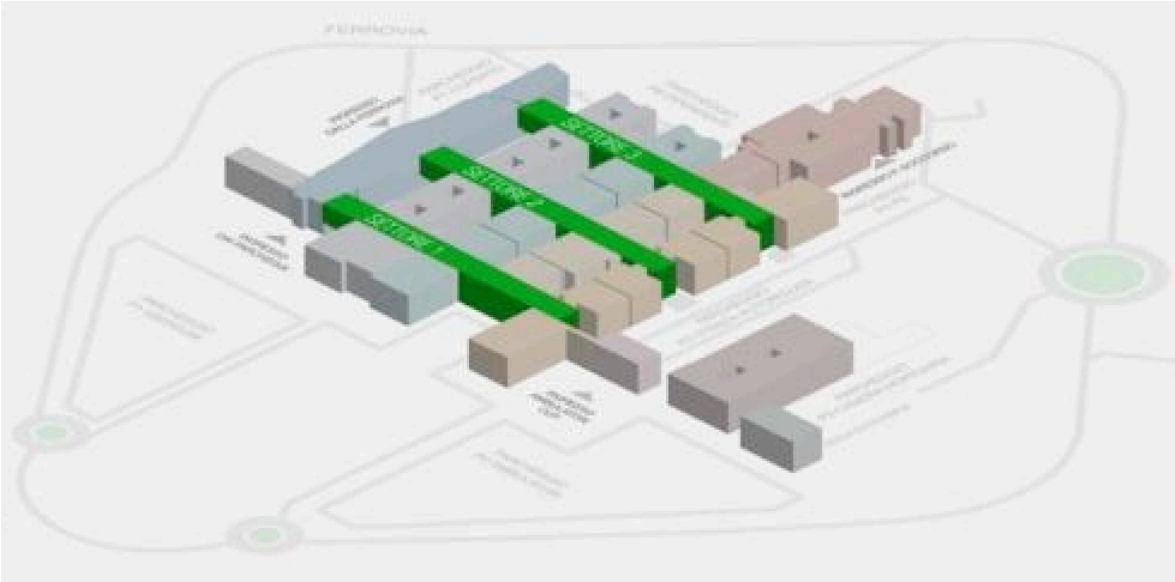


Figure 15 : North Hospital (2010) total hospital structure.

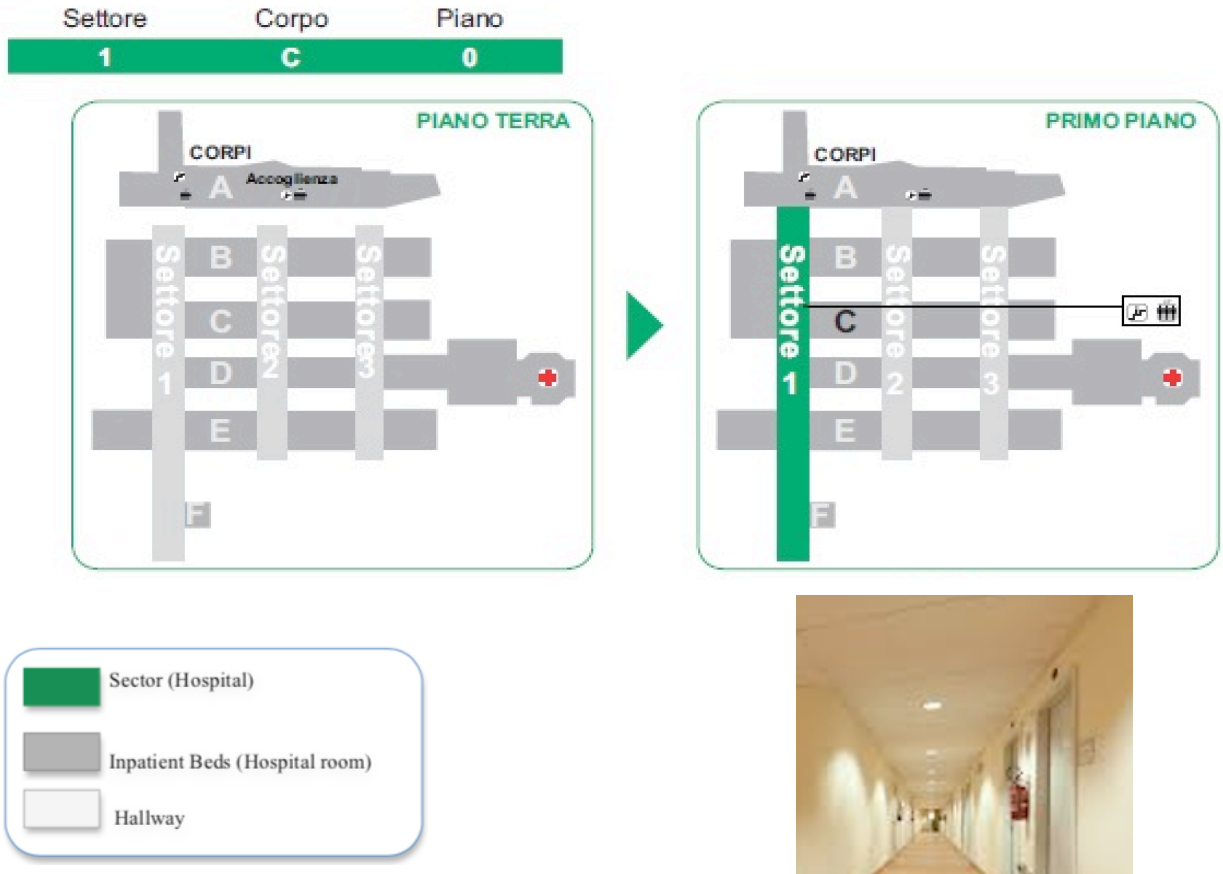


Figure 16: North Hospital (2010) First Floor.

Department of Medicine (1B3) probiotic-based sanitation procedure.
Oncology-Hematology Department (1B1) traditional chlorine-based chemical disinfectants procedure.

Figure 17: Long Corridor (epoxy flooring)

2.Hospital

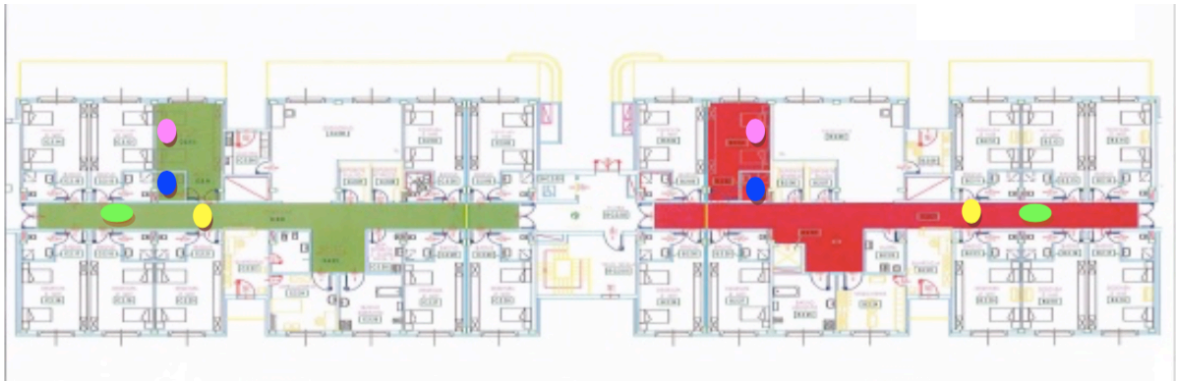


Figure 18: North Hospital (2004) first floor Traumatic Brain Injury Unit (UGC)

Figure 19: North Hospital (2004) second floor Rehabilitative Medicine Unit (UMR)
Green area: departments with probiotic-based sanitation procedure
Red area: departments with traditional chlorine-based chemical disinfectant procedure

3.Hospital

Figure 20: North Hospital first floor
Neurology Department probiotic-based sanitation procedure

3.Hospital

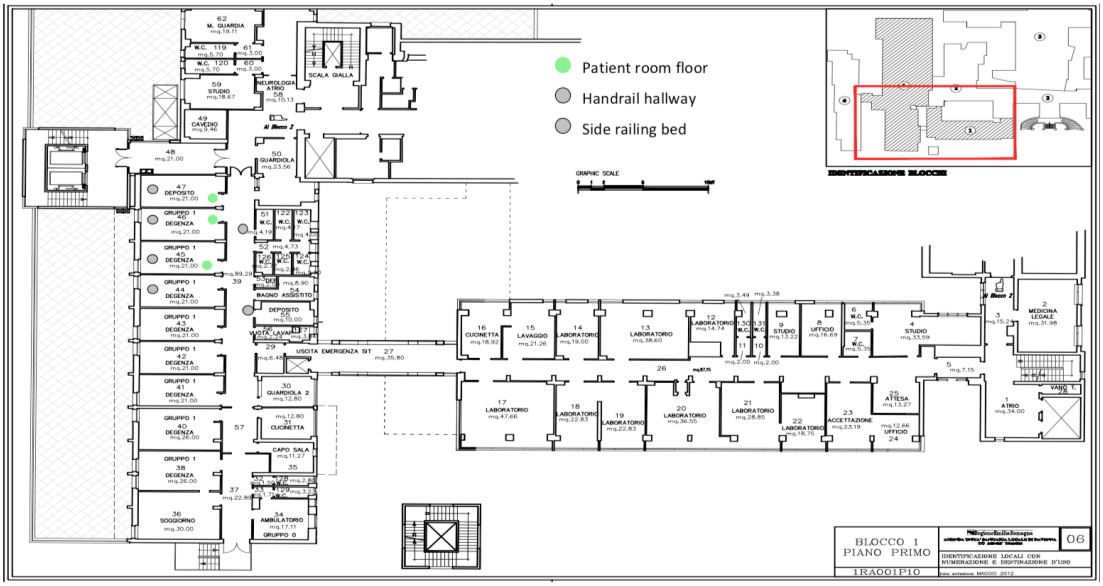


Figure 21: North Hospital first floor
Post Acute Care probiotic-based sanitation procedure

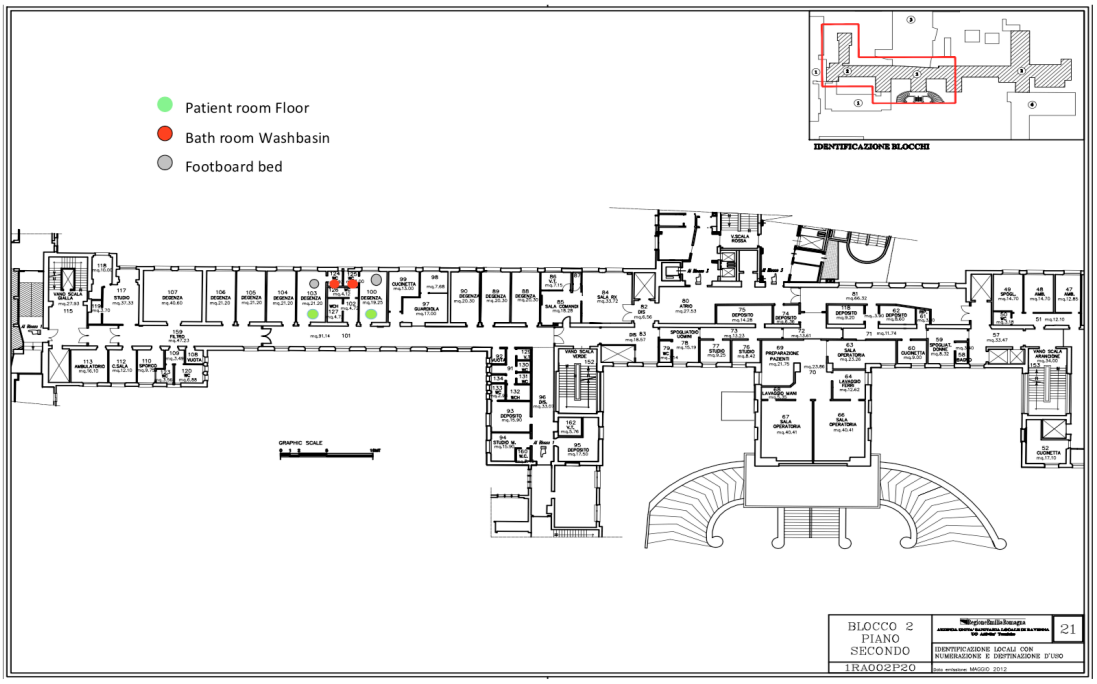


Figure 22: North Hospital second floor
Nephrology Department probiotic-based sanitation procedure

1.4 The features of probiotic bacteria*: Bacillus* spore-forming strains

Probiotics are collected in the Organic Database of the National Collection of Microorganisms of Agro-Industrial Interest (COL.MIA), and tested at the Research and Testing Authority (CRA), a national research and experimentation body with particular scientific expertise in the agricultural, agro-industrial, fisheries and forestry sector. The bacteria strains that compose probiotic-based cleaning are ATCC *Bacillus species* collected by the National Collection of Type Cultures and registered to the American Type Culture Collection in the Unites States. They are safe and healthy, their genome is highly analyzed and licensed by the FDA as dietary supplements. They do not need further verification of their safety [87; 88].

Probiotics have beneficial effects on both the balance of human intestinal flora and on human health.
Probiotics, like Lactobacilli and Bifidobacteria, are active in supporting defense mechanisms weakened by antibiotic treatments and various gastrointestinal tract infections [89]. The probiotic species of the genus *Bacillus* are stable, since they are aerobic spore-forming bacteria [90].
These bacteria survive in difficult conditions, as they form an internal spore which remains dormant until their revitalization (germination, defined sporulation) when the environmental parameters improve.
Bacillus is a genus of Gram-positive, rod-shaped bacteria and a member of the phylum Firmicutes [90].
Bacillus species can be obligate aerobes (oxygen reliant), or facultative anaerobes (having the ability to be aerobic or anaerobic). Ubiquitous in nature.

Many species of *Bacillus* can produce copious amounts of enzymes and batteriocines, which are used in different industries [91].

Bacillus subtilis is the most studied and well-known species [92].
Bacillus subtilis is a sporulating rod-shaped Gram-positive bacterium, which thrives in the soil. Like most of its closest relatives, *B. subtilis* is non-pathogenic, and has even been awarded the GRAS (Generally Recognized As Safe) status by the US Food and Drug Administration. The first known application of B. subtilis dates back more than a thousand years, when it was used to produce natto, a Japanese food product consisting of fermented soybeans [93; 94].
Nowadays, *B. subtilis* is best known as a source of useful enzymes and fine biochemicals, and as an attractive host for the production of heterologous proteins [95]. Many different enzymes originating from *Bacillus subtilis* and related *Bacillus species* like proteases and amylases, are being used in industries for a wide range of different applications [96]. Importantly, *B. subtilis* is able to produce and secrete large quantities of bacteriocins [97; 98].

Figure 23: Shape of the Bacillus spore (endospore): Under stressful environmental conditions, the bacteria can produce oval endospores that are not true 'spores', but to which the bacteria can reduce themselves and remain in a dormant state for very long periods.

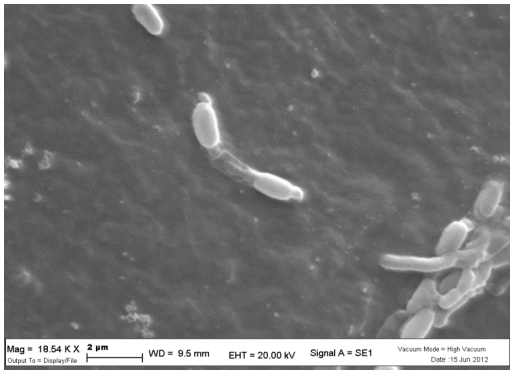
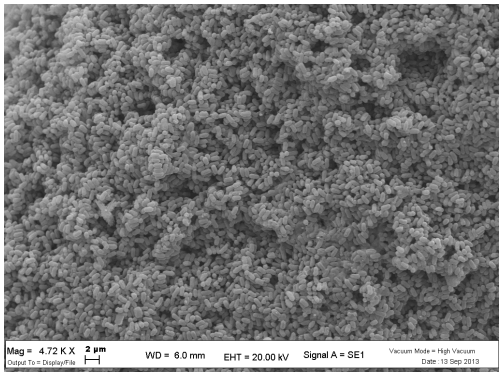


Figure 24: Probiotics *Bacillus spp.* colony



Probiotics also work several hours after their surface application due to their sporogenic ability and act by 'microbial management', counteracting the survival of potential pathogenic bacteria. Many hours after an application, the surfaces are hygienically safer due to the phenomenon of "competitive exclusion": they compete for space (colonization of surfaces) and nutrients through the production of proteolytic enzymes [31].

Through the use of quorum sensing, probiotics effectively counteract the proliferation and survival of other saprophytic bacterial species and/or pathogenic bacteria [99].

This phenomenon of bio-stabilization is called "bacterial antagonism," "bacterial interference," or "colonization resistance" [33; 34].

From several *'in vitro'* studies it was assessed that the *Bacillus* spores, contained in the probiotic detergent of this experimental study, were able to germinate on unanimated surfaces by germination assays after 24 and 72 hours [35] and produce bacteriocins [36].

In field trials *Bacillus spp.* was isolated by hospitals treated with probiotic cleaning for a period of 6 months to 2 years. This processed by Antimicrobial Susceptibility Testing (AST) and were not found to have other resistances apart from the natural to penicillin.

Their DNA extracted and processed by a quantitative real-time PCR (qPCR); this technology is used for confirming gene expression results obtained from microarray analysis.

In field trials *Bacillus spp.* was isolated and did not detect the presence of any antibiotic-resistant genes (R genes) [36]. In the field tests the DNA's bacteria *Bacillus spp*. isolated, analyzed by microarray did not detect the presence of antibiotic-resistant genes (R genes) [36].

2 Overview

In evaluating the phenomena of environmental pollution in hospitals, the microbial contamination of finishing and furnishing surfaces has been largely investigated [100; 101; 102; 103; 104; 105]. Although effective use of disinfectants may be an important factor in preventing both the transmission of microorganisms from surfaces to patients, and consequently the rise of HAIs, their routine use remains controversial because environmental surfaces, like those in patient rooms, present a low to medium risk of contamination [106; 107; 108]. Nonetheless, those surfaces potentially contribute to cross contamination through contact with the hands or skin of both operators and patients [109; 110; 111].

A suitable surface cleaning process procedure with detergents and disinfectants, especially hand-touch sites, is recommended by all major International Guidelines [112; 113]. Therefore, cleansing and disinfection must be considered fundamental components for the control of HAIs, and their application must be based on quantitative evidence such as the percentage reduction of the microbial load, or the low level of Microbial Build-up (MB) [114; 115;116 ;117].

Evidence that contaminated surfaces contribute to the transmission of hospital pathogens comes from studies modeling transmission routes, microbiologic studies, observational epidemiologic studies, intervention studies, and outbreak reports [118; 119].

Numerous studies concern the use of different chemical disinfectant molecules and chemical-based sanitation protocols to reduce the presence of pathogens or potential pathogens in healthcare facilities.

The chemical disinfectants produce an immediate reduction of the surface microbial contamination, but are ineffective in preventing recontamination phenomena, which are ultimately responsible for the persistence of pathogen contamination on hospital surfaces and for the associated onset of HAIs. They can provoke or select resistant microorganisms, similar to an antibiotic action [120; 121; 122].

In addition, a major global concern in HAI management is represented by the antimicrobial resistance of HAI-associated pathogens. Antimicrobial resistance (AMR) is a growing problem globally. The WHO has estimated that at present, about 700,000 deaths occur annually worldwide due to infections caused by drug-resistant microbes (MDROs). Antibiotic resistance has been increasing [123].

Some experimental studies have demonstrated the efficacy of microbial inhibition against potential nosocomial pathogens of an innovative probiotic cleaning procedure that uses spores of non-pathogenic probiotic bacteria of the Bacillus genus to detergents [33; 34].

Numerous studies of scientific importance demonstrate that *Bacillus subtilis* due to its great versatility and the absence of pathogenicity, may be used as a biocontrol agent for different fields and various applications (e.g. in aquaculture, agriculture and veterinary medicine). Many articles have been written on probiotics, such as preparations of viable microorganisms able to give health benefits to the host once recruited and, because of this, have been widely used as supplements as a result of alterations in both the qualitative and quantitative intestinal flora [124; 125].

Additionally, the process of "bacterial interference", according to which commensal bacteria are used to prevent the colonization of the host from pathogenic organisms, has benefits such as the activation of

the immune system and metabolic pathways that restore tissue homeostasis and promote a general state of health [127; 128].

In accordance with the criteria of the US Environmental Protection Agency (EPA) and classified as GRAS (Generally Recognized As Safe) by the Food and Drug Administration (FDA) the *Bacillus subtilis* is non-pathogenic and safe for the environment, animal feed and human consumption.

3. Objective

The aim of this experimental research is to provide an evaluation of the effectiveness of a probiotic-based cleaning procedure in comparison with traditional chlorine-based chemical disinfectants.

The objective is to create the conditions of bio-stabilization or biocontrol against the most common HAI-related bacteria and yeasts pathogens known to reside on surfaces, including *Staphylococcus species (*alert organism: *St. aureus* pathogen), *Enterobacteriacea* (*Escherichia coli* pathogen), *Pseudomonas species (Ps. aeruginosa* pathogen*), Acinetobacter species (Acinetobacter baumanii* pathogen*)*, *Clostridiun species* (*Clostridium difficile* pathogen) and *Candida species (*alert organism*: Candida albicans)*.

This is to stop the connection between microbiological contamination of surfaces and infectious events of patients during the period of hospitalization.

It is important to evaluate that the strategy of bio-stabilization by using probiotic cleaning can act as a sustainable alternative to chemical disinfectants for treating surfaces, by immediately and continuously compressing and reducing potential pathogens over long periods of time.

The application of probiotic cleaning in hospital inpatient wards, nursing homes or in RSAs is effective in reducing the microbial load and bringing it below alert levels, therefore contributing to the reduction of possible HAI occurrences.

4. Materials and Methods

According to the "CONTARP-INAIL Guidelines", 2005, to "UNI EN ISO 19698: 2004" and according to M. Pitzurra, A. Savino, C. Pasquarella "The microbiological environmental monitoring (MAM)", 1997 Ann.Ig., 9: 439-454 the surface sampling procedure and microbiological analysis methods are performed, to verify the degree of microbial pollution, defined index microbial surface (IMS) and the search for pathogens before and after cleaning [114].

Horizontal Surfaces:

a. Floor (Corridor / patient rooms / bathroom)
b. Touch hand surfaces:
   handrails, dialysis department armchairs, outpatient bed, door handle, washbasin;
c. surfaces bed unit: headboard, footboard bed, side railing bed, bedside table.

The materials kind of each test surface monitored is shown in Table 4.

Table 4: List critical points of microbiological sampling

Surface	Materials kind
Floor corridor / Floor patiens room / Floor bathroom	Gres
	Marble
	Rubber
	Epoxy
	Linoleum
Hospital corridor handrail / side railing bed	Metal
Armachair, ambulatory bed	Vinyl resin
Headboard and footboard bed	Plastic (Polymeric Material ABS / Epoxy)
Bedside table	PVC (Polymeric Material)
Washbasin	Gres

Figure 25: Critical point floor entrance (left) and in the middle of the patient room (right)

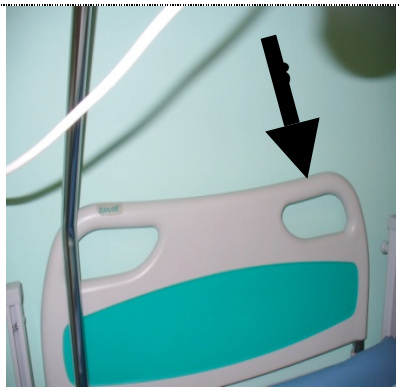


Figure 26: Critical point headboard bed

Figure 27: Critical point washbasin

Selection of enviroment surfaces for monitoring

Surface sampling was carried out by contact plate method to evaluate Microbial Accumulation (AM) [114; 115], which expresses the number of microbial colonies in CFU / cm^2, accumulated on a surface over a period of time 7 hours (Δt) from the time of cleaning. It is evaluated by pressing 30 seconds the convex side of the plate with the hands with sterile gloves. According to transport rules each plate is transported in laboratory.

Growth of all microbial strains was obtained by incubation at :

36°C for 48 h and, then, 25 °C for another 24 h.

The result is expressed in CFU / m^2.

Contact Plate method:

The microbiological testing by count plate Replicate Organism Detection And Counting (RODAC) is used for microbiological control of all surfaces.

RODAC was developed for environmental studies to determine levels of microbial contamination of surfaces. When plate is filled with 15 mL of media, the media surface is raised to a convex shape. Area of agar surface is approximately 24 cm^2.

Figure 28: Plate Count (source: http://www.e-scientia.in/product/ascda015-scda-with-lecithin-polysorbate-80-contact-plate-55-mm/ and https://www.merckmillipore.com)

Rodac plates are available with a lot of different growth media [129]:

- TSA (Tryptone Soya Agar) non selective medium, for determining the total amount of micro-organisms (general hygienic state) or total aerobic microbial count. Microbial limit: total aerobic microbial count
- BPA (Baird Parker Agar) for *Staphylococcus species.*
- Mac Conkey Agar for Gram-negatives bacteria Enterobacteriaceae.
- Cetrimide Agar for *Pseudomonas species*.
- Herellea Agar for *Acinetobacter species*
- Sabouraud Dextrose Agar (SDA) - CAF + Neutralizing for yeasts *Candida species* and moulds *Aspergillus species.*
- Clostridium difficile Agar in anaerobic conditions for *Clostridium difficile*.

The available microbial monitoring method by RODAC plate method has the following:

i. Advantages: direct growth on media in contact plate is convenient, neutralizers may be included in media and different media may be used;
ii. Provides indication of the presence of whatever pathogen is isolated, but needed then the typing strain microbial.
iii. Disadvantages: only applicable to surfaces that are smooth and have low counts.
iv. Provides indication of the presence of whatever pathogen is isolated, but needed then the typing strain microbial.
v. Expensive
vi. Prolonged time for results
vii. Requires suitable laboratory resources and equipment and trained personnel for interpreting results [130].

Calculation of the microbial load [131]:

- Calculation of vital units (ufc / plate)

visual count of colonies grown on RODAC (total colony number), then calculate the average and express the result in CFU / m^2.

- Calculation of the reduction of vitality:

$Red= (NT_0- T_{1(+n)}) / N T_0$

Where:

Red = reduction of vitality expressed in % value reduction

N = Total viable count for RODAC related to T_0

N_a = Total viable count for RODAC related to T_1

Interpretation of environmental surface monitoring / Microbiological sampling Acceptance criteria

In accordance with the Guidelines that are chosen, the results obtained from field trials can have different interpretations in relation to the reference acceptable limits. For exemple according to:

I. Pitzurra's criteria the acceptability limit value is defined by microbial risk of the hospital environment [114]:

≤5 CFU/ cm^2 (very microbiological risk environment such as Operating Rooms)

≤25 CFU/ cm^2 (high microbiological risk environment such as Intensive Care)

≤50 CFU/ cm^2 (medium-risk microbiological environment such as Patient Wards)

≤75 CFU/ cm^2 (environmentally low microbiological risk such as Hospital storage).

II. According to the table recommended by the "Microbiological contamination of surface of the Laboratory section of APHA" [XVI]:

Number of colonies for contact plate / CFU /24 cm^2		
Very acceptable (good cleaning effectiveness)	Acceptable (effective cleaning efficiency) sufficient	Not acceptable (not effective cleaning) insufficient
0 - 25	**26 - 50**	**> 51**

III. Microbial Acceptability Limit according to the ISPESL Guidelines (Higher Institute for Prevention and Safety at Work) related to pre and post operative patient room [116]:
Aerobic colony counts of <2.08 CFU/ cm^2
without pathogens, such as *St. aureus*, *Enterobactereriacea*, *Pseudomonas spp.* and *Aspergillus spp.*

Description of the Rodac type:

Tryptone Soya Agar (TSA) + Tween 80 + Lecithin (Merck):

TSA is richer in nutrients and containing the Tween and Lecithin for the inactivation of inhibitory substances.

Composition:

Ingredients	Grams/Litre
Tryptone	15.0
Soytone (enzymatic digest of soybean meal)	5.0
Sodium Chloride	5.0
Agar	15.0
Final pH 7.0 ± 0.2	

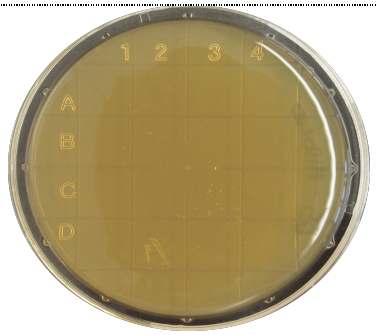


Figure 29:

TSA before sampling

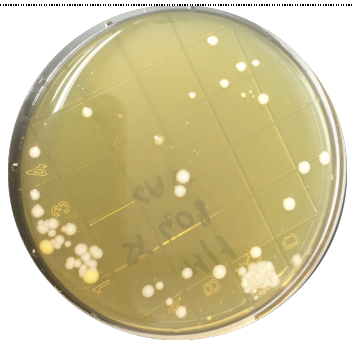


Figure 30: TSA after sampling and incubation. Bacterial mix of the traditional procedure

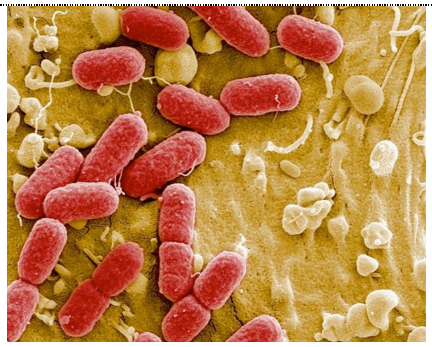


Figure 31: Scanning electron microscopic image (SEM) of bacterial mix (Source:http://www.lettera43.it/it/articoli/scienza-e-tech/2016/05/23/salute-virus-e-batteri)

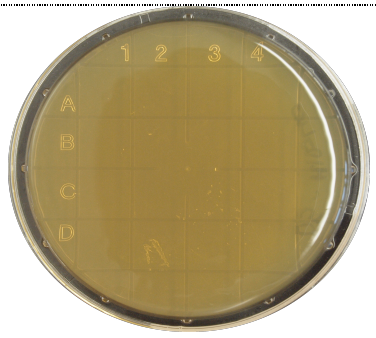


Figure 32:

TSA before sampling

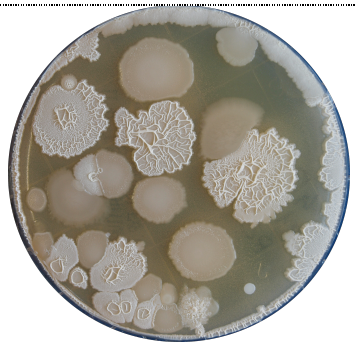


Figure 33: TSA after sampling and incubation. Bacteria spore-forming of the probiotic-based procedure

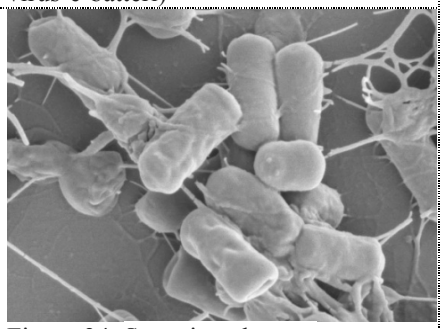


Figure 34: Scanning electron microscopic image (SEM) of B. subtilis 168 (Source:https://openi.nlm.nih.gov/detailedresult.php?img=PMC2323362_1475-2859-7-10-1&req=4

Baird Parker Agar (BPA) + Neutralizing (Merck):

selective medium for *Staphylococcus aureus* isolation with inactivation of disinfectants.

Composition:

Ingredients	Grams/Litre
Casein Peptone	10.0
Meat Extract	5.0
Yeast Extract	1.0
Lithium Chloride	5.0
Glycine	12.0
Sodium pyruvate	10.0
Potassium tellurite	0.1
Egg yolk solution	10.0 ml/L
Agar	15.0
Final pH 6.8 ± 0.2	

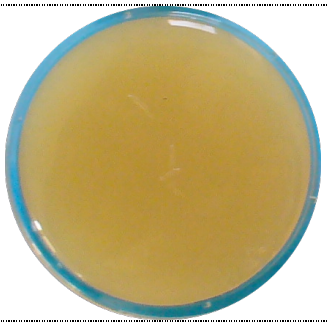


Figure 35: BPA before sampling

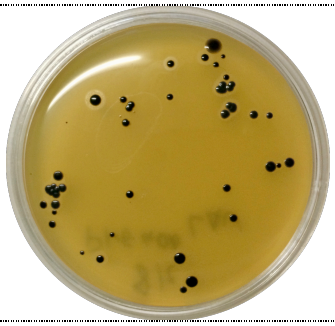


Figure 36 Baird Parker Agar: *S. aureus* colonies after sampling and incubation.
Double area around colonies, opaque inside, transparent outside

Figure 37:
Coloured scanning electron micrograph (SEM).
(Source: https://it.123rf.com/photo_72390229_staphylococcus-aureus-mrsa-batteri.html)

Baird Parker Agar is used for the isolation and differentiation of coagulase-positve staphylococci.

The medium contains lithium and tellurite, which inhibits other bacteria, while glycine and pyruvate enahnce Staphylococus growth [132; 133].

Staphylococci can reduce tellurite to telluride, which results in grey to black coloration of the colonies. The additon of gg yolk, the medium becomes yellow, slighty opaque. A clear halo develops around colonies from coagulase positive Staphylococcus aureus, and upon further incubation may produce an opaque zone due to an egg yolk: lecithinase reaction (Lypolytic activity). Grey-black colonies and a halo on this medium are presumed to be indicative of coagulase test and lipophylitic activity was found.

Organisms test ATCC	**Color of Colony**	**Growth**	**Lecithinase**	**Coagulase**
Staphylococcus aureus (25923)	Grey-black shiny	+	+	+
Staphylococcus aureus (6538)	Grey-black shiny	+	+	+
Staphylococcus epidermidis (12228)	Black (small colonies)	+/-	-	-
Micrococcus luteus (10240)	Very small in shade of brawn-black	+/-	-	-
Bacillus subtilis (6633)	Dark brown matt	+/-	-	-

In addition Coagulase test is used for confirming pathogenic bacteria *Staphylococcus aureus*.