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The potential of bacteriocin AS-48 in the control of *Propionibacterium acnes*

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Background Global reports show that the antimicrobial-resistance of *Propionibacterium acnes* isolated from patients with acne vulgaris is becoming a large problem, making it necessary to find new therapeutic drugs.

Methods In this study, 23 clinical isolates of *P. acnes* have been identified by MaldiToff and specific PCR. The susceptibility of theses strains to antibiotics (clindamicin, erytromycin and tetracicline) and to bacteriocin (AS-48) has been established, using the CECT 5684 strain as reference. Moreover, we have investigated the potential of several chemical compounds to bolster the activity of AS-48. Finally, the effectivity of four different formulations containing AS-48 and lysozyme have been evaluated on the surface of swine-ear skin previously inoculated with *P. acnes* CECT5684 strain.

Results. The results presented in this work probe that AS-48 has a significant bactericidal activity against the 23 clinical isolates of *P. acnes*, including isolates resistant to one or more common antibiotics used in the treatment of acne. Antibacterial synergy of AS-48 with other chemical compounds has been demonstrated, as was the effect of lysozyme and to a lesser extent with palmitic acid. Likewise, the use of a combination therapy into a cream formulation, resulted in large decrease in the number of viable *P. acnes* counts in an experiemental model.

Conclusion. Once more these studios support that compositions comprising bacteriocins displaying antibacterial activity, must be considered an approach for medical and pharmaceutical purposes. These applications are particularly promising in light of emerging antibiotic resistance across bacteria involved in treatment of dermatological disease as acne vulgaris.

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26
27 **Keywords** Acne vulgaris, Resistance antibiotic, Circular bacteriocin, Bacterial antagonism,
28 Synergism, Topical acne treatment.
29
30 **Subjects** Microbiology, Public Health
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35 **Background** Global reports show that the antimicrobial-resistance of *Propionibacterium acnes*
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41 tetracicline) and to bacteriocin (AS-48) has been established, using the CECT 5684 strain as
42 reference. Moreover, we have investigated the potential of several chemical compounds to
43 bolster the activity of AS-48. Finally, the effectivity of four different formulations containing
44 AS-48 and lysozyme have been evaluated on the surface of swine-ear skin previously inoculated
45 with *P. acnes* CECT5684 strain.
46

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50 common antibiotics used in the treatment of acne. Antibacterial synergy of AS-48 with other
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52 with palmitic acid. Likewise, the use of a combination therapy into a cream formulation, resulted
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54
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56 **Conclusion.** Once more these studios support that compositions comprising bacteriocins
57 displaying antibacterial activity, must be considered an approach for medical and pharmaceutical
58 purposes. These applications are particularly promising in light of emerging antibiotic resistance
59 across bacteria involved in treatment of dermatological disease as acne vulgaris.
60

61
62 ## Introduction
63
64 The human skin, the first barrier against attack by foreign organisms and toxic substances, is a
65 balanced ecosystem that harbors a broad range of beneficial microorganisms that act against
66 pathogens and participate in T-cell education (Kong, 2011). The human-skin microbiota consists
67 of resident and transient populations of bacteria and various fungal species. Recent advances in
68 sequencing techniques have enabled an accurate characterization of the skin microbiome (Grice
69 & Segre 2011; Lee et al., 2008; Fitz-Gibon et al., 2013; Grice, 2014). Generally, the 4 dominant
70 phyla of bacteria residing on the skin are the *Actinobacteria, Proteobacteria, Firmicutes,* and
71 *Bacteroidetes*. Although the composition and diversity of bacteria colonizing the skin is
72 depending on the microenvironment (hair follicles, ecrine and apocrine glands and sebaceous
73 glands), with high variability between individuals, the dominant types of bacteria that reside on
74 the skin appear to be relatively stable (primarily *Staphylococcus, Propionibacterium,* and
75 *Corynebacterium*) (Grice, 2014).
76
77 *Propionibacterium acnes* is member of the commensal skin microbiota of virtually every human,
78 and is by far the most prevalent in pilosebaceous follicles whereits association with the acne
79 vulgaris has been well-established (Fitz-Gibon et al., 2013). This bacteria plays a critical role in
80 the development of inflammatory acne when it overgrows and colonizes the pilosebaceous unit,
81 having a high psychosocial impact (Cunliffe & Gollnick, 2001; Bojar & Holland, 2004;
82 Dessinioti & Katsambas, 2010). Acne occurs in areas with higher densities of pilosebaceous
83 units, as a multifactorial response that include hormonal, microbiological, and immunological
84 mechanisms (Ki & Rotstein, 2008), and effective acne treatments need to target as many of the
85 causal factors as possible. Moreover, there are certain features leading *P. acnes* to be considered
86 as an opportunistic pathogen in implant-associated infections (prosthetic joint infections, breast
87 fibrosis, cardiovascular device-related infections or spinal osteomyelitis) (Perry et al., 2011;
88 Achermann et al., 2014; Aubin et al., 2014). Today, antimicrobial drug resistance is a growing
89 threat to global public health and the widespread use of antibiotics has been associated with the
90 increase in the occurrence of resistant organisms. Many causes are involved in the emergence of
91 resistance to antibiotics (prolonged administration, poor compliance, subdosing, or monotherapy
92 treatment) making it necessary to find new therapeutic drugs and targets (Aubin et al., 2014).
93 Various alternatives are currently under consideration, including antimicrobial peptides (Vaara,
94 2009; Cotter, Ross & Hill, 2013).
95
96 Bacteriocins are a family of ribosomally synthesized antimicrobial peptides/proteins secreted by
97 bacteria that inhibit the growth of closely related species (narrow spectrum) or across genera
98 (broad spectrum). Bacteriocins are biotechnologically relevant since show low toxicity and can
99 be used in the food industry as natural preservatives (Abriouel et al., 2010; Khan, Flint & Yu,
100 2010; Grande et al., 2014). Furthermore, some bacteriocins have a notable therapeutic potential
101 in local and systemic bacterial infections, highlighting its value as viable alternative to antibiotics
102 (Montalbán-López et al., 2011; Cotter, Ross & Hill, 2013). In fact, there are several publications
103 on the ability of some bacteriocins to inhibit *P. acnes*, reducing inflammatory lesions caused by
104 this bacterium, such as those produced by *Lactococcus s*p. HY 449 (Oh et al., 2006),
105 *Streptococcus* (Bowe et al., 2006) or *Enterococcus faecalis* SL-5 (Kang et al., 2009).
106

107 AS-48 is a circular bacteriocin produced by *Enterococcus* species that consists of 70 natural
108 amino acids. This peptide exerts a bactericidal action on sensitive cells (most of the Gram-
109 positive tested and some Gram-negative bacteria) (Maqueda et al., 2004). Its target is the
110 cytoplasmic membrane, in which it opens pores, leading to the dissipation of the proton motive
111 force and cell death, a mechanism similar to that proposed for the action of defensins and, most
112 generally, the cationic antibacterial peptides. The high proportion of basic amino acids confers a
113 strongly basic character upon this peptide and the combination of a net positive charge with a
114 large proportion of hydrophobic residues, also confers an amphipathic character (reviewed by
115 Maqueda et al., 2004). AS-48 is a model molecule on how proteins can evolve and adopt unique
116 structures in order to achieve a higher stability and greater antibacterial activity (Grande et al.,
117 2014). In fact, this molecule is recognized as one of the most effective bacteriocins, due to its
118 broad spectrum of action, stability throughout a range temperatures, pH values, and surfactant
119 treatments.These properties make AS-48 extremely promising compound for biotechnological
120 applications in food processing and phamaceuticals.
121
122 In fact, our results confirm the strong bactericidal activity of AS-48 to fight against clinical *P.*
123 *acnes* strains, even against those strains more resistant to the antibiotics, which could be a
124 promising alternative treatment to this drugs. Furthermore, the application of different
125 formulations containing AS-48 plus lysozyme, in a swine-skin model previously infected with
126 this bacteriumconfirms that cream exhibited much higher potential, being rapidly bactericidal
127 and may be the procedure of choice for topical applications of the active principles These results
128 open novel prospects for developing topical treatments with bacteriocin AS-48 in the control of
129 this bacterium.
130
131
132 **Methods and material**

133 **Bacterial strains**
134 The bacterial strains used in this study are summarized in Table 1. Regardless of the Spanish
135 Collection of Culture Type CECT 5684 (P0) used as reference, the other 23 samples were of
136 clinical source: 15 strains from different origins were isolated in the Microbiology Service of
137 Virgen de las Nieves Hospital (VNH) (Granada, Spain) and 8 more in the Regional Hospital of
138 Málaga (RUH) (Spain), 6 of them were blood contaminants.
139
140 **Identification of clinical samples by MALDI-TOF**
141 Clinical samples were inoculated onto blood agar plates (5% horse blood) for anaerobic
142 cultivation (37 ºC for 48 h). Then, from each plate, colonies with the appropriate morphologies
143 were picked up and identified by Matrix-assisted laser desorption ionization-time of flight mass
144 spectrometry (MALDI-TOF) according to (Clark et al., 2013). Briefly, colonies were transferred
145 onto a target plate and 1 µL of HCCA Matrix solution (10 mg/ml cyano-4-hydroxycinnamic
146 acid) was subsequently added and dried at room temperature. Mass spectra were acquired with a
147 Microflex LT mass spectrometer using Flex Control 3.3 software (Bruker Daltonics GmbH,
148 Bremen, Germany). For spectrum analysis the Flex Analysis 3.3 program (Bruker Daltonics
149 GmbH, Bremen, Germany) and MALDI Biotyper 3 library were used.
150
151 **DNA isolation and RNA16s sequencing**

152 The bacterial genomic DNA was extracted according to Martín-Platero et al. (2007). PCR or
153 rDNA16S was carried out using a 50-µl mixture containing 25 µl of Jump Start Taq Ready Mix
154 (Sigma-Aldrich), 2 µl of 20 µM WO1 (5'-AGAGTTTGATC[A/C]TGGCTC-3') and WO12 (5'-
155 TACGCATTTCACC[G/T]CTA
156 CA-3) primers, 1 µl of DNA (100µg/ml) and completing to 50µl with bi-distilled water (Ogier et
157 al., 2002). The PCR conditions were 1x 94 ºC 4m, 30x (94 ºC 30s, 50 ºC 30s, 72 ºC 1m), 72 ºC
158 2m). PCR products were purified with a MEGA quick-spin Total Fragment DNA Purification
159 Kit (iNtRON Biotechnology). DNA was sequenced using an ABI PRISM dye terminator cycle
160 sequencing ready reaction kit (Perkin Elmer, Applied Biosystems). To identify the species of the
161 isolated, a search for homology to the DNA sequence was made using the BLAST algorithm
162 (Altschul & Lipman, 1990) available at the National Center for Biotechnology Information
163 (NCBI), from the "16S ribosomal DNA sequences (Bacteria and Archaea)" database, optimized
164 for the "highly similar sequences" (Megablast). A target sequence was assigned to the species
165 with the highest identity value (>99% in all the cases). The similarity between the different
166 sequences was analyzed by Multiple Sequence Alignment with ClustalW2 (Larkin et al., 2007).
167
168 **Antibiotic resistance assays**
169 To check the resistance of the isolates to the erythromycin (Sigma), tetracycline, and
170 clindamycin (Duchefa Biochemia), the antibiotics were separately assayed by limit dilutions
171 (from 200 to 0.195 µg/ml) into 96-well plates, inoculated with each test *P. acnes* strain at 10^5
172 CFU/ml in 100 µl Wilkins-Chalgren anaerobe liquid medium. Then, the plates were incubated in
173 anaerobic jars for 48 h at 37ºC. The minimum inhibitory concentration (MIC) of the individual
174 drug was established by the lowest concentration, in which absence of growth was detected at
175 620 nm, in a Sunrise (Tecan) plate reader.
176
177 **Bacteriocin AS-48 purification**
178 AS-48 was purified from supernatant of the probiotic enterococcal UGRA10 strain (Cebrián et
179 al., 2012). Cultures were carried out on a food-grade whey-derived susbtrate, Esprion 300 (DMV
180 Int., Veghel, Netherland), supplemented with 1% glucose as described Ananou et al. (2008). The
181 bacteriocin was purified as described Abriouel et al. (2003). The protein concentration of the AS-
182 48 samples were determined by measuring UV absorption at 280 nm in a Nanodrop 1000
183 (Thermo Scientific).
184
185 **Biological activity assays and determination of the Minimal Inhibitory Concentration**
186 **(MIC)**
187 The susceptibility of the *P. acnes* isolates against samples of known AS-48 concentration (32 to
188 0.125 µg/ml) was assayed by limit dilutions using the spot-assay method on plates of Wilkins-
189 Chalgren solid medium (Oxoid) grown in anaerobiosis for 48 h at 37ºC. This method was
190 selected due to the difficulty of testing in liquid medium where some chemical compounds here
191 used were precipitated and sometimes difficult to dissolve.
192
193 We determined the MIC for AS-48 onto plates previously overlaid with 6 ml of Wilkins-
194 Chalgren soft agar inoculated with the indicator *P. acnes* strain (10^5 CFU/ml). For this, we
195 assayed 3 µl of half decreasing concentrations from RP-HPLC AS-48 purified samples of known
196 concentration, in sterile distilled water according to Sánchez-Hidalgo et al. (2008). The plates

197 were incubated in anaerobic jars (GasPak; BBL Microbiology System, Baltimore, MD, USA) for
198 48 h at 37ºC to be examined for the appearance of the inhibition halos.
199
200 In parallel, the activity of AS-48 in presence of the following chemical compounds used at
201 subinhibitory concentration, was assayed to check the enhanced, neutral or antagonistic
202 interaction on the inhibitory activity of this bacteriocin:
203 -Salicylic acid (Duchefa Biochemie) dissolved in hot sterile water (50 ºC) at 2 mg/ml.
204 -Muramidase (4 mg/ml) (crystalline lysozyme from egg, Sigma) prepared in sterile water.
205 -Free fatty acids (100 µg/ml): lauric acid (Merck), palmitic acid (Merck) and oleic acid (VWR)
206 dissolved in DMSO (5 %).
207 To determinate the MIC, a series of half-decreasing concentrations of AS-48 samples in solution
208 containing each chemical compound prepared at the afore mentioned concentration, was assayed.
209 In both cases, two independent experiments were performed starting from two different protein
210 stocks.
211
212 **Determination of the cell viability in presence of AS-48**
213 A 96-well plate was filled with 100µl of sterile Wilking-Chalgren medium. Then, the first row
214 (row A) was inoculated with the P27 strain (10^5 UFC/ml) and incubated during 24h in
215 anaerobiosis before AS-48 addition of half decreasing concentrations (from 32 to 0.031µg /ml)
216 and re-incubated for 48 hours. Then, different percentages of the cultures grown in row A, were
217 used to inoculate the following rows of the plate, in order to establish the concentration of AS-48
218 where there was not survivors of P27 that could restart growth after incubation.
219
220 **Statistical analysis**
221 The experimental results were subjected to statistical analysis using the IBM SPSS statistics 20
222 (IBM, Spain). Data relative to the antimicrobial activity of AS-48 alone or in different
223 combinations with active chemicals were subjected to normality test date Sapiro-Wilks and non-
224 parametric Wilcoxon test to compare averages between them. The criterion $p<0.05$ was used to
225 determine the statistical significance.
226
227 ***Ex-vivo* activity of different formulations containing AS-48 and lysozyme**
228 *P. acnes* P0 strain was applied on the surface of swine-ear skin to give a concentration of approx.
229 4 logs of CFU/cm^2 and incubated under anaerobic conditions for 1 h to allow absorption and
230 adherence of bacteria to the skin surface, Then, four different formulations containing 10 µg/ml
231 of AS-48 plus lysozyme (4 mg/ml) were applied to the skin samples (cream, hydro-alcoholic
232 solution, gel and cream plus urea). The treated skin formulation were incubated under anaerobic
233 conditions and samples removed every hour to make a count of the survivors, rinsing with 1 ml
234 cold saline twice to collect any viable bacteria still existing on the skin, diluting in saline solution
235 to be plated onto Wilkins-Chalgren medium, and incubating anaerobically at 37°C (Pannu et al.,
236 2011). A control without treatment was also carried out.
237
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239 **Results and Discussion**
240
241 **Identification of the clinical samples as *Propionibacterium acnes***
242
243 A total of 24 samples have been used in this work (Table 1). Of these, 23 were fresh clinical
244 isolates identified by MALDI-TOF method in the routine microbiological hospital laboratories
245 (Clark et al., 2013). The identification was confirmed by 16s DNA sequencing and BLAST
246 similarity analysis. *Propionibacterium acnes* sp. was assigned with >99% identity in all cases
247 and the sequences were deposited in GeneBank (Table 1). No differences between the sequences
248 were detected by ClustalW2 analysis (Larkin et al., 2007).
249
250 **Range of antibiotic resistance of the isolates**
251
252 *P. acnes* is naturally susceptible to various antimicrobial drugs including β-lactams, macrolide,
253 lincosamide, quinolone, tetracycline, and aminoglycoside. Nevertheless, since the 1970s,
254 antimicrobial resistance gradually accumulated in the skin isolates, especially in European
255 countries, with 51 to 94% of strains showing some antibiotic resistance (Ross et al., 2001).
256 Erythromycin (Ery) resistance is the most common one detected in *P. acnes*, with rates ranging
257 between 17.1 to 52%, while resistance against tetracycline (Tet) is lower than against macrolides,
258 affecting 26% of the isolates (Gübeli et al., 2006). For these reasons, and with the consensus of
259 the Global Alliance to Improve Outcomes in Acne Group and the S3 guideline of the European
260 Dermatology Forum (Gollnick, 2015), antibiotic treatments should no longer be recommended as
261 monotherapy.
262
263 Here we have investigated the susceptibility of the 23 fresh clinical samples, 9 of which from
264 wound exudates of inflammatory acne (P1, P3, P4, P11, P12, P13, P14, P24, and P25) (Table 1)
265 against the most commonly used antibiotics for acne treatment: tetracycline (Tet), erythromycin
266 (Ery), and clindamycin (Clin), using the broth microdilution method. For Ery, we adopted the
267 susceptibility breakpoint recommended by the European Committee on Antimicrobial
268 Susceptibility Testing (EUCAST) (Song et al., 2011) and for Clin and Tet, we used the Clinical
269 and Laboratory Standards Institute (CLSI, 2015) guidelines for anaerobic bacteria.
270
271 Table 2 shows that 4 of the 24 strains tested showed resistance to the antibiotics, while around
272 80% of the strains were susceptible to Ery and Clin (with MIC <0.5 and <8 μg/ml, respectively).
273 Four strains were resistant to Ery (P3, P5, P11, P12), two of them shown combined resistance to
274 Clin (P11, P12), and no one to Tet. According to the history of usage (clinical data from
275 Dermatology Service), the Ery resistance of P11 and P12 could be in relation to previous
276 treatment with this antibiotic.
277
278
279 **AS-48 is bactericidal against clinical *P. acnes* isolates**
280 All the *P. acnes* isolates, including the multidrug-resistant P11 and P12 strain, were examined
281 for susceptibility to bacteriocin AS-48 alone and in combination with different chemical
282 compounds. In a previous assay, a time-kill study of AS-48 samples against exponential-phase
283 culture of the P0 strain (CECT 5684) had been performed. In such assay, the minimal bactericide
284 concentration of AS-48 was dependent on the concentration used. Thus, with 1μg/ml, no

survivors after 144 h of incubation, whilst in the presence of 10 µg/ml of AS-48, no *P. acnes* survivors remained after 48 h of growth, this requiring 96 h of incubation with 5 µg/ml of AS-48 (Maqueda et al., 2012).

Here, the minimum inhibitory concentration (MIC) for AS-48 was determined in solid media, assaying half-decreasing concentrations of purified AS-48 samples, in spite of the susceptibility is generally lower that in liquid media. It is noteworthy that the MIC was strain-dependent (Table 3): the susceptibility to AS-48 ranged from the most sensitive strains with MIC of 2-3 µg/ml (P_1, P_3, P_4, P_{14}, P_{23}, P_{25}, P_{27}) or 4-5 µg/ml (P_0, P7, P9, P10, P11, P13, P15, P17, P18, P20, P22, P24, P26) to 6-7 µg/ml (P6 and P19) or even 12 µg/ml of AS-48 for the P5 and P12 strains. It was remarkable that of the four resistant antibiotic strains (P3, P5, P11, P12) only two P3 and P11 showed significant susceptibility to AS-48 (Table 3).

Determination of interactions between AS-48 and other compounds

MIC for AS-48 combined with fatty acids

The use of lauric, palmitic, and oleic acids against *P. acnes* is attracting attention as potential therapeutic antimicrobial agents due to their potency, broad spectrum of activity and the lack of pathogen resistance to them (Nakatsuji et al., 2009; Desbois & Lawlor, 2013). The aim of these assays was to compare the dose–response effect of AS-48 combined with these substances to improve its efficacy in controlling this pathogen. To this end, we assayed separately combinations of AS-48 with each of these fatty acids (100 µg/ml).

Comparisons of the combined effect of the three fatty acids assayed showed a reduction in the MIC in all cases, but the *p* value was not significant, with the exception of palmitic acid (*p*=0.002; Table 4), which exerts synergism in 17/24 cases. Although the antibacterial activity of these fatty acids remains unclear, the prime target is the cell membrane where these compounds disrupt the electron-transport chain and oxidative phosphorylation, together with cell lysis, enzyme inhibition, impairment of nutrient uptake, and generation of toxic peroxidation or autooxidation products (Desbois & Smith, 2010).

MIC for AS-48 combined with salicylic acid or lysozyme

Salicylic acid was selected due to its keratolytic effect (Zander & Weisman, 1992; Boutli et al., 2003). This combination exhibited a strain-dependent response with MIC values ranging between 14-2.5 µg/ml. Notably, in all cases where the statistical analysis had a significant "*p*" value, the salicylic acid had an antagonistic effect on the AS-48 activity (Table 4).

The increase of AS-48 activity was noticeable in the presence of lysozyme (4 mg/ml) in all the assays performed, with MIC consistently lower than AS-48 alone (*p*-value 0.000; Table 4) in agreement with those recently reported (Maqueda et al., 2012). These formulations were actives against all *P. acnes* isolates tested, including those that were Ery and Ery-Clin resistant as P5 and P12 strains, where the MIC of AS-48 decreased from 12 to 3.5 and 1.25 µg/ml, respectively. Overall, the activity of AS-48 in presence of lysozyme had the most powerful synergy found in all the combinations assayed.

Activity of AS-48 against clinical *P. acnes* isolates, alone and in combination with different chemical compounds

Proliferative capacity of *P. acnes* after AS-48 treatment.

In order to determine whether in the cultures treated with AS-48 there are left living cells able to restart the growth, we conducted a study on the proliferative capacity of the P7 strain (selected by its intermedium susceptibility) post-treatment. Thus, to cultures of 24 h performed in the row A of a microtiter plate, we added serial half decreasing concentrations of AS-48 (from 32 to 0.031µg /ml) to each well. After 48h of incubation, the remaining rows, containing only fresh culture media, were inoculated with different percentages (1, 3, 5, 8, 10, 15 and 20 %) of the cultures grown in row A.

The results showed that after addition of 1µg/ml of AS-4 or plus, was not possible to recover bacterial growth, even in those wells inoculated with 20 % of P27 culture. Thus, confirming that the minimal bactericide concentration for AS-48 was 1µg/ml, a concentration that effectively kills all the cell.

Activity *ex-vivo* of different formulations with AS-48 and lysozyme

We assayed *ex-vivo* the activity of four different formulations (gel, hydro-alcoholic solution, cream, and cream plus urea) containing AS-48 (10 µg/ml) plus lysozyme (4 mg/ml) in a model of ear skin of swine, previously infected with 10^4 CFU/cm^2 of the P0 strain.

The kinetic of bactericidal activity of the active principles incorporated into four different formulations has been evaluated in a swine-skin model. Figure 1 shows how the use of a combination therapy into different formulations, resulted in large decrease in the number of viable *P. acnes* counts and this reduction was significantly more effective with the cream.This study depicted that AS-48 plus lysozyme in cream was the most rapidly bactericidal, causing more than a 4-log reduction after 1 h of exposure time in the experimental model, followed by cream + urea (2 h) and finally by the hydro-alcoholic solution that required 6 h to give the same result.

Conclusions

The results presented prove the widespread susceptibility of the *P. acnes* strains tested to the AS-48 bacteriocin. We suspect that this activity could be favoured by the amphiphatic nature of the AS-48 and the hydrophobic characteristics of the cell wall of these bacteria, belonging to the phylum *Actinobacteria* (Cebrián et al., 2015).

The synergism, indifferent or antagonistic effects on AS-48 activity of combinations with several chemical compounds assayed at sub-inhibitory concentrations are shown in Table 3 and Figure 2. The strong synergism between AS-48 and lysozyme, and to a lesser extent with palmitic acid, is of special relevance because such combinations could provide greater effectiveness even against multi-resistant *P. acnes*. The synergy between these compounds highlights once again the importance of combining antimicrobial agents that act on different targets ("hurdle technology"):

376 the simultaneous application of multiple barriers acting on different targets is more effective than
377 just one, and allows the application of the active principles in more moderate doses (Leistner
378 2000). Besides, these active principles have the advantage of avoiding the development of
379 resistance by pathogens that colonize and infect the skin (Bowe et al., 2006; Lee et al., 2008;
380 Kang et al., 2009; Pannu et al., 2011).
381
382 In summary, this study supports that AS-48 exhibits propierties that make it particularly
383 promising in light of emerging antibiotic resistance across bacteria involved in treatment of
384 dermatological disease as acne vulgaris. The results in an *ex vivo* model suggest that the active
385 principes in the cream were rapidly bactericidal and may be the procedure of choice for topical
386 applications. The results confirm once more that compositions comprising bacteriocins
387 displaying antibacterial activity, would be considered an approach for medical and
388 pharmaceutical purposes (Parisien et al., 2008; Cotter, Ross & Hill, 2013).
389

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395 Research Group general (BIO160, UGR).
396

397 **Competing Interests**
398 Mercedes Maqueda, Eva Valdivia, Manuel Martínez-Bueno, Samir Ananou and Rubén
399 Cebrián have the Spanish Patent "Composition for treatments of skin and mucosal
400 bacterial infection" referred in this paper (Spanish Patent ES-2 387 425; PCT/ES2013/070461)
401

402 **Ethics**
403 The study protocol was carried out in accordance with the Declaration of Helsinki. This was a
404 non interventional study with no additional investigation to routine procedures. Biological
405 material was only used for standard diagnostics following physicians' prescriptions. No
406 additional sampling or modification of the routine sampling protocol was performed. Data
407 analyses were carried out using an anonymous database.
408

409 **Author Contributions**
410 Conceived and designed the experiments: Maqueda M and Valdivia E. Performed the
411 experiments: Cebrián R, Arévalo S and Ananou S. Analyzed the data: Maqueda M and Martínez-
412 Bueno M. Statistical analysis: Cebrián R. Contributed reagents/materials/ analysis tools: Valdivia
413 E and Martínez-Bueno M. Clinical samples were supplied by Arias-Santiago S, Riazzo C, and
414 Bermúdez P. Identification of the clinical samples was carried out by Rojo MD and Bermúdez P.
415 Wrote the paper: Maqueda M and Valdivia E. Data interpretation: Maqueda M.
416

417 **References**

418

419 **Abriouel H, Valdivia E, Martinez-Bueno M, Maqueda M, Galvez A**. 2003. A simple method
420 for semi-preparative-scale production and recovery of enterocin AS-48 derived from
421 *Enterococcus faecalis* subsp. *liquefaciens* A-48-32. *Journal of Microbiological Methods*
422 **55**:599–605 DOI 10.1016/S0167-7012(03)00202-1-

423 **Abriouel H, Lucas R, Ben Omar, N, Valdivia E, Gálvez A**. 2010. Potential applications of the
424 cyclic peptide enterocin AS-48 in the preservation of vegetable foods beverages. *Probiotic*
425 *and Antimicrobial Protein* **2**:77–89

426 **Achemann Y, Goldstein EJC, Coenye T, Shirtliff M**. 2014. *Propionibacterium acnes*: from
427 commensal to opportunistic biofilm-associated implant pathogen. *Clinical Microbiological*
428 *Reviews* **27**:419-440

429 **Altschul SF, Lipman DJ.** 1990. Protein database searches for multiple alignments. Proceeding
430 of the National Academic of Sciences USA **87:**5509–5513 DOI 10.1073/pnas.87.14.5509

431 **Ananou S, Muñoz A, Gálvez A, Martínez-Bueno M, Maqueda M, Valdivia E**. 2008.
432 Optimization of enterocin AS-48 production on a whey-based substrate. *International Dairy*
433 *Journal* **18**:923–927 DOI 10.1016/j.idairyj.2008.02.001

434 **Aubin GG, Portillo ME,Trampuz A, Corvec S**. 2014. *Propionibacterium acnes*, an emerging
435 pathogen: from acne to implant-infections, from phylotype to resistance. *Médecine et*
436 *Maladies Infectieuses* **44**:241–250 DOI10.1016/j.medmal.2014.02.004

437 **Bojar RA, Holland KT.** 2004. Acne and *Propionibacterium acnes*. *Clinical Dermatology*
438 **22**:375–379 DOI10.1016/j.clindermatol.2004.03.005

439 **Boutli F, Zioga M, Koussidou T, Ioannides D, Mourellou O**. 2003. Comparison of
440 chloroxylenol 0.5% plus salicylic acid 2% cream and benzoyl peroxide 5% gel in the
441 treatment of acne vulgaris: a randomized double-blind study. *Drugs Under Experimental*
442 *and Clinical Research* **29**:101–105

443 **Bowe WP, Filip JC, DiRienzo JM, Volgina A, Margolis DJ**. 2006. Inhibition of
444 *Propionibacterium acnes* by bacteriocin-like inhibitory substances (BLIS) produced by
445 Streptococcus salivarius. *Journal of Drugs Dermatology* **5**:868–870

446 **Cebrián R, Baños A, Valdivia E, Pérez-Pulido R, Martínez-Bueno M, Maqueda M**. 2012.
447 Characterization of functional, safety, and probiotic properties of *Enterococcus faecalis*
448 UGRA10, a new AS-48-producer strain. *Food Microbiology* **30**:59–67 DOI
449 10.1016/j.fm.2011.12.002

450 **Cebrián R, Martínez-Bueno, M, Valdivia E, Albert A, Maqueda M, Sánchez-Barrena MJ.**
451 2015. The bacteriocin AS-48 requires dimer dissociation followed by hydrophobic
452 interactions with the membrane for antibacterial activity. *J. Struct. Biol.* **190**:162–172 DOI
453 10.1016/j.jsb.2015.03.006

454 **Clark AE, Kaleta EJ, Arora A, Wolk DM**. 2013. Matrix-assisted laser desorption ionization-
455 time of flight mass spectrometry: a fundamental shift in the routine practice of clinical
456 microbiology. *Clinical Microbiological Reviews* **26**:547–603 DOI 10.1128/CMR.00072-12

457 **Cotter PD, Ross RP, Hill C**. 2013. Bacteriocins - a viable alternative to antibiotics? *Nature*
458 *Reviews Microbiology* **11**:95–105 DOI 10.1038/nrmicro2937

459 **Cunliffe WJ, Gollnick HP**. 2001 Microbiology of acne. In: Acne (Cunliffe WJ, Gollnick H.P.,
460 eds). Martin Dunitz: Kent, 29–36.

461 **Desbois AP, Smith VJ**. 2010. Antibacterial free fatty acids: activities, mechanisms of action and
462 biotechnological potential. *Applied Microbiology and Biotechnology* **85:**1629–1642 DOI
463 10.1007/s00253-009-2355-3

464 **Desbois AP, Lawlor KC**. 2013. Antibacterial activity of long-chain polyunsaturated fatty acids
465 against *Propionibacterium acnes* and *Staphylococcus aureus*. *Marine Drugs* **11**:4544–4557
466 DOI 10.3390/md11114544

467 **Dessinioti C, Katsambas AD**. 2010. The role of *Propionibacterium acnes* in acne pathogenesis:
468 facts and controversies. *Clinical Dermatology* **28**:2–7 DOI
469 10.1016/j.clindermatol.2009.03.012

470 **Fitz-Gibbon S, Tomida S, Chiu BH, Nguyen L, Du C, Liu M, Elashoff D, Erfe**
471 **MC, Loncaric A, Kim J, Modlin RL, Miller JF, Sodergren E, Craft N, Weinstock**
472 **GM, Li H**. 2013- *Propionibacterium acnes* Strain Populations in the Human Skin
473 Microbiome Associated with Acne. *Journal of Investigative Dermatology* **133**:2152–2160
474 DOI 10.1038/jid.2013.21

475 **Gollnick HPM**. 2015. From new findings in acne pathogenesis to new approaches in treatment.
476 *Journal of the European Academy of Dermatology and Venerology* **29**:1–7

477 **Grande MJ, Pérez-Pulido R, López-Aguayo MC, Gálvez A, Lucas R**. 2014. The Cyclic
478 Antibacterial Peptide Enterocin AS-48: Isolation, Mode of Action, and Possible Food
479 Applications. *International Journal of Molecular Science* **15**:22706-22727

480 **Grice EA, Segre JA**. 2011. The skin microbiome. *Nature Reviews Microbiology* **9**:244–253

481 **Grice EA**. 2014. The skin microbiome: potential for novel diagnostic and therapeutic approaches
482 to cutaneous disease. *Seminary of Cutaneous Medical Surgery* **33**:98-103

483 **Gübelin W, Martínez MA, Molina MY, Zapata S, Valenzuela ME**. 2006. Antimicrobial
484 susceptibility of strains of *Propionibacterium acnes* isolated from inflammatory acne.
485 *Revista Latinoamericana de Microbiología* **48**:14–16

486 **Kang BS, Seo JG, Lee GS, Kim JH, Kim SY, Han YW, Kang H, Kim HO, Rhee**
487 **JH, Chung MJ, Park YM**. 2009. Antimicrobial activity of enterocins from *Enterococcus*
488 *faecalis* SL-5 against *Propionibacterium acnes*, the causative agent in acne vulgaris, and its
489 therapeutic effect. *Journal of Microbiology* **47**(1):101–109 DOI 10.1007/s12275-008-0179-y

490 **Khan H, Flint S, Yu P-L**. 2010. Enterocins in food preservation. *International Journal of Food*
491 *Microbiology* **141**:1–10

492 **Ki V, Rotstein C**. 2008. Bacterial skin and soft tissue infections in adults: A review of their
493 epidemioloy, pathogenesis, diagnosis, treatment and site of care. *Canadian Journal of*
494 *Infection Diseases Medical Microbiology* **19**:173–184

495 **Kong HH**. 2011. Skin microbiome: genomics-based insights into the diversity and role of skin
496 microbes. *Trends in Molecular Medecin* **17**:320–328

497 **Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H,**
498 **Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG**.
499 2007. ClustalW and ClustalX version 2. *Bioinformatic* **23**:2947-2948

500 **Lee YJ, Choi HJ, Kang TW, Kim HO, Chung MJ, Park YM**. 2008. CBT-SL5, a bacteriocin
501 from *Enterococcus faecalis*, suppresses the expression of interleukin-8 induced by
502 *Propionibacterium acnes* in cultured human keratinocytes. *Journal of Microbiology and*
503 *Biotechnology* **18**:1308–1316

504 **Leistner L.** 2000. Basic Aspects of food preservation by hurdle technology. *International*
505 *Journal of Food Microbiology* 55:181–186 DOI 10.1016/S0168-1605(00)00161-6

506 **Maqueda M, Valdivia E, Martínez-Bueno M, Ananou S, Cebrián R.** 2012. Composición para
507 el tratamiento de infecciones bacterianas de la piel y mucosas Patent nº: ES-2 387 425
508 (PCT/ES2013/070461)

509 **Maqueda M, Gálvez A, Martínez-Bueno M, Sánchez-Barrena MJ, González C, Albert A,**
510 **Rico M, Valdivia E**. 2004. Peptide AS-48: prototype of a new class of cyclic bacteriocins.
511 *Current Protein and Peptide Sciences* **5**:399–341 DOI 10.2174/1389203043379567

512 **Martín-Platero AM, Valdivia E, Maqueda M, Martínez-Bueno M**. 2007. Fast, convenient,
513 and economical method for isolating genomic DNA from lactic acid using a modification of
514 the protein "salting-out" procedure. *Analytical Biochemistry* **366**:102–104 DOI
515 10.1016/j.ab.2007.03.010

516 **Montalbán-Lopez M, Sánchez-Hidalgo M, Valdivia E, Martínez-Bueno M, Maqueda M**.
517 2011. Are bacteriocins underexploited? Novel applications for old antimicrobials. *Current*
518 *Pharmaceutical Biotechnology* **12**:1205–1220 DOI 10.2174/138920111796117364

519 **Nakatsuji T, Kao MC, Fang JY, Zouboulis CC, Zhang L, Gallo RL, Huang CM**. 2009.
520 Antimicrobial property of lauric acid against *Propionibacterium acnes*: its therapeutic
521 potential for inflammatory acne vulgaris. *Journal of Investigative Dermatology* **129**: 2480–
522 2488 DOI 10.1038/jid.2009.93

523 **Ogier JC, Son O, Gruss A, Tailliez P, Delacroix-Buchet A**. 2002. Identification of the
524 bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis.
525 *Applied and Environmental Microbiology* **68**:3691–3701 DOI 10.1128/AEM.68.8.3691-
526 3701.2002

527 **Oh S, Kim SH, Ko Y, Sim JH, Kim KS, Lee SH, Park S, Kim YJ**. 2006. Effect of bacteriocin
528 produced by *Lactococcus* sp. HY 449 on skin-inflammatory bacteria. *Food and Chemical*
529 *Toxicology* **44**:1184–1190 DOI 10.1016/j.fct.2005.08.008

530 **Pannu J, McCarthy A, Martin A, Hamouda T, Ciotti S, Ma L, Sutcliffe J, Baker JR Jr**.
531 2011. In Vitro Antibacterial Activity of NB-003 against *Propionibacterium acnes*.
532 *Antimicrobial Agents and Chemotherapy* **55**: 4211–4217 DOI 10.1128/AAC.00561-11

533 **Parisien A, Allain B, Zhang J, Mandeville R, Lan CQ**. 2008. Novel alternatives to antibiotics:
534 bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. *Journal of*
535 *Applied Microbiology* **104**: 1e13.

536 **Perry A, Lambert P**. 2011. *Propionibacterium acnes*: infection beyond the skin. *Expert Review*
537 *of Anti-Infective Therapy* **9**:1149–1156 DOI 10.1586/eri.11.137

538 **Ross JI, Snelling AM, Eady EA, Cove JH, Cunliffe WJ, Leyden JJ, Collignon P, Dréno**
539 **B, Reynaud A, Fluhr J, Oshima S**. 2001. Phenotypic and genotypic characterization of
540 antibiotic-resistant *Propionibacterium acnes* isolated from acne patients attending
541 dermatology clinics in Europe, the U.S.A., Japan and Australia. *British Journal of*
542 *Dermatology* **44**:339–346 DOI 10.1046/j.1365-2133.2001.03956.x

543 **Sánchez-Hidalgo M, Martínez-Bueno M, Fernández-Escamilla AM, Valdivia E, Serrano L,**
544 **Maqueda M**. 2008. Effect of replacing glutamic residues upon the biological activity and
545 stability of the circular enterocin AS-48. *Journal of Antimicrobial Chemotherapy* **61**:1256–
546 1265

547 **Song M, Seo S-H, Ko H-C, Oh C-K, Kwon K-S, Chang CL, Kim M-B**. 2011. Antibiotic
548 susceptibility of *Propionibacterium acnes* isolated from acne vulgaris in Korea. *Journal of*
549 *Dermatology* **38**:667–673

550 **Vaara M**. 2009. New approaches in peptide antibiotics. *Current Opinion in Pharmacology*
551 **9**:571e576.

552 **Zander E, Weisman S**. 1992. Treatment of acne vulgaris with salicylic acid pads. *Clinical*
553 *Therapeutics* **14**:247–53

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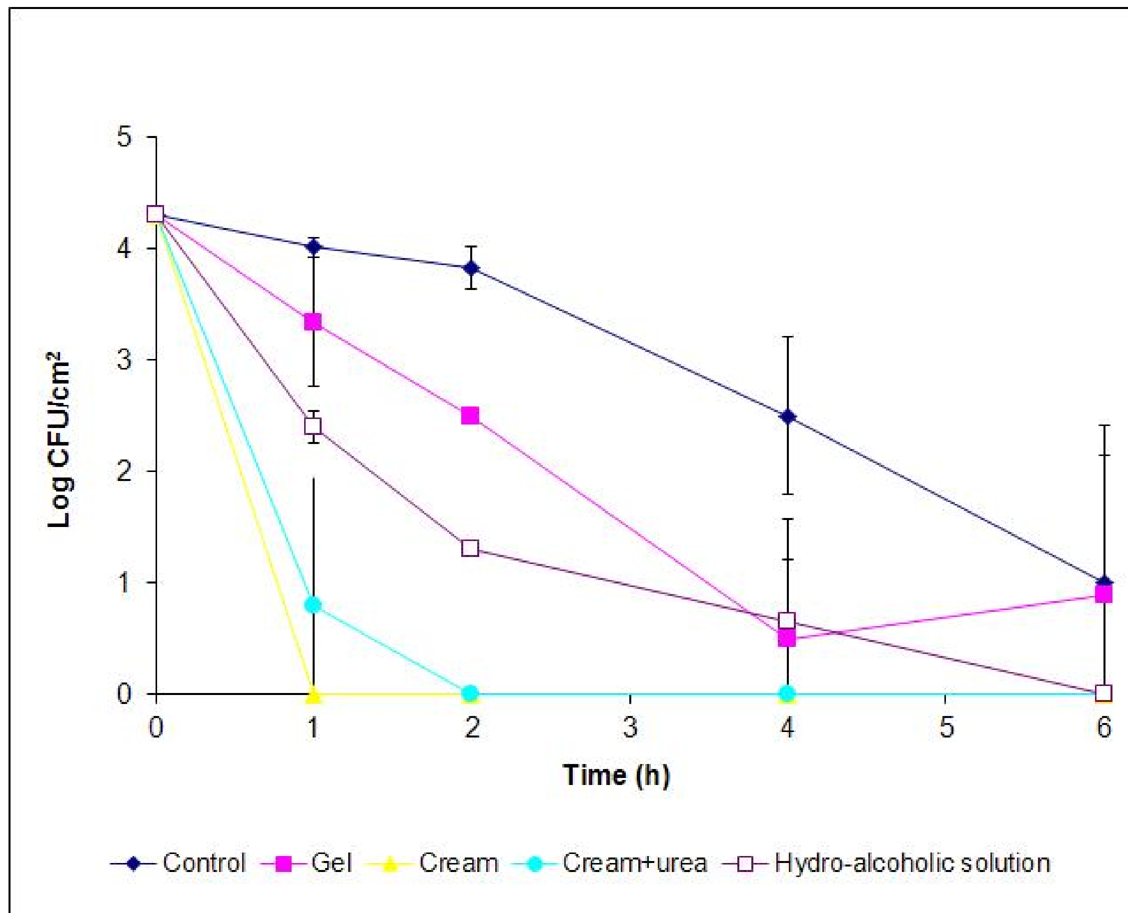
556



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559 **Figure 1.** Activity of four formulations containing AS-48 (10 µg/ml) plus lysozyme (4 mg/ml)
560 applied "*ex vivo*" on previously infected ear skin swine with the P0 strain (10^4 CFU/cm^2).

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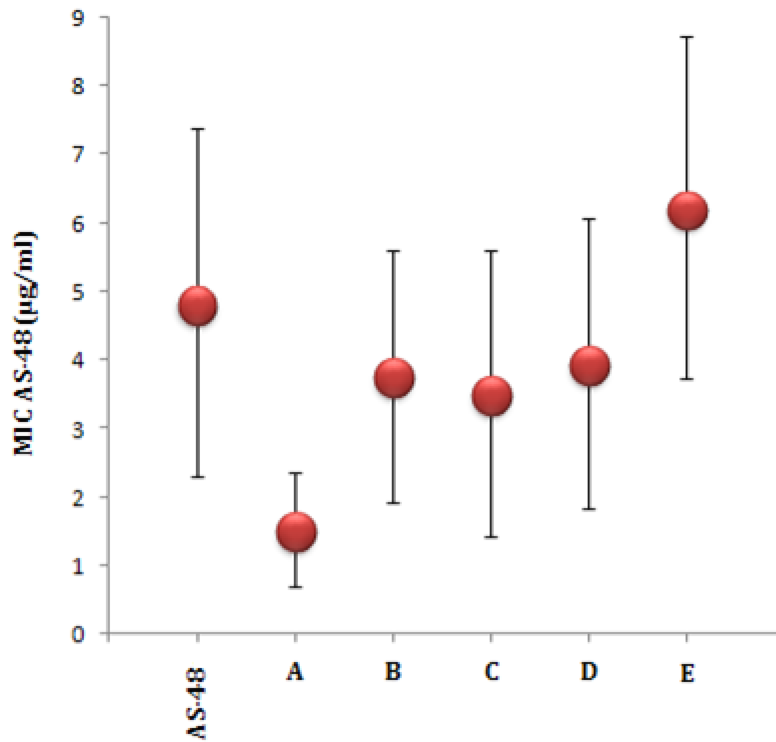

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565 **Figure 2.** Statistic descriptive for AS-48 and the different combinations assayed against the *P.*
566 *acnes* isolates.
567
568

569 Table 1

570 Bacterial strains used in this study.

Strains	Characteristics	Source	
Enterococcus faecalis UGRA-10	AS-48^{+}, AS-48^{R}	*Cebrián et al., 2012*	
Clinical samples	**Source**	**Source**	**GenBank accession number**
P0	Facial acne	CECT 5684	-
P1	Wound exudate from inflammatory acnes (papules or pustules)	VNH	KT957276
P3	Wound exudate from inflammatory acnes (papules or pustules)	VNH	KT957277
P4	Wound exudate from inflammatory acnes (papules or pustules)	VNH	KT957278
P5	Blood	RUH	KT957279
P6	Bone biopsy	RUH	KT957280
P7	Blood	RUH	KT957281
P9	Blood	RUH	KT957282
P10	Pustule exudate	VNH	KT957283
P11	Wound exudate from inflammatory acnes (papules or pustules)	VNH	KT957284
P12	Wound exudate from inflammatory acnes (papules or pustules)	VNH	KT957285
P13	Wound exudate from inflammatory acnes (papules or pustules)	VNH	KT957286
P14	Wound exudate from inflammatory acnes (papules or pustules)	VNH	KT957287
P15	Abscess	VNH	KT957288
P17	Abscess	VNH	KT957289
P18	Abscess	VNH	KT957290
P19	Blood	RUH	KT957291

P20	Blood	RUH	KT957292
P22	Synovial fluid	RUH	KT957293
P23	Blood	RUH	KT957294
P24	Wound exudate from inflammatory acnes (papules or pustules)	VNH	KT957295
P25	Wound exudate from inflammatory acnes (papules or pustules)	VNH	KT957296
P26	Aspirate contamination	VNH	KT957297
P27	Abscess isolation	VNH	KT957298

571
572 VNH=Virgen de las Nieves Hospital. Granada (Spain).
573 RUH= Regional University Hospital of Málaga (Spain).
574

575
576 Table 2
577 Minimal inhibitory concentration (MIC) of the antibiotics tested in this work against the *P. acnes*
578 isolates. Resistance to Ery (EUCAST) (*Song et al., 2011*) and Clin and Tet antibiotic (CSLI,
579 2015), are marked in dark.
580

Strain	**MIC (µg/ml)**		
	Erythromycin R ≥0.5	**Tetracycline** S<4, I=8, R≥16	**Clindamycin** S<2, I=4, R≥8
P0	<0.195	0.781	<0.195
P1	<0.195	0.781	<0.195
P3	**>200**	12.50	**6.250**
P4	<0.195	0.390	<0.195
P5	**1.562**	0.390	<0.195
P6	<0.195	0.390	3.125
P7	<0.195	<0.195	<0.195
P9	<0.195	0.781	<0.195
P10	<0.195	<0.195	<0.195
P11	**>200**	0.390	**100**
P12	**>200**	0.781	**50**
P13	<0.195	0.390	<0.195
P14	<0.195	0.781	<0.195
P15	<0.195	0.781	<0.195
P17	<0.195	<0.195	1.562
P18	<0.195	<0.195	<0.195
P19	<0.195	<0.195	<0.195
P20	<0.195	<0.195	<0.195
P22	<0.195	0.390	<0.195
P23	<0.195	<0.195	<0.195
P24	<0.195	0.390	<0.195
P25	<0.195	0.390	<0.195
P26	<0.195	0.390	0.781
P27	<0.195	<0.195	<0.195

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584 Table 3. Minimal inhibitory concentration (MIC) of AS-48 alone and in combination with
585 different compounds (added at subinhibitory concentrations) carried out in solid medium. A)
586 Lysozyme (8 mg/ml); B) Lauric acid (0.1 mg/ml); C) Palmitic acid (0.1 mg/ml); D) Oleic acid
587 (0.1mg/ml); E) Salicylic acid (2 mg/ml). The synergism is marked in dark
588
589

	AS-48	**+A**	**+B**	**+C**	**+D**	**+E**
P0	4	3	5	2	7	6
P1	2	1,5	2,5	4	1,5	6
P3	2	1,5	2,5	2,5	2	4
P4	3	1	1	5	3	5
P5	12	3,5	5,5	8	12	14
P6	6	2	4	3	1,75	4
P7	5	0,75	3	3	3	6
P9	5	1,5	2,25	2	2,5	6
P10	4	2,5	5	2	4	5
P11	5	1,5	6	4	3	10
P12	12	1,25	4	10	4	2,5
P13	5	3	6	3	5	4
P14	3	0,75	3	3	4	5
P15	3,5	2	5,5	2,5	4	6
P17	5	2	8	2,5	5	4
P18	4,5	1,37	4,5	2,25	5	8
P19	7	1	3	2,25	3	8
P20	5	1,68	2	4,75	3	8,5
P22	5	0,62	3	3	4	8,5
P23	2	1	0,87	0,31	2,5	6
P24	4,5	1	4	6	3	8,5
P25	3,5	1	6	2,75	5	5
P26	4,5	0,625	2	3	4	5
P27	3	0,25	1,5	3	3	4

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593
594 Table 4. No parametric Wilcoxon test for average comparison between AS-48 and the different
595 combinations assayed. A) Lysozyme (8 mg/ml); B) Lauric acid (0.1 mg/ml); C) Palmitic acid
596 (0.1 mg/ml); D) Oleic acid (0.1mg/ml); E) Salicylic acid (2mg/ml). Significant results for *p* value
597 (<0.05) is shown in dark.
598

	AS-48	**A**	**B**	**C**	**D**	**E**
AS-48	--------	0.000	0.076	0.002	0.067	0.003
A	--------	--------	0.000	0.000	0.000	0.000
C	--------	--------	0.501	--------	0.298	0.000

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