



Università di Ferrara

fondata nel 1391

The University of Ferrara Study At the St. Anne Hospital



The Research Centre For Pollution Control In High Sterile Rooms

Conducted A Year-Long Study (2010 - 2011) On The

“TESTING OF BIO-STABILIZATION
TECHNIQUES USING CHRISAL PROBIOTIC
PRODUCTS FOR CLEANING AND SANITIZING
OF HOSPITAL WARDS”



Medical Sciences Group

JE Translation Copy

October 13, 2011

TESTING OF BIO-STABILIZATION TECHNIQUES FOR THE CLEANING AND SANITIZING OF HOSPITAL STAYS

SUMMARY OF REPORT

1- INTRODUCTION

Hospital acquired infections represent one of the most common problems of health care and influence the prolongation of hospital stay. This results in human and economic costs that very significant.

In Italy it is estimated that the number of such events is equal to about 8% of patients hospitalized or about 700,000 cases per year. Approximately 25% of the cases appeared post-surgery with the remaining 75% occurring during hospitalization or after the discharge of a patient. In the latter case the infection was not clinically apparent at the time of discharge.

The percentages above are in line statistics in Europe and North America.

These events can be caused by microorganisms like salmonella or hepatitis, but increasingly also by opportunistic microorganisms. They may be poorly virulent and common in the environment however can become clinical in susceptible patients such as the elderly, trauma patients, burns, premature babies, diabetes, cancer, and those recovering from major surgery.

The cleaning procedures of the hospital environment, together with the antibiotic prophylaxis are carried out with the aim of reducing and containing the proliferation of microorganisms, and reducing the potential development of opportunistic infections.

Traditionally, these procedures are done through the use of chemical disinfectants; however there are several disadvantages to this protocol:

- There is a limit to the biocidal effectiveness of disinfectants over time. This usually ends within 20-30 minutes after application, which is followed by exponential growth of microbiological agents.
- Micro-organisms are continuing to develop genetic mutations and defenses that are designed to render ineffective the biocidal action. This has created the consequent problems of antibiotic resistance and/or multi-drug resistance (MDR) which has been well documented in the literature.
- The problems of environmental pollution generated by the massive use of toxic chemicals that can accumulate in a persistent manner.

This has led to a process of natural selection of pathogenic microbial strains increasingly resistant to disinfection procedures, among which include:

- Staphylococcus aureus
- E. coli
- Pseudomonas aeruginosa
- Candida albicans
- Acinetobacter
- Clostridium difficile

For these reasons there has been intensifying international research regarding methods of sanitation that are based on the biological principle of competition. This involves in the use of

non-pathogenic microbial products can colonize the surfaces on which they are applied to counter the proliferation of other bacterial species. This is based on the principle of competitive exclusion (Gause's law).

This approach to the problem of sanitation is a complete reversal of the objectives of traditional procedures where disinfection is defined by a minimal presence of microorganisms on surfaces of any kind. While the traditional approach seeks control of pathogenic strains it does so without tolerating the presence of microorganisms are not harmful to human health.

These new procedures can be defined as "bio-stabilization techniques" of one species over another, thus implying no general biocidal action, except as a final effect against certain microbial species.

The principle of action is that two different species (bacterial and/or fungal), who seek to live on the same ecological microcosm, cannot coexist in a stable equilibrium if they require the same nutritional substrates, Ultimately one of the two will become dominant over the other, and can even cause extinction.

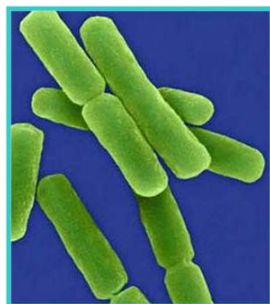
The use of the products with a non-pathogenic microbial load is also able to inhibit the activity of transcriptional regulation (quorum sensing) among pathogenic bacteria, i.e. those activities of information exchange which are spread even among microorganisms belonging to different strains. This is a method of defense against environmental pressure (sanitizers, disinfectants and antibiotics).

2- PROBIOTICS

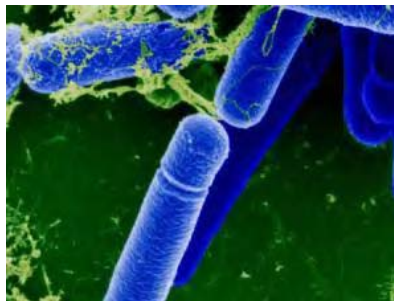
There has been a recent introduction to the market of a new range of products designed to clean the environment, based on the use of a mixture of probiotic bacteria (Fig. 1 and 2). They are called PIP (Probiotics in Progress).

These products contain a mixture of spores of the genus *Bacillus*. They are formulated in very high concentration, and can foster a competitive action against all other microorganisms, without distinction whether they are Gram-positive, Gram-negative or spore-forming fungi.

These bacteria occur two forms. One is the vegetative form and the other is as spores. The vegetative form is metabolically aerobic as well as a facultative anaerobe. It has very little nutritional needs, and is able to multiply and colonize the environment by competing with other potentially pathogenic bacteria. When adverse conditions arise the vegetative cells produce spores that allow the survival of the organism. The spores retain the ability to germinate and return to the vegetative state as soon as a favorable environment returns.



PIP Probiotic bacteria



Bacillus subtilis

The bacteria of the genus *Bacillus* are not normally pathogenic to humans and animals. They are used in agriculture, especially horticulture, for humans, in veterinary practices (as a dietary supplement), and are considered safe. The genus *Bacillus* is considered sensitive to antibiotics.

The use of *Bacillus* in medicine dates back to the second half of the 1800s, mainly in alternative medicine, because of claimed immune-boosting effects, a valuable function in the absence of antibiotics. It was noted that their presence facilitates the production of secretory immunoglobulin type A which is present particularly on the mucous membranes of the digestive system. Immunoglobulin A has been shown to prevent or slow the colonization of the gut by other microorganisms that could affect the digestive health.

Several *Bacillus* species have been classified as "GRAS" ("Generally Regarded As Safe"), for their use in food processing or pharmaceutical preparations and are approved by the FDA (Food and Drug Administration) for use by humans.

In addition, bacteria of the genus *Bacillus* are classified in class 1 by the American Type Culture Collection (ATCC), defining them as have a low pathogenicity.

Because of their ability to be stable for many hours on dry surfaces, and to form spores they have many potential uses. They do not induce the formation of resistant pathogenic bacteria, are biodegradable and are environmentally safe.

3 - PURPOSE OF THE RESEARCH

The recent availability of these products for cleaning and sanitizing of surfaces, including the control of microbial residents, led to a proposal for experimental research, aimed at verifying the quantitative *in vitro* and field testing of the effectiveness and efficiency of these products compared to the use of traditional treatments such as chemical disinfectants.

The study was funded by COPMA SCRL, which has also provided its expertise and personnel.

The experiment was designed to evaluate the effectiveness of the PIP products compared to traditional cleaning products. This was done by determining the numbers of pathogenic bacteria found on surfaces treated with both products then calculating the percentage difference.

The microorganisms under investigation were *Staphylococcus* spp (particularly MRSA), *Pseudomonas* spp, *Escherichia coli* spp, *Candida albicans*, *Clostridium difficile*, and the *Acinetobacter* spp. They are all implicated in hospital-acquired infections.

4 - THE CONDUCT OF THE RESEARCH

The study was conducted from autumn 2010 until autumn 2011. The "in vitro" work was done at the laboratories of the University of Ferrara, and the field work was at the Hospital Santa Anna di Ferrara.

The purpose of the "in vitro" tests (made in accordance with ISO 13697:2001) was to allow us to verify the effectiveness of the competitive exclusion properties of PIP compared to other bacterial species, in the absence of external factors, i.e. those processes of re-contamination of surfaces which occur naturally in human occupied environments.

The results of the "in vitro" tests are shown in section 6.

The field experiments had the purpose of verifying the action exerted by PIP when the surfaces of interest were subject to constant re-contamination. This contamination is attributable to the activities that commonly occur in a nosocomial environment because of the presence of people and the contact with floors, furniture, and sanitary devices.

The intent of these experiments was also related to expectations, later confirmed, about the potential that the PIP products were capable of a continuing action that controlled pathogens over time when compared to the more traditional use of disinfectants. The typical instant biocide action characterized by disinfectants is often followed by an increase in bacteria by 100 to 1000 times in following 24 hours.

In order to control the critical operating conditions in which the experiment was run, we chose to intervene in two areas of the Santa Anna of Ferrara Hospital (see Figures 6, 7 and 8). The experimental site is not newly built has a range of different health treatment areas, and has no air filtration and ventilation plant.

One area was the General Medicine Ward. It was divided into two sections, one of which was treated with a traditional regimen of cleaners and disinfectants, and the other cleaned with Chrisal PIP products.

The other area is called the Outpatient Ward, and is characterized by continuous change of persons and materials, resulting in a regular contribution of pathogens. There are two specific treatment areas; one is Ophthalmology and Cardiology, and in the second is Orthopedics.

TABLE 3- A summary of the trials.

	General Medicine		Outpatient Ward	
	Sala T	Sala S	Cardiology Ophthalmology	Orthopedics
Phase 1 16.03.2011 to 16.04.2011	PIP	Traditional Disinfectants	PIP	Traditional Disinfectants
Phase 2 16.04.2011 to 16.07.2011	Traditional Disinfectants	PIP	Traditional Disinfectants	PIP
Phase 3 16.07.2011 to 28.08.2011	PIP 1	PIP 2		

It was therefore possible to conduct parallel experiments by applying the protocol in each area for one phase then making the changes in the second and third phases. It involved the use of PIP probiotics in one of two departments, and the use of traditional products in the remaining areas of the department.

Both the experimental and control departments were very similar in terms of the type of user and the characteristics of contamination. It was therefore possible to measure the time intervals and to set the values of bacterial load obtained from the two different cleaning systems. This gave us the ability to evaluate the reduction of microbial load for the use of PIP compared to traditional methods.

To test the reliability of the results, the experimental sites were reversed after 1 month (Phase 2).

As is customary, the cleaning procedures for the departments took place at 06:30 am. The microbiological monitoring campaigns were carried out at regular time intervals (about every 2-3 days), both at 07:00 am to 14:00 am, in order to assess the state of contamination of the area after 7 hours of use.

Each sampling was performed in triplicate, using Rodac contact plates to determine growth and evaluate the changes in terms of CFU/cm².

- Total microbial (TVC);
- Staphylococcus aureus,
- Coliform bacteria (Escherichia coli),
- Pseudomonas spp,
- Candida

For some cases it was also possible to evaluate the presence of:

- Acinetobacter
- Clostridium difficile

The samples were collected in different parts of the departments concerned. The locations are as follows:

- The floor of the corridor near its entry point.
- The floor of the corridor at its end point.
- The floor of the toilet (bathroom).
- The sink in the toilet (bathroom);
- The floor beside the beds in the hospital rooms (one for the Care Medicine)

On March 11, 2011, before the experiment commenced microbiological samples were performed for the evaluation of the initial total microbial load. This was called T0 (14,00).

With regards to the sample points, is to be noted that while sites on the floor of the corridors were fixed during the entire trial, those relating to the floor and sink of the toilets, and floor beside the beds in the hospital rooms were chosen randomly.

It was therefore possible to obtain a good representation of the state of contamination for each pathogen of interest and for each of the two experimental areas (General Medicine and Outpatient Ward).

The trial then continued with a third phase, which started on 16.07.2011. This was approximately one month after the completion of Phase 2.

In the third phase, which lasted from 16.07 to 23.08.2011, probiotic products are used in both departments of the Medical Care, in order to check for further containment of pathogenic loads after prolonged application of PIP.

Microbiological samples taken during the experiment:

- 6804 samples in Phase 1
 - 4212 samples Phase 2 (concentrated in the first month)
 - 1512 samples in Phase 3.
- for a total of 12,528 samples.

It worth noting however that the types of floor finishes in the 4 departments involved in the trial are different, and therefore the results may also reflect the difference in those finishes.

TABLE 4- List of the sampled surfaces

	General Medicine		Outpatient Ward	
	Sala T	Sala S	Cardiology Ophthalmology	Orthopedics
Corridor entry	Gray tile floor	Rubber floor	Red tile floor	Rubber floor
End of the corridor	Gray tile floor	Rubber floor	Red tile floor	Rubber floor
Floor beside the toilet	Tile floor	Tile floor	Gray tile floor	Tile floor
Sink beside the toilet	Porcelain	Porcelain	Porcelain	Porcelain
Beside beds in the hospital room	Hard plastic	Hard plastic	Not determined	Not determined

The procedure for sampling and microbiological testing of the surfaces were carried out according to "Guidelines CONTARP-INAIL", 2005, the "UNI EN ISO 19698:2004" and coded according to the custom in the literature [M. Pitzurra, A. Savino, C. Pasquarella "The Microbiological Environmental Monitoring (MAM)", 1997, Ann.Ig., 9:439-454] to verify the degree of microbial contamination and the search for pathogens.

The culture media used are as follows:

- 1-Rodac TSA for total microbial counts (TVC)
- 2-Rodac BAIRD PARKER AGAR for Staphylococcus aureus
- 3-Rodac MacConkey Agar for Escherichia coli
- 4-Rodac CETRIMIDE AGAR for Pseudomonas aeruginosa
- 5-Rodac SABOURAUD AGAR + CFL for Candida albicans
- 6-Rodac HERELLA AGAR for Acinetobacter spp.

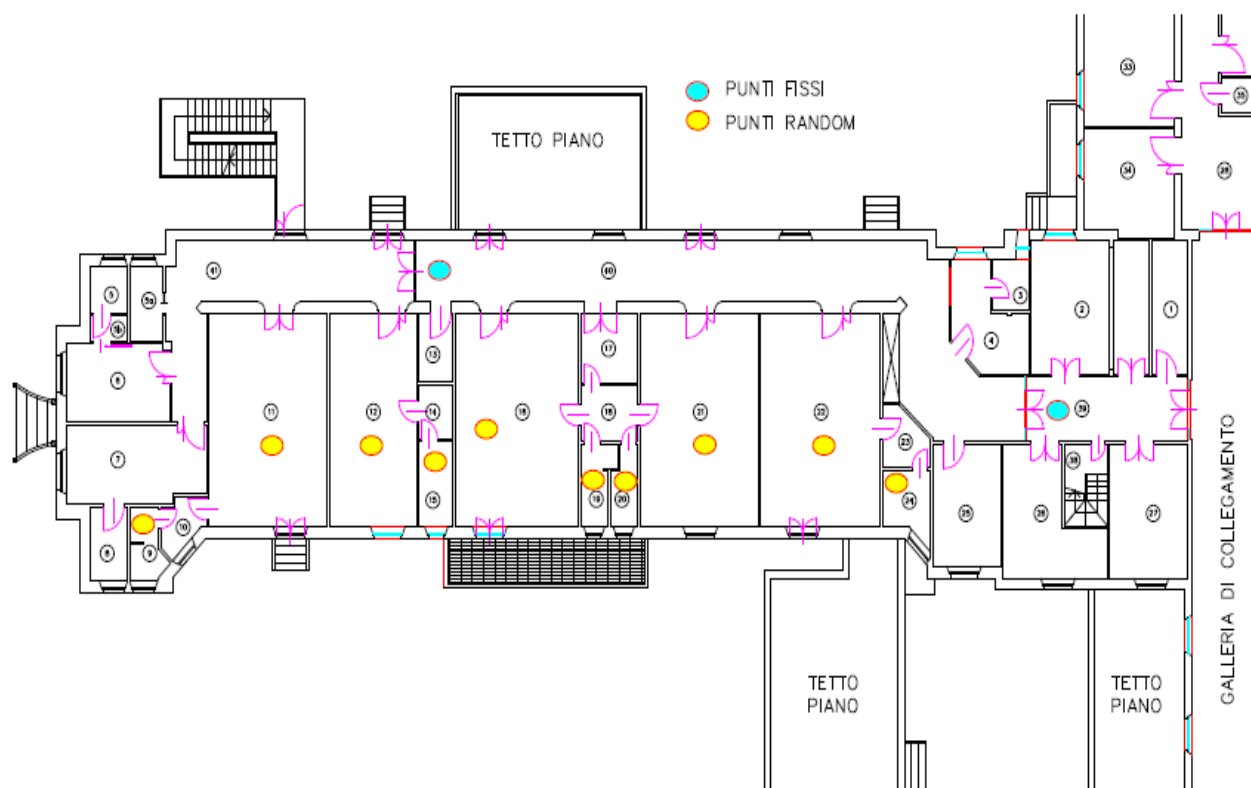


Figura 3 – Sala S

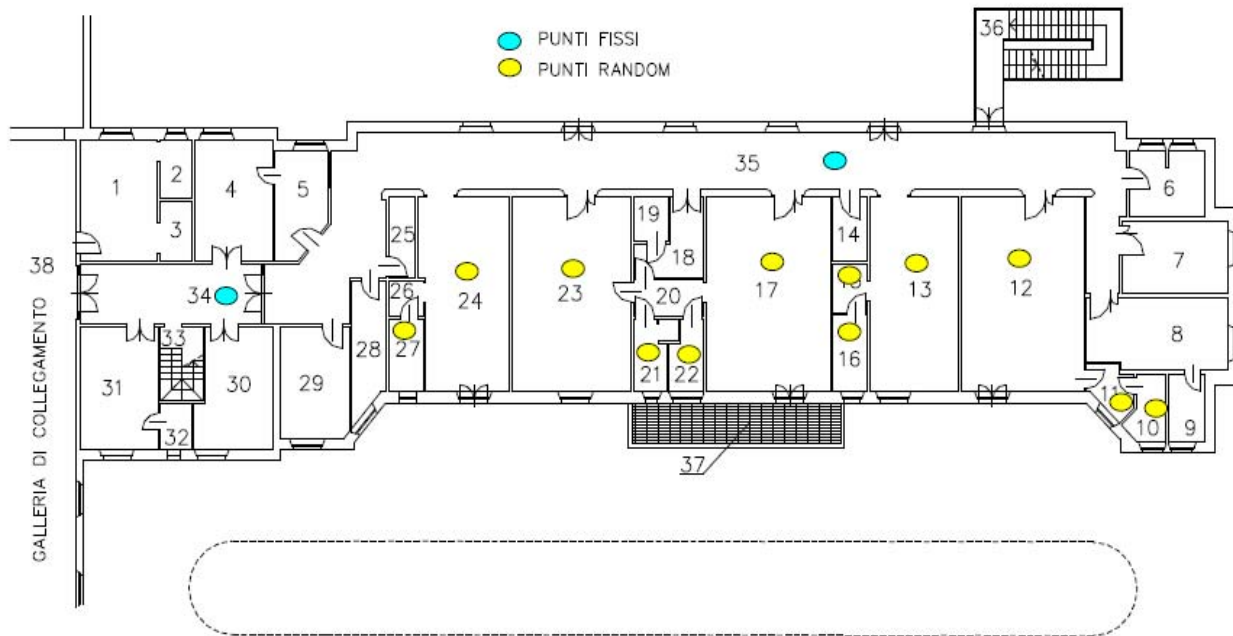


Figura 4 – Sala T

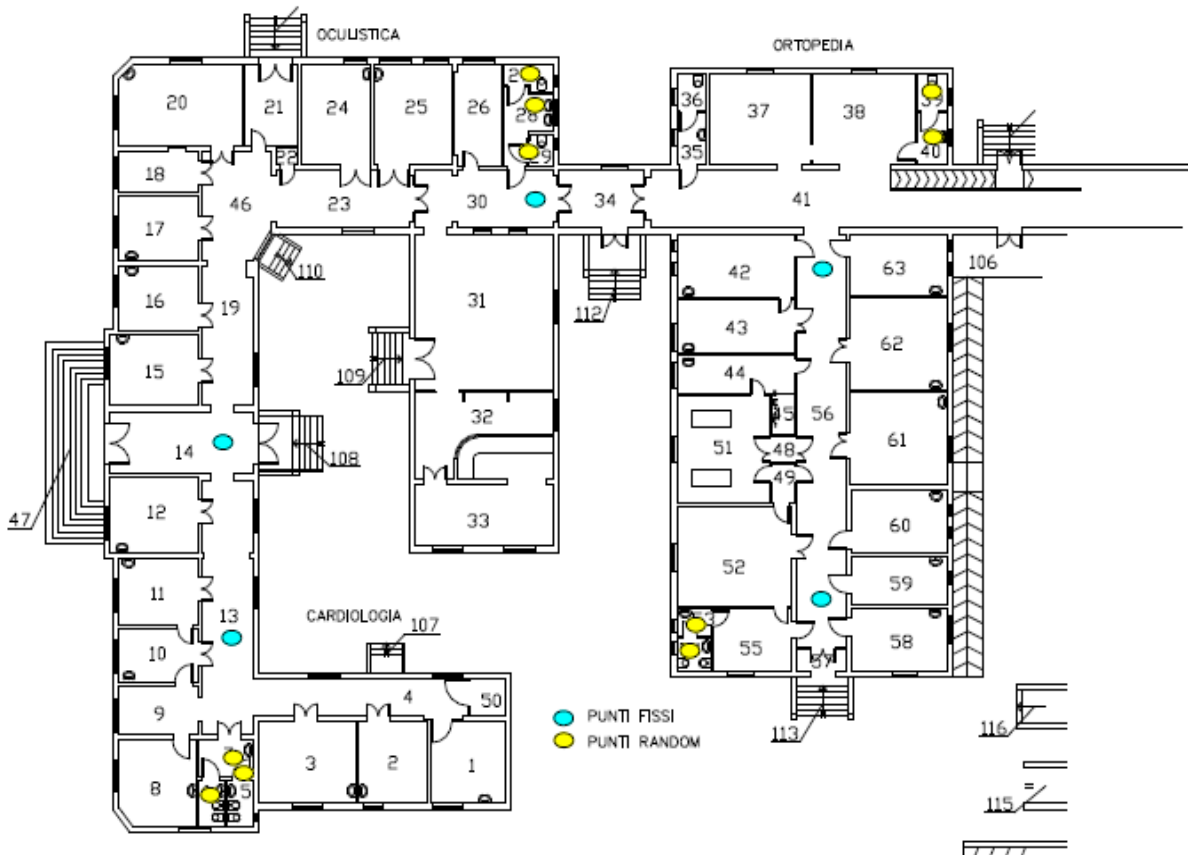








Figura 5 – Poliambulatori

The products used are listed in the following diagram:

<p>No. 1</p>		<p>PIP Floor Cleaner For floors. Containing 30 million CFU of probiotic bacteria per ml Concentration of use: 1% Dilution with water at 40 ° C.</p>		<p>Biospot For floors. Active Chlorine disinfectant tablets. Contains 33% active chlorine which are dissolved in water (1 3.25 g tablet in 5 liters) Free hypochlorous acid. For Medical and Surgical Use.</p>
<p>2</p>		<p>PIP All Purpose Cleaner For varying surface materials. Containing 30 million CFU of probiotic bacteria per ml Concentration of use: 1% Dilution with water at 40 ° C.</p>		<p>Task Actichlor For washable surfaces: furniture, walls, surfaces and sanitary use. . Disinfectant cleaner for all washable surfaces. A 1% solution contains 300 ppm of available chlorine.</p>
<p>3</p>		<p>PIP Sanitary Cleaner For Sanitary Cleaning Containing 50 million CFU of probiotic bacteria / ml Concentration of use: 1% Dilution with water at 40 ° C.</p>		<p>Sanikal detergent Sanitizing cleaner for healthcare. Contains anionic and nonionic surfactants for cleaning. Concentration-Ready to use.</p>

The procedure for cleaning and disinfection was the same for both of these protocols. The cleaning tools used (mops and cloths) were of the same materials.

5 - REFERENCE STANDARDS

The international reference values for the classification of surface contamination are somewhat fragmentary.

In the literature, it is consolidated using the index IMS of Pitzurra index (microbial surface), which represents the value of the total contamination (TVC) acceptable in the operating room per cm² of surface.

This index, however, is representative of the state of contamination of a surface in the moments immediately following a treatment of disinfection. It should be understood that this is disinfection (chemical) of the surfaces of interest and the reduction of microbial loads does not define non-pathogenic loads.

It clear that this index does not lend itself to the evaluation of the findings from this study, for several reasons:

1. While the operating room environment is considered at high risk of infection because sterile organs of the human body are exposed to air. The risk of infection in a ward or clinic may be even more so if the areas examined are represented by our values taken from the floor of these same.
2. Once the surfaces of an operating room are sanitized, the room is air-conditioned and partitioned with absolute filtration and ventilation rates of at least 15 vol / h (Decree Bindi). The processes of re-contamination that occurs are therefore solely attributable to the natural growth of microorganisms that survived disinfection. In contrast, in the environments considered in this study such processes of microbial growth are primarily attributable to the phenomena of contamination by the passage of people and materials (which occurs without interruption over 24 hours) and the phenomena of gravitational sedimentation of atmospheric dust. There is no controlled mechanical ventilation system. Ventilation is ensured only through the opening and closing of exterior doors and the air is not filtered.
3. The complexity of the phenomena that affect microbial growth in the areas makes testing difficult, and it is not useful to set a maximum threshold value of contamination at the time immediately following the time of cleaning. These processes are due to the dynamic nature of growth of microorganisms and result in an increase of the initial amount of bacterial species by 10-30 times over a few hours. This has been confirmed in the results obtained in this study and that data is also available in literature [5].
4. In the case of cleaning with PIP products, the microbial population on the sanitized surfaces is largely formed by *Bacillus* spp., which are harmless to human health. Only small percentage of the bacteria present is made up of other bacterial species. The evaluation of surface contamination through the use of the method of counting Total (TVC) is therefore not at all descriptive of the actual risk of the patient contracting an infection.

It is clear that there is a comprehensive methodology present in the literature and the law for assessing an acceptable level of contamination of surfaces. However there is a gap in the description and identification of parameters that completely describe the level of hygiene that should be present in a general hospital setting.

6 - TRIAL RESULTS IN VITRO

The in vitro experiments occurred under laboratory conditions. (Figures 6, 7 and 8)

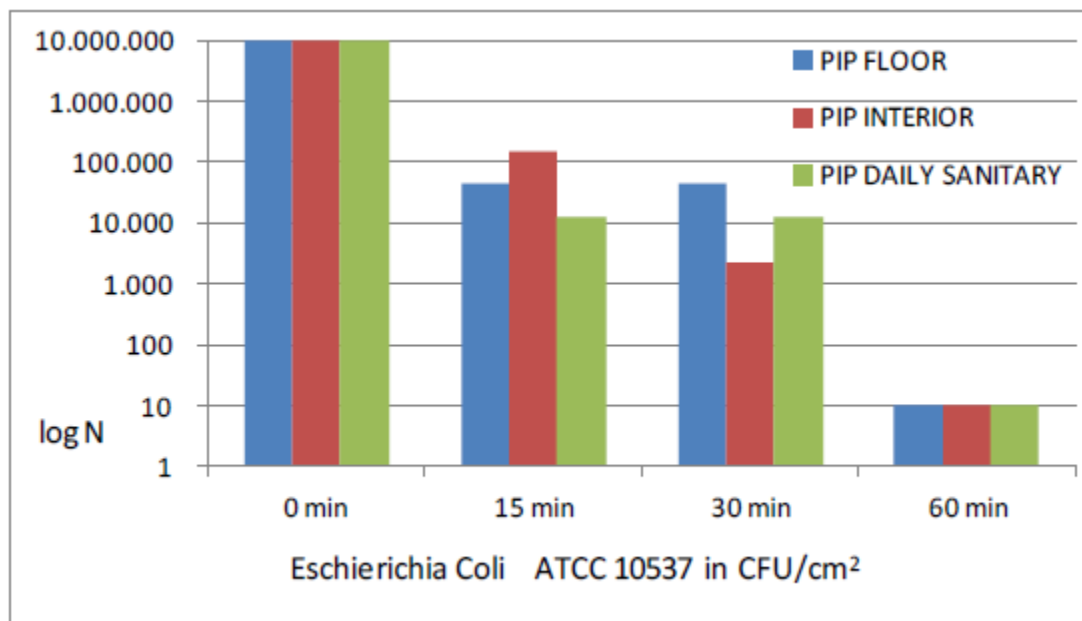


Figure 6 - Reduction of the original dose of Escherichia coli "in vitro" by applying the 3 different PIP products. Changes were recorded over 3 different time periods

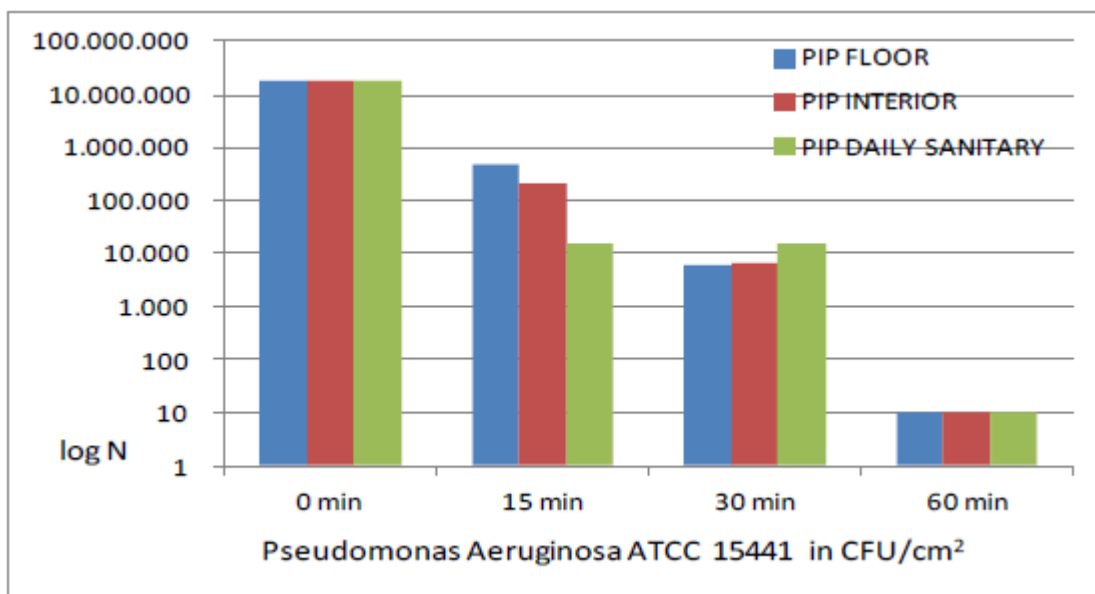


Figure 7 - Reduction of the original dose of Pseudomonas aeruginosa "in vitro" by applying the 3 different PIP products. Changes were recorded over 3 different time periods

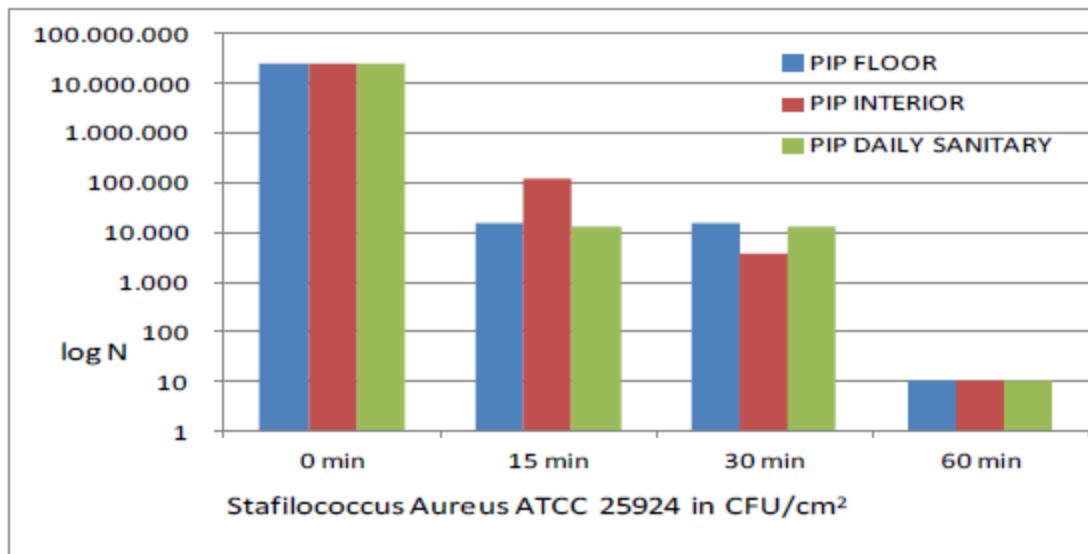


Figure 8 - Reduction of the original dose of Staphylococcus aureus "in vitro" by applying the 3 different PIP products. Changes were recorded over 3 different time periods

It was found that 1 hour after application of the PIP products on the sample surfaces that had been previously contaminated with various microbial strains, the reduction in the concentration of pathogens was 6 logs, or 1,000,000 times lower than the initial counts.

It is obvious that, in laboratory conditions, probiotics effectively prevent the survival of microorganisms under test.

7 – RESULTS OBTAINED FROM TESTING IN THE HOSPITAL

As described above, it was possible to evaluate the effectiveness of PIP probiotic products compared to traditional disinfectants. In this study, there was sampling for 5 separate pathogens, and in different areas of hospital wards that are homogeneous and intended for regular use and health activities. Areas were treated with two different protocols, one based upon the use of PIP probiotics, and the other based upon the use of traditional disinfectants.

Figure 7-13 shows the trend in the values of various pathogens, detected at 14:00, during the three phases.

The graphics depict the changes observed at the sampling sites in terms of CFU/m² of the specific microorganisms of interest. The traditional disinfectant values are indicated with a solid line, and with PIP values are indicated with a dashed line.

The same trends are also graphically distinct in terms of color of the line, which is always the same for each department, regardless of the period of observation.

It is obvious that the use of probiotics leads to a general reduction of the pathogen loads compared to traditional procedures.

This decrease was then evaluated in terms of percentage reduction, and is expressed both in the caption of the same graphs and the summary in Table No. 7.

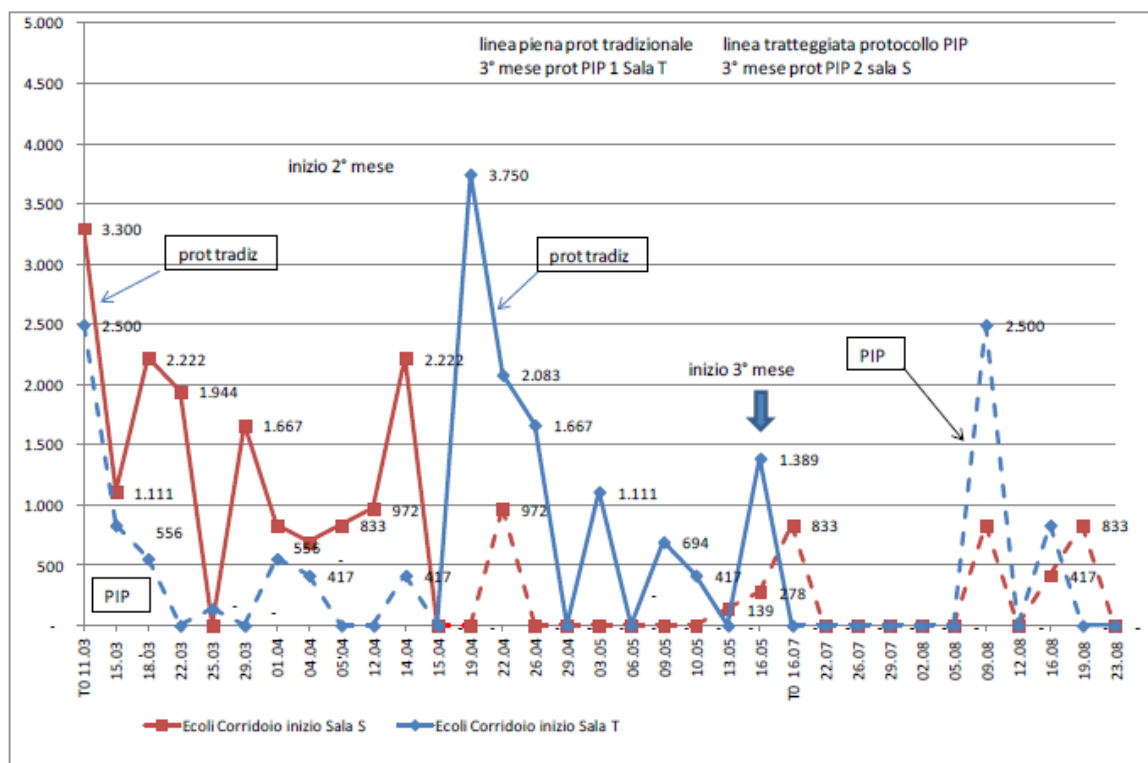


Figure 8 – Reductions of Escherichia coli in patient areas for S and T ;
PIP 1 month -76.67% PIP 2 months - 87.5%, PIP 3 months - 79.72%

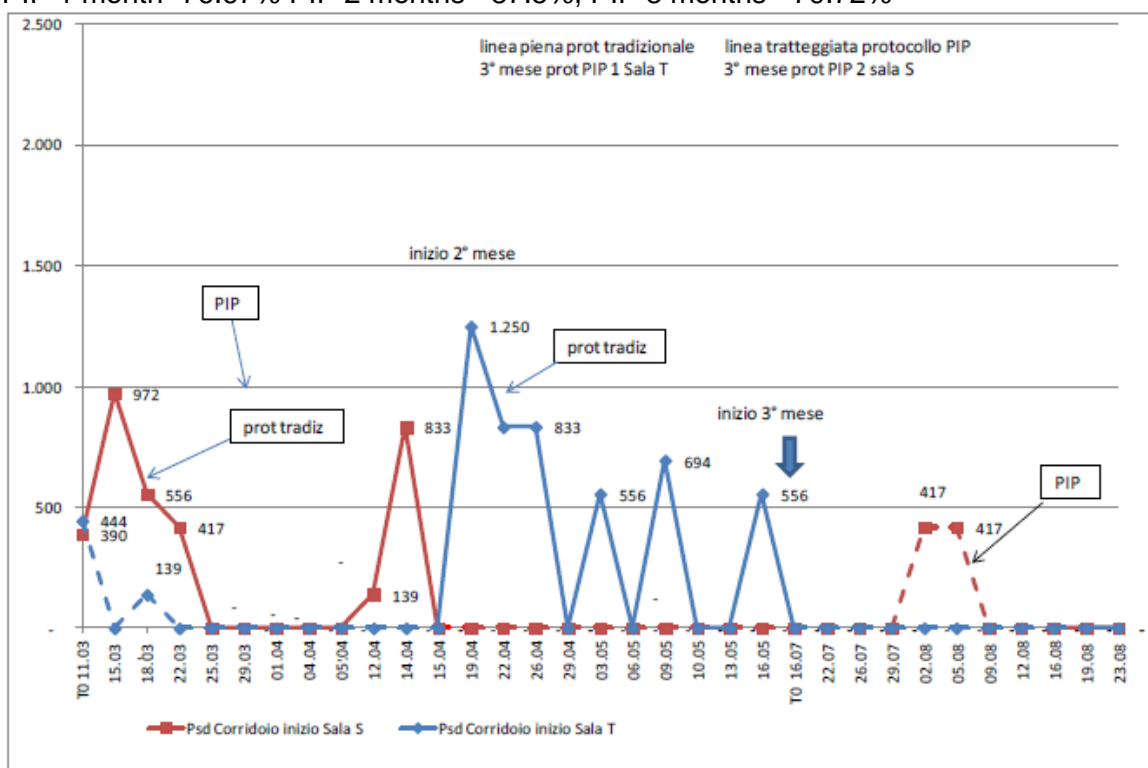


Figure 9 - Reductions of Pseudomonas spp in patient areas for S and T ;
PIP 1 month -95.2%, PIP 2 months - 100.0%, PIP 3 months - 88.4%

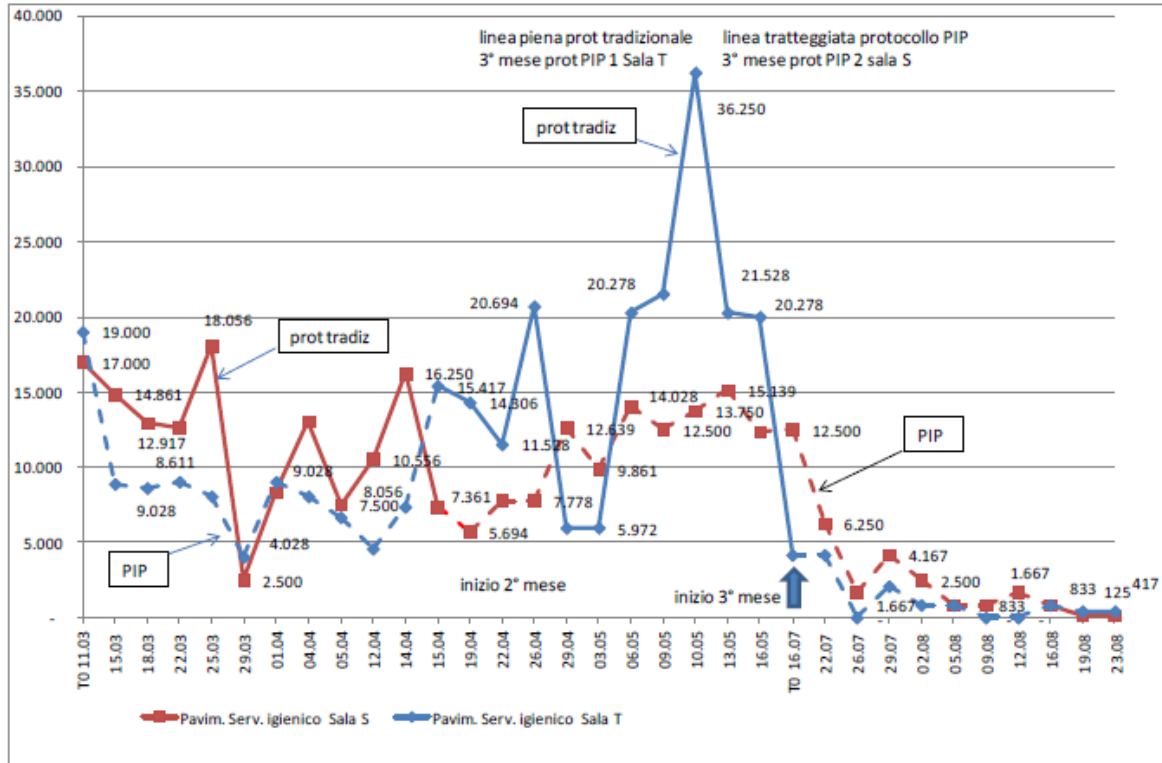


Figure 10 – Reductions of Staphylococcus aureus on the floor of the toilets of S and T; PIP 1 month -36.3% PIP 2 months - 38.1%, PIP 3 months - 85.8%

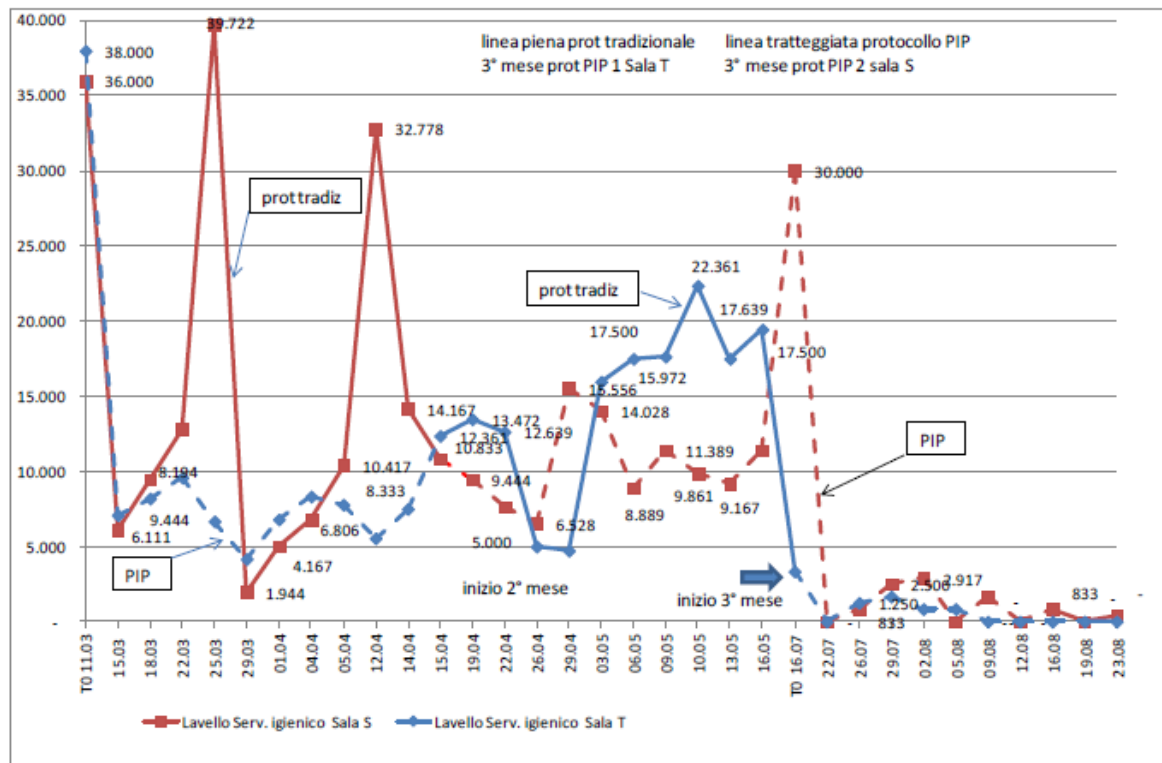


Figure 11 - Reductions of Staphylococcus aureus on the toilet sinks of S and T; PIP 1 month -48.5% PIP 2 months - 27.6%, PIP 3 months - 84.89%

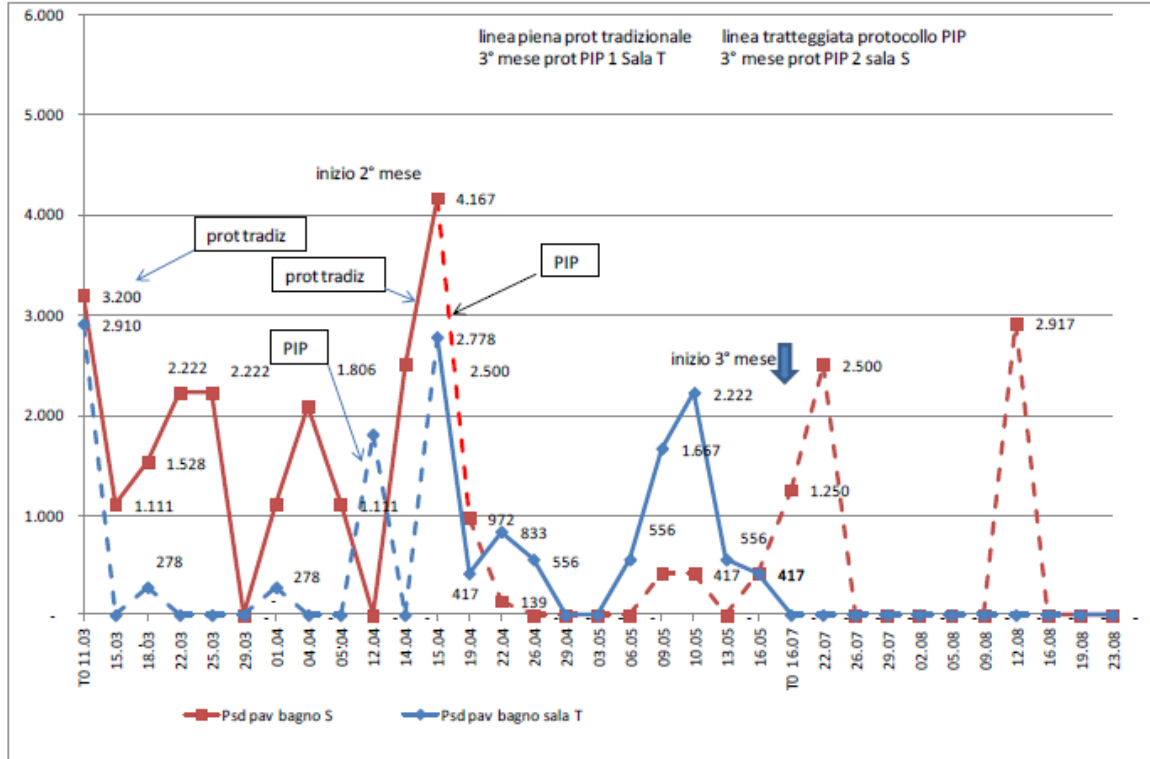


Figure 12 - Evolution of the charge of Pseudomonas to the bathroom floor Salt T and S; rid perc PIP month 1 -83.0%; PIP 2 months - 34.7%; 3 months -78.5

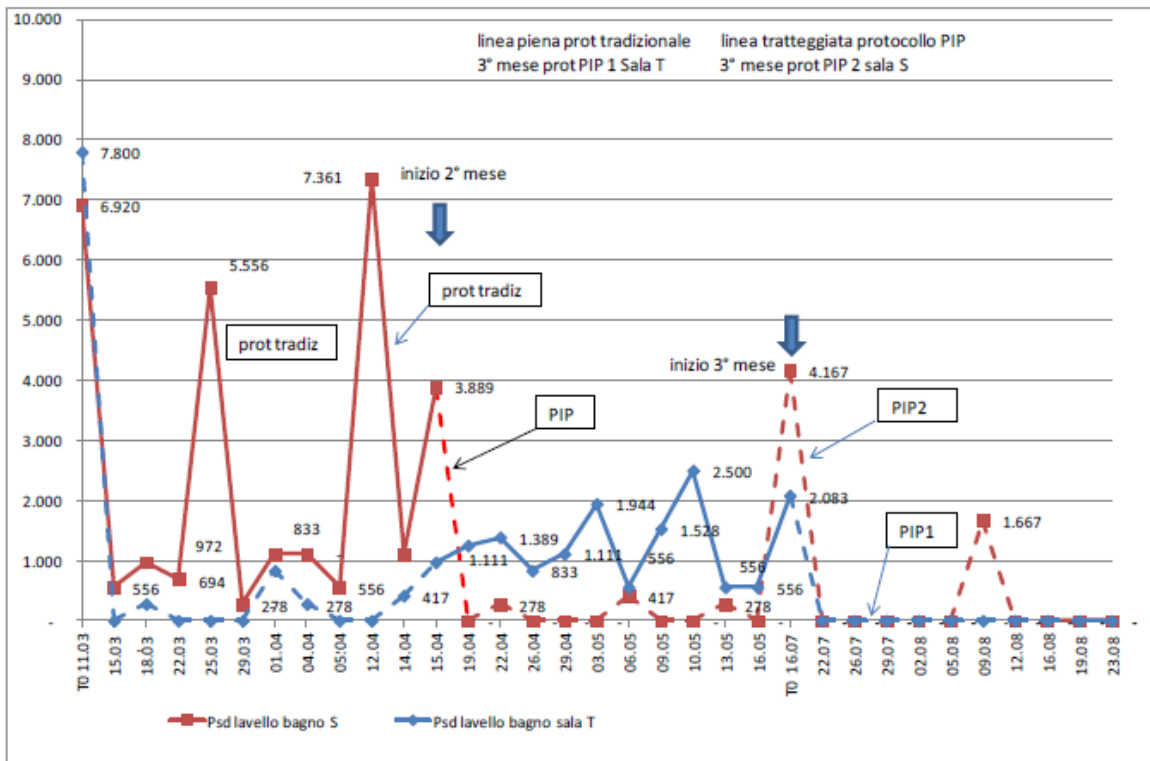


Figure 13 - Evolution of the office of the bathroom sink for Pseudomonas Salt T and S; rid perc PIP month 1 -90.6% PIP 2 °months - 63.1% -95.1% 3 month

We also calculated the average value of the pathogen loads relative to each species and always at 14:00 for the period corresponding to the third phase. The only products used in this phase were the PIP probiotics. The average values thus obtained were then compared with similar average values of these pathogens obtained for departments treated with traditional products.

It was found (last column of Table 7) that there was a further reduction in pathogens by well over 80%.

The same values are shown in Figure 14 for all three phases of the study.

TABLE 7 - PERCENTAGE OF TOTAL REDUCTION OF PATHOGEN PROCEDURES WITH RESPECT TO THE PROCEDURES PIP VS TRADITIONAL EXPRESSED IN ABSOLUTE PERCENTAGES

<i>Sampling Points</i>	<i>Pathogen</i>	<i>General Medicine Phase 1 & 2</i>	<i>Outpatient Ward Phase 1&2</i>	<i>Mean value of General Medicine Ward Phase 3 (*)</i>
<i>Entry and end of corridor</i>	<i>Staphylococcus aureus</i>	29,56%	36,64%	81,03%
	<i>Coliforms spp</i>	72,38%	46,62%	79,72%
	<i>Pseudomonas spp</i>	93,09%	64,49%	88,44%
	<i>Candida spp.</i>	68,88%	56,21%	68,47%
	<i>Acinetobacter</i>		44,74%	
<i>Toilet floor</i>	<i>Staphylococcus aureus</i>	58,75%	51,33%	85,88%
	<i>Coliforms spp</i>	89,15%	78,13%	78,31%
	<i>Pseudomonas spp</i>	55,28%	75,94%	78,57%
	<i>Candida spp.</i>	82,90%	67,80%	71,78%
	<i>Acinetobacter spp.</i>	74,25%		
<i>Toilet sinks</i>	<i>Staphylococcus aureus</i>	55,74%	52,50%	95,59%
	<i>Coliforms spp</i>	81,56%	75,83%	85,12%
	<i>Pseudomonas spp</i>	67,53%	50,41%	95,16%
	<i>Candida spp.</i>	50,38%	27,93%	94,86%
	<i>Acinetobacter</i>	16,39%	31,25%	75,99%

It was found that after two months of use, the prolonged action of the PIP probiotics caused a substantial decrease in the pathogenic microbial load compared to the areas that were treated with traditional products. In many cases the populations of the microorganisms of interest were reduced by close to 90%. One example was from the sink in the toilets, which is a critical surface for patients.

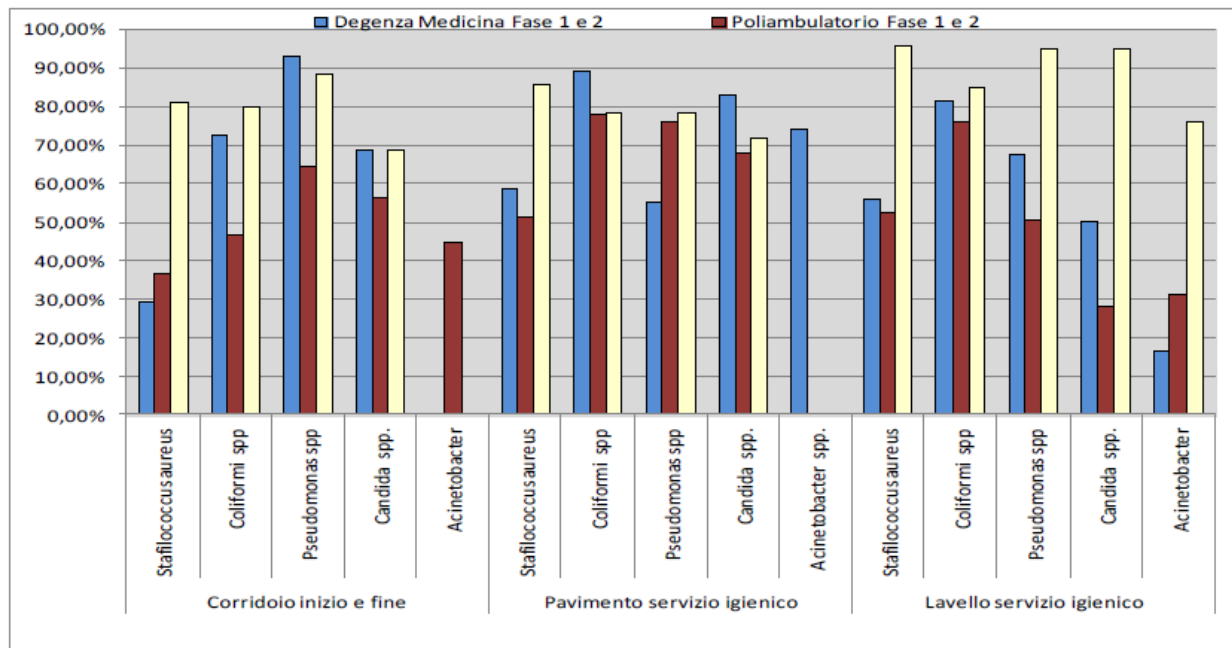


FIGURE 14 - REDUCTION OF PERCENTAGE POINT FOR VARIOUS PATHOGENS AND SAMPLING FOR PHASES 1, 2 AND 3 (the values of the corridor were calculated as the arithmetical average at the entry and end of the corridor)

8 – CONCLUSIONS

Comparing the values shown in Table 7, the mean overall percentage of reduction of pathogens by using the Chrisal PIP Probiotic Products protocol compared to the use of traditional disinfectants is more than a 70 - 80% reduction in pathogens.

Therefore, these results are statistically significant because they have been obtained from the results of the testing of more than 12,000 microbiological samples.

Further, that these samples were taken in many different areas of the hospital, and that were subjected to important everyday recontamination.