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Harmless Bacterial by Products for Chronic Wound Treatment. A Clean Production Experience.

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ABSTRACT: Our research team develops topic formulations based on harmless bacterial by products for chronic wound treatment. The entire scaling up process was designed to facilitate clinical trials, while Cleaner Production (CP) technologies were implemented in order to improve the process. By defining our own critical process points and increasing the efficiency of production processes, we expect reentering our own wastes in the process line, to replace expensive constituents maintaining all pharmacological properties. Four new media were designed and characterized, all activities previously reported were tested *in vitro* and a new quality control was designed. It is possible by reentering in the process line to minimize environmental damage, to use resources more efficiently; and to increase business profitability and competitiveness.In countries where research budgets are meager as Argentina, the tendency to innovation and improvements in the designs prototypes ‘‘*made in Argentina*’’ marks a growing trend adopted by researchers. The entering process and products were patented on INPI (National Institute of Industrial Property), INPI AR*20170123114.*
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I. INTRODUCTION

Our research team develops topic formulations based on harmless bacterial by products for chronic wound treatment [1, 2]. Chronic wounds are those that remain in a chronic inflammatory state and therefore fail to follow normal healing process patterns [3, 5], being infection one of the most important causes of the chronicity [4]. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are frequently isolated from chronic infections [6]. These bacteria are extremely refractory to therapy and host immune attack when living in biofilm phenotype. Previous works show that *Lactobacillus plantarum* ATCC 10241 cultures supernatants (LAPS) interfere with the *P. aeruginosa* and *S. aureus* pathogenic capacity by inhibiting *in vitro* adhesion, quorum sensing and virulence factors [7, 8]. In addition, LAPS showed bacteriostatic and bactericide properties and a great biofilm disrupting capacity [8, 9]. Safety use of LAPS on humans was demonstrated in *ex vivo* model, and inflammatory response *in vivo*, on a mouse model [10]. The chemical composition of LAPS was determined; allowing us to propose a correlation between the chemical constituents and their biological activity [11]. Given direct and indirect pro-healing properties and safety demonstrated by LAPS, we design pharmaceutical dosage forms to facilitate their administration on humans [1, 2]. The entire scaling up process was designed to facilitate clinical trials, while Cleaner production (CP) technologies were implemented in order to improve the process. CP is a preventative approach to managing the environmental impacts of processes and products. CP uses changes in processes, resources and practices to reduce waste, environmental and health risks [12]. The trend towards environmental sustainability and development of renewable resources has significantly increased interest in the recovery of fermentation products, organic acids, and industrial chemicals. A range of products produced by fermentation is expanding beyond the traditional high value low-volume compounds, like pharmaceuticals, competing with traditional synthetic production of commodity chemicals. As fermentation moves into higher values and low-volume chemicals, it becomes necessary to maximize efficiency and minimize costs and waste to compete effectively against traditional options. But, when products are highly

specific such as an Active Pharmaceutical Ingredient, the options are significantly reduced. However, waste by products represents a sustainable option for diminishing cost and increase profits.

The aims of this work were: To define our own critical process points and increase the efficiency of production processes by using CP technologies. Furthermore, we expect by a reentering our own wastes in the process line, to replace expensive constituents maintaining LAPS´s antipathogenic properties. On this process we want to minimize environmental damage, to use resources more efficiently; and to increase business profitability and competitiveness.

II. MATERIALS AND METHODS

2.1. *Bacterial strains and culture conditions*

A strain of *Lactobacillus plantarum* ATCC 10241 was used to obtain all tested supernatants. *Lactobacillus plantarum* reaches stationary phase in de Man Rogosa Sharpe (MRS-Britania-CABA-Argentina) broth and kept at 37°C and maximum concentration of bioactive metabolites at 12 h [11].

For antimicrobial and anti-biofilm activity tests (inhibition and disruption) two of the main pathogenic strains were used. *Pseudomonas aeruginosa* ATCC 27853 was grown in Luria – Bertani (LB-Britania-CABA-Argentina) broth, *Staphylococcus aureus* ATCC 6738 was grown in Brain Hearth Infusion (BHI-Britania-CABA-Argentina) broth.

2.2. LAPS Production process

2.2.1. L. plantarum supernatant (LAPS)

After 12 h of static cultivation $DO_{600} \approx 0.1$ (Spectrophotometer Biotraza 172) in MRS broth, supernatants of *L. plantarum* were recovered after centrifugation (8000 rpm, 10 min) and subsequent filtration through 0.22 μm membranes. The pH was determinated as 5.22±0.43 (Lutron PH-206).

2.2.2. Hazard Analysis and Critical Control Points (HACCP)

Critical control points on pilot scale production process of a drug of biological origin were identified and safety measures were taken.

2.2.3. CP implementation

Scaling up unit operations was defined and Residual Bacterial Cells (RBC) was quantified in two of them. This critical point of residual generation was chosen as first pilot experience in order to design our own CP.

2.3. Biomass production

2.3.1. Lysis and Drying process

RBC, were washed on distillate water twice and then centrifuged 10 min at 8000 rpm (Thermo Scientific Sorvall ST 8). Supernatants were separated for chemical analysis and RBC precipitated was used for lysis tests. Thermal lysis, at high pressure was chosen, as the most effective process for major proteins concentration. Proteins concentration was determinated by UV methods [13] by using bovine albumin as standard for calibration curve. For dehydration RBC were put on stove (Donelab DL-723) at 80°C for 12 h.

2.3.2. Chemical and physical characterization

For physical characterization dry weight was determinated by heated at 80°C for 24 h or to constant weigh. The percentage of dry matter was calculated by weighing difference. Proteins concentration was determinated by UV methods [13] by using bovine albumin as standard for calibration curve. pH measurements were made after sterilization.

2.4. Clean production protocol application and culture media design

As an important step on CP implementation, our research team develops new media for LAPS production.

2.4.1. *Media design*

By using the MRS media as reference of growth for *L. plantarum*, four new media were designed. Protein sources such as: yeast extract, peptone and beef extract, were replaced total or partially by RBC. All so, glucose was partially replaced in one off them. For its identification, four media designed were named SCM (Sesto Cabrera Moreno) one to four.

Protein and carbohydrates quantification was made in a semi-quantitative way by Fourier transform infrared (FTIR) spectroscopy measurements. A Perkin Elmer® GX1 with a DPGS detector was used for measurements. Samples of SCM1, SCM2, SCM3 and SCM4 (10 μl) and MRS broth control were processed by triplicate. Spectra were acquired with 64 scans and 4 cm^{-1} of resolution and previously dried by N_2 flow. Then, spectra were analyzed by OMNIC 8.3 and Origin Pro 8.5.1 software. Spectrum regions analyzed were: Amide I (1700-1600 cm^{-1}), Amide II (1600-1450 cm^{-1}), Carbohydrates and polysaccharides (1200-900 cm^{-1}). Spectra base line correction and smoothing were necessary to improve signal-to-noise ratio, specifically in Amide I region.

2.4.2. *L. plantarum growth*

All media designed were tested for Lactobacillus growth by using MRS media as positive control. *L. plantarum* was grown for 12 h at 37ºC in MRS media, then suspensions of $OD_{600} \approx 0.150$ were prepared using fresh media as diluent and blank. In 96-well polystyrene microtiter plates (Costar Corning Inc. USA), 200 µl of each media designed and controls were placed, and then 20 µl of bacterial suspension were added. The plates were incubated for 12 h at 37°C and growth curves were obtained by measuring the OD_{600} once per hour in a microplate reader (Thermo Scientific Multi scan Go). Each curve was performed by quintuplicate and every point was expressed as mean±SD.

2.5. ***Reentering SCM media on the production line***

2.5.1. *L. plantarum culture supernatants*

Bacteria-free supernatants were recovered as mentioned previously. Original LAPS was used as control of concentration and original activity. LAPSa, LAPSb, LAPSc and LAPSd were obtained from SCM1, SCM2, SCM3 and SCM4 cultures media, respectively. All selected supernatants should comply with the requirement of having a pH greater than 4.2.

2.5.2. *Antimicrobial activity*

The pathogenic strains (*P. aeruginosa* and *S. aureus*) were grown for 12 h at 37ºC in LB and BHI broth respectively and suspensions of $OD_{600} \approx 0.150$ were prepared using fresh medium as diluent and blank. In 96-well polystyrene microtiter plates, 150 µl of each growth media, 20 µl bacterial suspension were placed and 70 µl of inhibitors such as: LAPS (control for original activity), gentamicin (8 µg/ml) (antimicrobial positive control), LB or BHI (growth control), MRS (control for original media components activity), saline (dilution control), SCM1 to SCM4 (control for original media components activity) and selected LAPS, were added. The plates were incubated for 12 h at 37°C and growth curves were obtained by measuring the OD_{600} once per hour in a microplate reader. Each curve was performed by quintuplicate and every point was expressed as mean±SD.

2.5.3. *Biofilm inhibition*

The content of the wells of the above assay (see section 2.5.2) was discarded and the wells were washed three times with saline. The remaining attached biomass (biofilm) was stained during 15 min with 250 µl of crystal violet (0.1%). Cell-attached dye was solubilized with 250 µl of ethanol and the OD_{540} of the resulting solution was measured in a microplate reader. The measured OD is directly proportional to the biomass formed. Results were expressed as mean±SD (n=5).

2.5.4. *Biofilm disruption*

The pathogenic strains (*P. aeruginosa* and *S. aureus*) were grown for 12 h at 37ºC in LB and BHI broth respectively and suspensions of $OD_{600} \approx 0.150$ were prepared using fresh media as diluent and blank. In 96-well polystyrene microtiter plates, 150 µl of each growth media, 20 µl bacterial suspensions were placed at 37ºC for 6 h. Then 70 µl of LAPS (control for original activity), LB or BHI (negative control), MRS (control for original media components activity), saline (dilution control) and selected LAPSa to LAPSd, were added and placed at 37°C. After 6 h the content of the wells was discarded and the wells were washed three times with saline. The remaining attached biomass was stained during 15 min with 250 µl of crystal violet (0.1%). Cell-attached dye was solubilized with 250 µl of ethanol and the OD_{540} of the resulting solution was measured in a microplate reader. The measured OD is directly proportional to the biomass remaining after disruption [11, 14]. The results were expressed as mean±SD (n=10).

2.5.5. D-lactic and L-lactic acid concentration

A commercial D- and L-lactic acid determination kit (Boehringer Mannheim GmbH, Mannheim, Germany) was used to determine the concentration of lactic acid in LAPSa to LAPSd by using original LAPS like optimal concentration.

2.6. *Quality control*

Quality control of the supenatants (LAPS, LAPSa, LAPSb, LAPSc, LAPSd) production was analyzed by FTIR spectroscopy. Samples were previously freeze-dried and then processed as KBr solid tablets. Spectra for each sample were averaged. Normalization process was made for scale the spectra in a similar range [15]. Savitsky – Golay second derivative (9 points, order 3) from each spectrum average was obtained for the characteristic peaks searching within analyzed regions [16].

2.7. *Statistical analysis of data*

Data analysis results were expressed as a mean±SD. The student *t*-test was performed and $p<0.05$, $p<0.01$ and $p<0.001$ was considered statistically significant.

III. RESULTS AND DISCUSSION

Significant advantage over chemical synthesis is that biological ones take less production time but often can use more expensive raw materials. LAPS, is a biological origin API with a complex composition. Formulations design its simple, adequate for bioadhesion and easy to apply, allowing patient care on an outpatient level [2]. Scaling up process allow as to achieve humans trials. However, cost is one of the major factors in economics of biotechnological production. Refined sugars and protein sources, although costly, are the most commonly used substrates for commercial production of metabolites by fermentation process [17]. In this work, we design new culture media, by using waste from the same production scaling up process.

Within the CP framework, two critical biological waste generation points were identified in LAPS scaling up process. Seeking for a method for the efficient disposal of this residual material, several lysis methods were tested with the aim of releasing the cellular content and reusing this lysate. The total yield of bacterial pellet obtained from the critical point was calculated as dry weight 0.7636±0.0031 g/l.

Four lysis methods and modifications were tested and higher protein release was observed to the RBC supernatant, using the thermal lysis at high pressure method (data not shown). RBC was chemically characterized by pH measurement of the lysate supernatant (3.78±0.1) and protein concentration (7.55 g/l/Kg of RBC). pH values are critical, in order to maintain optimum growth conditions. The same buffers used on the commercial broth were added to regulate these values. By replacing one or more nutrients sources for a known weight of lysed bacteria four media were designed. Table 1 shows all media SCM1, SCM2, SCM3 y SCM4 composition. pH values for media designed were: SCM1=6.7±0.1; SCM2=6.6±0.12; SCM3=6.4±0.1; SCM4=6.6±0.1. These values were consistent with MRS pH reported for the commercial brand: Britania-Argentina (6.4±0.2). Proteins concentration of the four media was determinated by UV method and values were: 6.12±0.182 g/l; 3.98±0.145 g/l; 4.88±0.216 g/l; 4.57±0.186 g/l, respectively.

Protein and carbohydrates quantification was made in a semi-quantitative way by Fourier transform infrared (FTIR) spectroscopy measurements. Table 2 shows all media proteins relative amount. Under-curve area value in the spectrum bands at 1700 cm^{-1}-1600 cm^{-1} (Amide I) and different protein sources for each media.

Design growth media spectra profiles obtained were different from MRS broth used as control. This has been explained by Amide I absorption decrease. While Amide II spectrum region have information from different vibrations as N-acetyl ester of peptide linkage, C-N and COO^- stretching and N-H swing [18], it was reported that this information is not useful for protein quantification due to only ~40% of vibrations come from protein linkage [19]. Thus, relative protein amount was determinate based only Amide I areas value, where ~80% vibrations energy corresponds to the symmetric and antisymmetric stretching of the carbonyl group (C=O) specific of the peptide bond [20]. As well, it may note the relative protein amount by absorbance for Amide I (Fig. 1). When bands in Amide I is wider then specific protein bonds variety is higher [21]. Figure 1 shows absorbance for Amide I bands and its width variation. Therefore, it suggests that media designed have less protein variety, some of whom have presented higher growth of test strains used before (data not shown). It might indicate that greatest protein concentration is not necessary for bacteria growth. From spectra outcomes, MRS broth and SCM3, both would have the highest variety of proteins respect to SCM2. Therefore, yeast extract brings a great protein input that allows us to replace the protein and peptide sources from meat peptone. At the same time, spectra correlation value between SCM1 and MRS suggest that yeast extract has been correctly supplied for *L. plantarum* biomass.

Table 3 shows spectral area values on carbohydrates and polysaccharides region of C-OH, C-O-C stretching bonds and specific C-O carbohydrates bonds. C-O-H from carbohydrates deformation ring vibrates on Mid-IR carbohydrates and polysaccharides region [22, 23]. Finally, may find C-O-P and P-O-P bond vibrations from bacteria wall polysaccharides [24]. SCM3 spectrum shows great polysaccharides develop from glucans and mannans residues. In this case, outcomes suggest these compounds are not critical for *Lactobacillus* strains growth. Instead, necessary polysaccharides input for nutritional-demanding strains growth. Absorbance on carbohydrates region in spectra shows similar amount in SCM1 and MRS broth and both showed lower value than SCM3 media. Therefore, components from lysed biomass may supply MRS broth glucose and polysaccharides from yeast extract. Lack carbohydrates sources might avoid contaminants spores develop. Thus, media designed may replace rich-carbohydrates culture media properties, increasing media selectivity and decreasing contaminant develop. Therefore, SCM4 may be the most adequate media to avoid contaminants develop due to its glucose amount is half than SCM3.

By using the MRS media as positive control, all media were tested (16 h) for *L. plantarum* growth static cultivation. Figure 2, shows curves of *Lactobacillus plantarum* growth compared to optimal commercial media. SCM1 to SCM4 were more efficient that MRS. Proteins and sugar sources were replaced totally or partially for RBC and four media designed show similar pH and protein values that MRS media and could be reentered on the production line. However, LAPS previously reported properties should test in order to maintain or increase antimicrobial and biofilm inhibition. For these purpose, 12 h static cultivation of *L. plantarum* was centrifuged and filtered. New supernatants LAPSa to LAPSd were obtained and will be tested as potential chronic wounds API´s. pH values of the resulting APIs (5.31±0.01; 4.60±0.12; 4.37±0.20; 4.52±0.10 respectibly) were tested and compared to original LAPS pH (5.22±0.43), in all cases pH values were similar or higher that original LAPS. Wound pH is a dynamic factor that can change rapidly and affect healing. Minimum

pH value for wound skin accepted by National Regulatory Health Institution (Administración Nacional de Medicamentos, Alimentos y Tecnología Médica) is 4.2 and formulation processes increase this value as part of the production line to 5.26±0.20. It was reported that these values are in the acceptable range for topical skin formulation (4.0-7.2 depending on race, age, sex and environmental factors) [24]. This would be useful in the treatment of chronic wounds as it was demonstrated that wound healing is more effective at low pH (pH=5.0 or lower). Studies have shown that the presence of acidic pH correlates with early stages of healing process in compromised, chronic, and infected wounds. Alkaline environments in a wound (mean values of pH=7.42) are related to chronicity [26]. Clinical studies looked into the benefit of acidity in wound healing where patients were treated with a solution at a pH of 7.3 or 6.0, patients treated with the solution at pH 6.0 showed a much quicker healing time than if treated with the solution at pH 7 [27].

Antimicrobial activity was tested on two pathogenic strains [28]. Statistics of our own Public Health system shows that 14% of the most isolated bacteria are *S. aureus* and 16% of the isolated bacteria are *P. aeruginosa*. Even at seems small proportion, these pathogens are the main responsible for infection while they prepare wound bed for colonization of other bacteria. Once established in the patient, *P. aeruginosa* can be especially difficult to treat. The genome encodes a host of resistance genes, including multidrug efflux pumps and enzymes conferring resistance to beta-lactam and aminoglycoside antibotics, making therapy against this gram-negative pathogen particularly challenging due to the lack of novel antimicrobial therapeutics [29]. Antimicrobial and antipathogenic activities against both bacteria (*P. aeruginosa* y *S. aureus*) were tested for LAPSa to LAPSd, using as positive control the original LAPS. These assays allowed us to identify the supernatants that retain or increase the bacteriostatic and bactericidal activity present in the original LAPS. Growth inhibition test was measured as OD_{600} of 12 h of static cultivation. $p<0.01$ and $p<0.001$ were considered as statistically significant when we compared new media against positive growing control LB. Figure 3A shows *P. aeruginosa* growth inhibition, OD was significantly different in all cases LAPSa to LAPSd. Al so when were compared to original API activity, all show excellent growing inhibition. These data was demonstrated that four new culture supernatants could be used as API for *P. aeruginosa* growth inhibition. Figure 3B shows *S. aureus* growth inhibition. LAPSa, LAPSc and LAPSd OD were significantly different when samples were compared against positive growth control BHI media. LAPSc shows equal growing inhibition activity than original LAPS.

It was previously demonstrated that, LAPS interferes with the pathogenic capacity of *P. aeruginosa* inhibiting *in vitro* adhesion, quorum sensing, and biofilm production [7-9]. Biofilm formation inhibition, measured as OD_{540} of 12h of static cultivation figure 4A shows *P. aeruginosa* biofilm formation inhibition. All LAPS tested were shown optimal biofilm inhibition activity. Besides, the presence of planktonic bacteria, there is an increasing evidence to believe that biofilm formation in wounds is the best unifying explanation for the failure of wound healing [30, 31]. That is why one of the main properties sought in LAPSa to LAPSd is the increase or preservation of biofilm inhibitory activity shown by the original LAPS [14]. $p<0.001$ was considered as statistically significant. Figure 4B shows *S. aureus* biofilm formation inhibition. LAPSa to LAPSd OD_{540} were considered as significantly different whit respect to BHI medium. All so, modified growth media showed anti-biofilm formation action; this could be due to the content of broken bacterial cells used to prepare them.

Remaining attached biofilm, measured as OD_{540} of 12 h of static cultivation. $p<0.001$ was considered as statistically significant. Figure 5A shows *P. aeruginosa* remaining attached biofilm. Novel LAPSa to LAPSd shows optimal disruption of pre formed biofilm capacity maintaining original API activities. According to the hypothesis of Bjarnsholt et al. [32], *P. aeruginosa* would be responsible for the chronicity of wound infections and in turn, it would be the predisposing factor for other infections. If so, treatment with LAPS and our derivatives media would be extrapolated to any chronic wound. Figure 5B shows *S. aureus* remaining attached biofilm. LAPSa, and LAPSb maintains original LAPS disruption activity.

Table 4 shows Lactic acid concentration on LAPSa to LAPSd, compared to original LAPS. LAPSc and LAPSd, shows not significant differences concentration of total Lactic acid (g/l) than original LAPS. It was previously reported that low molecular weight molecules of organic acids such as D/L-lactic acid present antimicrobial activity on Gram-negatives and to synergize antimicrobial action with others components of LAPS [32, 33].

Figure 6 shows the principal peaks from second-derivative peaks analysis (Savitsky-Golay second derivative with 9 points and order 3) for Amide I and carbohydrates regions. Through standardized spectra acquisition, areas value at 1650 cm^{-1} may use as a protein-amount index referred to patrons in a calibration curve for a standardized process. Further, biomass amount might be controlled by area value at 1740 cm^{-1}. Peaks found in this work could be used for a green quality control in the scaling up production process, due to reactive or additional materials is unnecessary [34].

IV. CONCLUSIONS

We define our own critical process points. By replacing sugar and protein sources it is possible to maintain or increase the efficiency of production processes. By reentering our own wastes in our own the process line, we manage to replace expensive constituents maintaining LAPS´s antipathogenic properties. The use of harmless bacteria by-products, such as LAPSa to LAPSd, to antagonize infectious pathogens that have ability to form biofilm is an efficient and economic approach to treat infected chronic wounds. We achieve our main purpose to minimize environmental damage, to use resources more efficiently; and to increase business profitability and competitiveness.

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Figure and Table legends

Fig. 1 Amide I absorbance (1700 cm^{-1}-1610 cm^{-1}) for design-culture media and control media.

Fig. 2*L. plantarum* growth measured as OD600 vs time in media samples: SCM1, SCM2, SCM3, SCM4 and MRS commercial media as positive control.

Fig. 3. *P. aeruginosa* and *S. aureus* growth inhibition test measured as OD600 vs time. In both cases, original LAPS and Gentamicine were used as positivecontrol. Comercial and designed growth media were tested as negative inhibition controls:MRS, SCM1, SCM2, SCM3, SCM4. **A)***P. aeruginosa* growth inhibition for samples LAPSa, LAPSb, LAPSc, LAPSd, and LB as media for growth control. **B)***S. aureus* growth inhibition all samples LAPSa, LAPSb, LAPSc, LAPSd and BHI as media for growth control.

Fig. 4 *P. aeruginosa* and *S aureus* biofilm formation inhibition test measured as OD540 vs time. In both cases, original LAPS was use as positivecontrol. Comercial and designed growth media were tested as negative inhibition controls: MRS, SCM1, SCM2, SCM3, SCM4. **A)***P. aeruginosa* biofilm formation inhibition for samples: LAPSa, LAPSb, LAPSc, LAPSd and LB as media for growth control. **B)***S. aureus* biofilm formation inhibition for samples: LAPSa, LAPSb, LAPSc, LAPSd and BHI as media for growth control.

Fig. 5 *P. aeruginosa* and *S aureus* biofilm disruption test measured as OD540 vs time. In both cases, original LAPS was use as positive control. Comercial and designed growth media were tested as negative inhibition controls: MRS, SCM1, SCM2, SCM3, SCM4. **A)***P. aeruginosa* preformed biofilm disruption for samples: LAPSa, LAPSb, LAPSc, LAPSd and LB as media for growth control. **B)***S. aureus* preformed biofilm disruption for samples: LAPSa, LAPSb, LAPSc, LAPSd and BHI as media for growth control.

Fig. 6 Shows the principal peaks from second-derivative peaks analysis (Savitsky-Golay second derivative with 9 points and order 3) for Amide I and carbohydrates regions. (a) Show a peak at 1650 cm^{-1} which increase when protein sources diversify. (b) Show a peak at 1740 cm^{-1} which may related to *L. plantarum* biomass presence. (c) Show a peak at 950 cm^{-1} which increase when carbohydrates sources diversify.

Table 1 Composition of media designed, SCM 1 to SCM4. The MRS (Britania- Bs As. Argentina) commercial media was used as reference.

Table 2 Under-curve area value in the spectrum bands at 1700 cm^{-1}-1600 cm^{-1} (Amide I) and different protein sources for each media.

Table 4 Lactic acid concentration on g/l for LAPSa to LAPSd tested compared to original LAPS.

Culture media composition (g/l)				
Compounds	**SCM1**	**SCM2**	**SCM3**	**SCM4**
Meat peptone	10.0	10.0	-	-
Yeast extract	-	-	5.0	5.0
Beef extract	10.0	-	-	-
Glucose	20.0	20.0	20.0	10.0
Polysorbate 80	1.08	1.08	1.08	1.08
Potassium hydrogen phosphate	2.0	2.0	2.0	2.0
Sodium acetate	5.0	5.0	5.0	5.0
Sodium citrate	2.0	2.0	2.0	2.0
Manganesum sulphate	0.05	0.05	0.05	0.05
Magnesium sulphate	0.2	0.2	0.2	0.2
RBC	**2.5**	**7.5**	**10.0**	**10.0**

Media	Area (Amide I)	Protein Source
MRS (control)	1.767	YE, MP, ME
SCM1	1.845	B, MP, ME
SCM2	0.915	B, MP
SCM3	1.394	B, YE
SCM4	0.871	B, YE
MRS (protein-free)	0.111	-

YE (Yeast extract) **MP** (Meat Peptone) **ME** (Meat Extract) **B** (*L. plantarum* biomass)

Table 3 Spectral area values on carbohydrates and polysaccharides region (1180 cm^{-1}–940 cm^{-1}) for design-culture media and control media

Culture media	Carbohydrates and Polysaccharides area
MRS (control)	10.502
SCM1	10.878
SCM2	11.534
SCM2	22.021
SCM4	13.755

Sample	D-Lac. (g/l)[a]	L-Lac. (g/l)[b]	Total Lac. acid (g/l)[c]
LAPS	3.06±0.24	2.98±0.12	6.04±0.36
LAPSa	1.84±0.05	0.65±0.05	2.49±0.10
LAPSb	1.29±0.21	1.65±0.13	2.93±0.34
LAPSc	4.11±0.11*	2.73±0.06*	6.84±0.17*
LAPSd	2.75±0.11*	2.96±0.10*	5.51±0.21*

[a]D-lactic acid, [b]L-lactic acid, [c]D+L lactic acid sumatorie

Figures 1

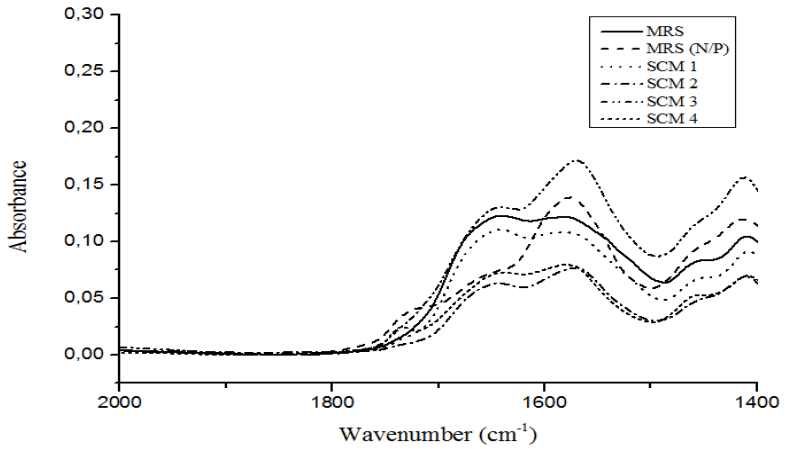


Figure 2

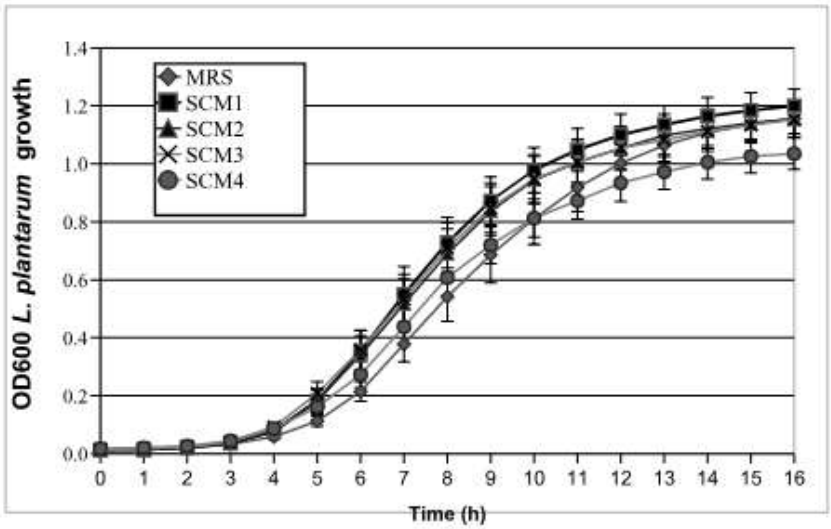


Figure 3

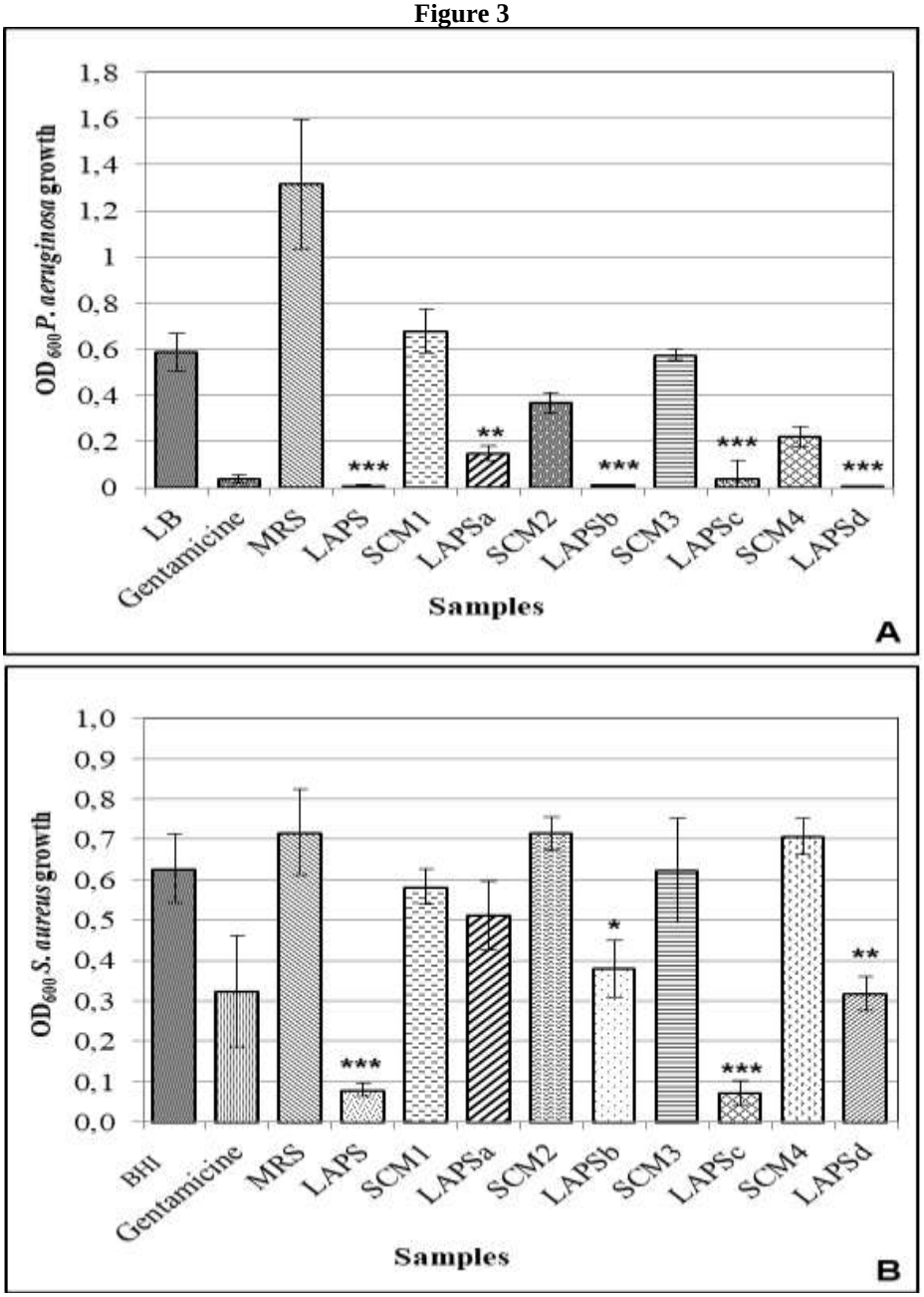
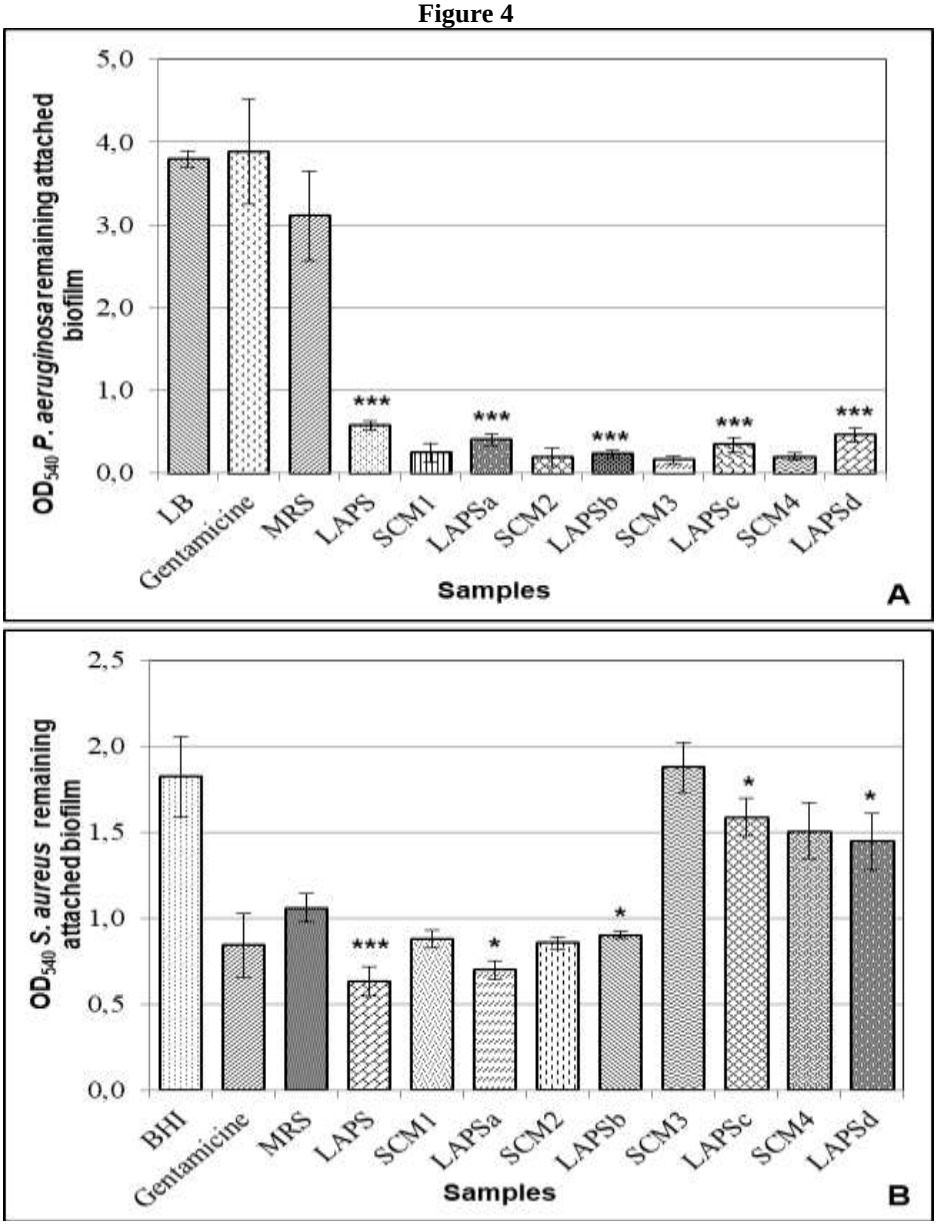

1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
OD600 P. aeruginosa growth
**
***
***
***
***
LB
Gentamicine
MRS
LAPS
SCM1
LAPSa
SCM2
LAPSb
SCM3
LAPSc
SCM4
LAPSd
Samples
A
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
OD600 S. aureus growth
*
**
***
***
BHI
Gentamicine
MRS
LAPS
SCM1
LAPSa
SCM2
LAPSb
SCM3
LAPSc
SCM4
LAPSd
Samples
B

Figure 4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OD540 P. aeruginosa remaining attached biofilm
***
LB
Gentamicine
MRS
LAPS
SCM1
LAPSa
SCM2
LAPSb
SCM3
LAPSc
SCM4
LAPSd
Samples
A
2,5
2,0
1,5
1,0
0,5
0,0
OD540 S. aureus remaining attached biofilm
*
BHI
Gentamicine
MRS
LAPS
SCM1
LAPSa
SCM2
LAPSb
SCM3
LAPSc
SCM4
LAPSd
Samples
B

Fig 5

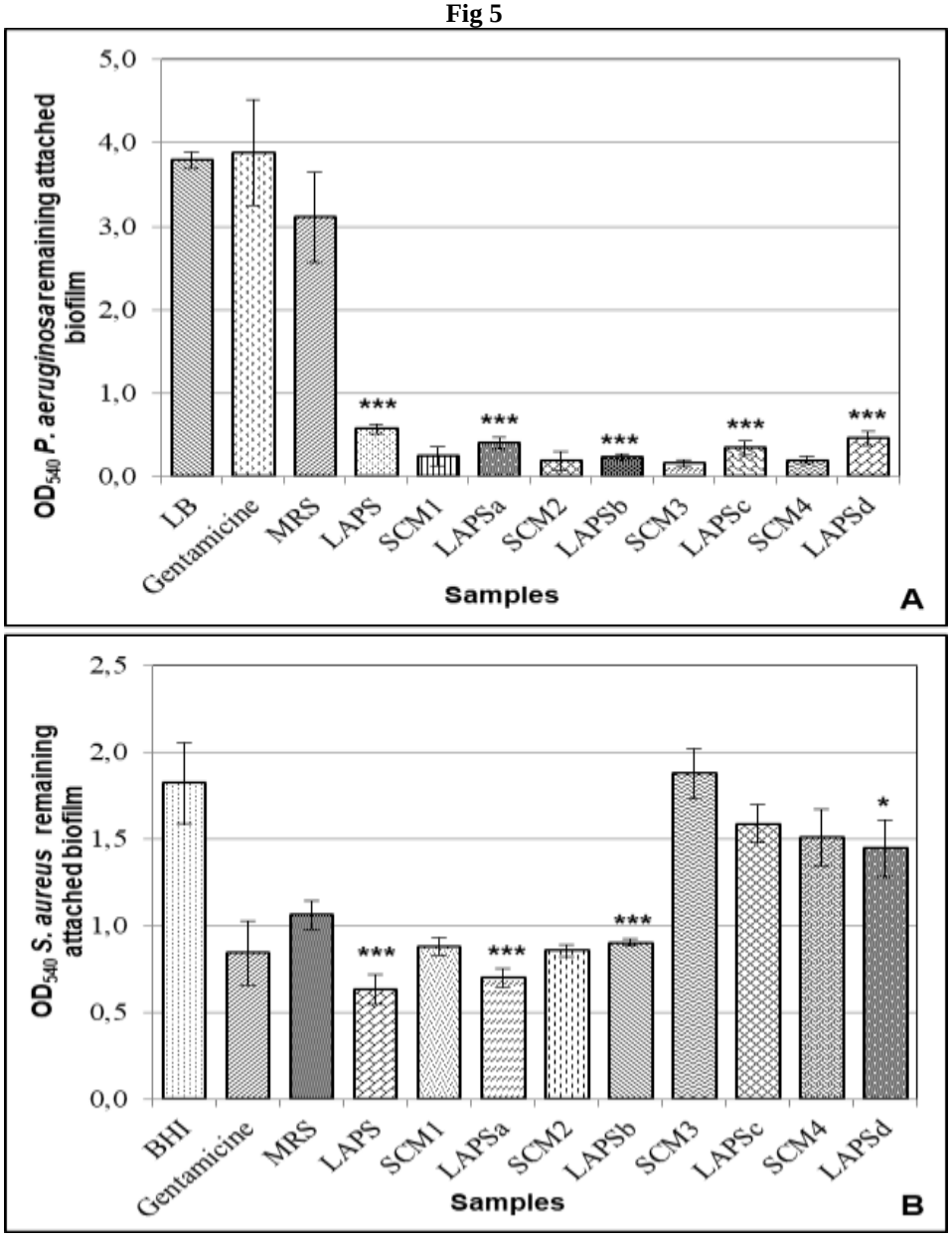
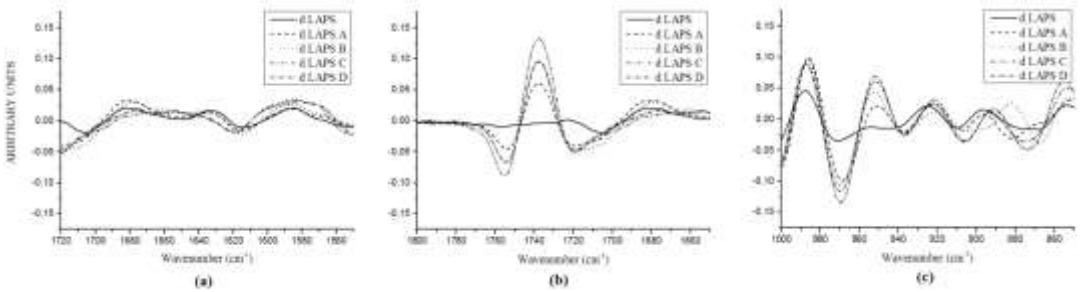


Fig 6



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