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(Article begins on next page)

1 **Organo-modified bentonite for gentamicin topical application: Interlayer structure and in vivo**
2 **skin permeation**

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5

6 **Highlights**

7 An organo-modified raw bentonite was developed as novel antibacterial material.

8 Thermal reactions could support drug intercalation occurrence.

9 MD simulations showed gentamicin monolayer arrangement within Mnt interlayer.

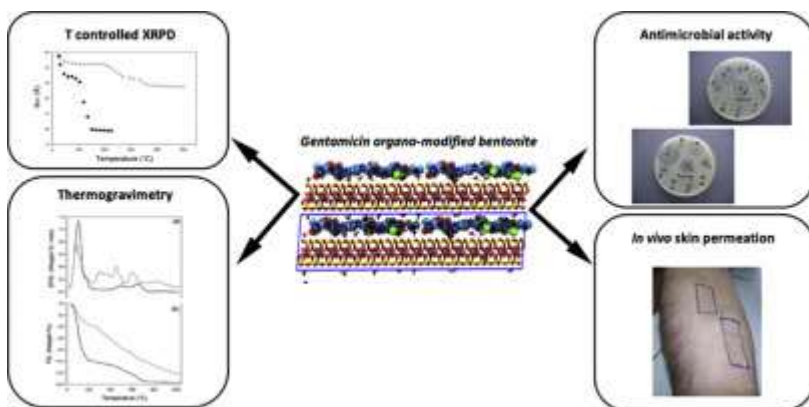
10 *Trans*-epidermal route was favored by drug intercalation as arisen from *in vivo* data.

11

12 **Abstract**

13 Recent [biomedical applications](#) of clay materials have included organically modified clays or clay minerals
14 with the purpose of modifying and improving drug biological activity. The present research aims to explore
15 the potential benefits provided by a raw [bentonite](#) (Bt) modified by gentamicin (GM) adsorbed within
16 [montmorillonite interlayers](#) in the management of cutaneous infectious diseases. Information arisen from
17 controlled X-ray powder diffraction, [thermogravimetry](#) coupled with evolved gas [mass spectrometry](#), and
18 molecular dynamics simulations pointed out GM monolayer arrangement within montmorillonite
19 framework without producing substantial effects on the layer periodicity. Concerning skin biomedical
20 application, unlike the pure antibiotic permeating along the trans-follicular pathway across *stratum*
21 *corneum*, the organo-modified Bt/GM would favor the trans-epidermal route along inter-cluster [corneocyte](#)
22 region, as *in vivo* skin penetration studies by means of tape stripping test indicated. Based on the results
23 obtained, GM [intercalation](#) could represent a potential advantageous approach allowing a long-term
24 Bt/GM reservoir for sustained antibacterial activity.

25 **Graphical abstract**



26

27 **Keywords**

28 Bentonite, Gentamicin, Intercalation, Thermal analyses, Molecular dynamics, *In vivo* skin penetration

29

30

31 1. Introduction

32 There is a strong demand to identify new strategies in order to set optimal drug delivery systems for
33 antibiotic treatments. [Intercalation](#) of organic molecules into layered inorganic solids provides a useful and
34 convenient approach to prepare hybrids that show properties of both the inorganic host and organic guest
35 in a single material ([Aguzzi et al., 2007](#); [Rodrigues et al., 2013](#)). In the last five decades the ability of both
36 raw and synthetic [smectites](#) to exchange cations with several organic compounds has been exploited in
37 many application fields. An archetypical example of such [versatility](#) is represented by the polymeric
38 [nanocomposites](#) employing organo-modified [bentonites](#) ([Benelli et al., 2017](#); [Franchini et al., 2008](#); [2011](#);
39 [Morgan and Wilkie, 2007](#)).

40 More recently, smectites have been proposed as materials for modulating drug delivery or improving
41 dissolution of poorly water-soluble drugs ([Aguzzi et al., 2005](#); [Iannuccelli et al., 2015](#); [Joshi et al., 2009](#)).
42 Among smectites, the 2:1 layered montmorillonite is probably the most investigated clay mineral. The
43 reasons that drive this interest mainly arise from its high specific surface area, swelling and [adsorptive](#)
44 [capacity](#), high cation exchange capacity (CEC), specific rheological properties, drug-carrying capability and
45 ability to modulate drug release ([World Health Organization, 2005](#)). Montmorillonite is mainly used as
46 auxiliary material in the [pharmaceutical industry](#) for oral or topical dosage forms, recorded in the United
47 States, European, and British Pharmacopeias. Montmorillonite, following to its high [swelling behavior](#), can
48 intercalate therapeutic compounds between the layers generating a host for oral or topical drug delivery
49 ([Aguzzi et al., 2005](#); [Bello et al., 2015](#); [Bonina et al., 2007](#), p. 200; [de Paiva et al., 2008](#); [Forni et al., 1987](#);
50 [Iannuccelli et al., 2015](#); [Iliescu et al., 2011](#); [Joshi et al., 2009](#); [Kant and Datta, 2016](#); [Katti et al., 2010](#); [Kim et](#)
51 [al., 2016](#); [Mohamed et al., 2014](#); [Rapacz-Kmita et al., 2015](#)). Concerning topical use, montmorillonite has
52 beneficial effects in dermatological and cosmetic applications (geotherapy, paleotherapy) ([Carretero, 2002](#);
53 [López-Galindo et al., 2007](#)).

54 The present work focuses on the assessment of a raw bentonite (Bt), a montmorillonite rich clay recently
55 characterized in a previous work ([Iannuccelli et al., 2016](#)), for the development of a novel gentamicin/clay
56 hybrid material for the topical use. Gentamicin (GM) is an aminoglycoside antibiotic widely used in the
57 treatment of severe infections, caused by many Gram-negative and Gram-positive bacteria, such as
58 meningitis, nephritis, and post-operative infections. Although it presents a very broad spectrum of action,
59 its use is limited to serious infections caused by Gram-negative bacteria because of its high toxicity.
60 Gentamicin is commonly administered as injections, topical and ophthalmic dosage forms because of poor
61 absorption following the oral administration. The well-known poor gastrointestinal membrane permeability
62 and the consequent low [bioavailability](#) (class III of the biopharmaceutical classification system) are likely
63 connected to the high polarity of this cationic compound. Various approaches have been investigated in
64 order to increase GM oral bioavailability, including the co-administration of absorption-enhancing agents
65 such as surfactants ([Hu et al., 2001](#); [Ito et al., 2005](#)), bile salts and glucosteroids ([Axelrod et al., 1998](#)), and
66 liposaccharides ([Ross et al., 2004](#)). Although good gastrointestinal absorption enhancing effects were
67 demonstrated, cytotoxicity and damage to the mucosa have been reported ([Aungst, 2000](#); [Ross et al., 2004](#);
68 [Swenson et al., 1994](#)). Another strategy aiming to promote GM oral bioavailability could involve the use of
69 microparticulate carriers to be taken up by the intestinal lymphoid tissue ([des Rieux et al., 2007](#); [Hussain et](#)
70 [al., 2001](#); [Iannuccelli et al., 2011](#); [McClellan et al., 1998](#); [Moyes et al., 2007](#)) or to be implanted for bone
71 infection treatment also exploiting drug interaction with anionic polymers ([Iannuccelli et al., 1996, 2011](#)).
72 Gentamicin is extensively used topically against severe microbial infections especially in burns and wounds
73 ([Chang et al., 2006](#)), but also in the treatment of impetigo, infected bed sores, nasal staphylococcal carrier
74 state, pyoderma, infections of the external eye, and adnexa ([Nishijima and Kurokawa, 2002](#)). Gentamicin
75 applied to the skin has only a low systemic absorption due to the difficult penetration through the deep
76 layers of the skin, related, probably, to its cationic nature; for this reason, its use is limited to the local
77 effect that involves mainly the most superficial skin layers. Despite its benefits, GM short-life, bacterial

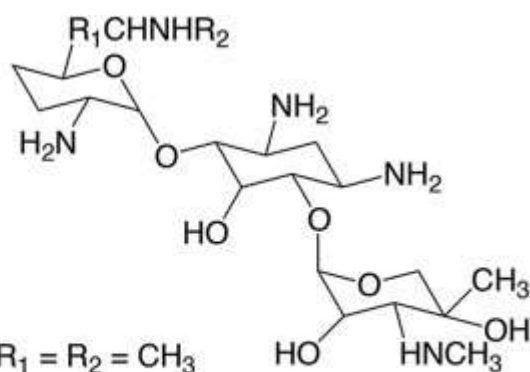
78 barriers and adverse effects such as nephrotoxicity, ototoxicity, and neurotoxicity upon prolonged use limit
 79 GM daily dosage (Roberts, 2007). In fact, many clinicians are reluctant to use it, even for a short term
 80 (Drusano, 2007). Efforts have been made to reduce toxicity associated with prolonged use by means of
 81 liposomes, [micellar systems](#), hydrogels, [microgels](#), or [nanospheres](#) (Ahangari et al., 2013; Ayhan and Ozkan,
 82 2007; Changez et al., 2003; Eljarrat-Binstock et al., 2004; Jia et al., 2008; Nnamani et al., 2013; Sökmen et
 83 al., 2008; Umeyor et al., 2012). Local delivery of GM can solve the major disadvantages of the [systemic](#)
 84 [administration](#) by maintaining a high local antibiotic concentration for an extended time (Zalavras et al.,
 85 2004). Particularly, drug delivery systems exhibiting high initial release rate followed by a sustained release
 86 at an effective antibiotic concentration may allow local control of infection while minimizing side effects
 87 and preventing bacterial resistance (Aviv et al., 2007; Persson et al., 2006).

88 The preparation of a GM-based organo-modified bentonite (Bt/GM) may therefore represent a valuable
 89 alternative to assure safer and more effective utilization of GM for topical treatment. Based on these
 90 premises, the present research includes a thorough characterization of Bt/GM by means of several
 91 instrumental analyses as well as the comparison of the experimental results with Molecular Dynamics
 92 simulations (MD modeling) to provide a more detailed understanding about the [interlayer](#) arrangement
 93 and interactions promoted by the organic guest molecules confined in the montmorillonite framework.
 94 Moreover, GM [antimicrobial activity](#), *in vitro* desorption, and *in vivo* skin [permeation](#) on human beings
 95 were assessed in the perspective of contribution to a novel antibiotic material.

96 2. Experimental part

97 2.1. Materials

98 A [bentonite](#) (Bt) of volcanic origin from Iglesias (Sardinia, Italy) deposit (average [mineralogical composition](#)
 99 from the producer's [datasheet](#): [montmorillonite](#) 80%, quartz 13%, illite-kaolinite 5%, [plagioclase](#) 2%) was
 100 donated by Eurit srl (Colorobbia Group, Sovigliana Vinci, Italy). Gentamicin sulfate (GM, [Fig. 1](#)), composed
 101 of gentamicin C1 ($C_{21}H_{43}N_5O_7 \cdot H_2SO_4$, <45%), gentamicin C1a ($C_{19}H_{39}N_5O_7 \cdot H_2SO_4$, <35%), and gentamicin C2
 102 ($C_{20}H_{41}N_5O_7 \cdot H_2SO_4$, <25%), pKa = 12.55 in acidic condition; 10.18 in basic condition, was purchased by
 103 Polichimica (Bologna, Italy). All the chemicals and reagents were of analytical grade (Sigma-Aldrich, Milan,
 104 Italy).



C₁ (< 45%) R₁ = R₂ = CH₃

C₂ (< 25%) R₁ = CH₃; R₂ = H

C_{1a} (< 35%) R₁ = R₂ = H

105

106 Fig. 1. Molecular structure of gentamicin [sulfate](#).

107

108 2.2. Bt activation

109 Bt activation and thus the implementation of its organophilic behavior are provided by the saturation of the
 110 montmorillonite [interlayers](#) with a homogeneous cationic population through the cation exchange reaction

111 hereafter detailed. A defined amount of Bt was grinded by a vibratory [ball mill](#) (Fritsch GmbH, Idar-
112 Oberstein, Germany) for 10 h to remove particle aggregates. Batches of dispersions were prepared mixing
113 1 g of milled Bt and 25 mL of NaCl 0.1 M and were shaken with a [magnetic stirrer](#) at room temperature for
114 24 h. The supernatant was centrifuged (mod. 4235, 188 ALC International, Milan, Italy) at 2115 ×g for
115 20 min and the solid was twice subjected to the same treatment. The separated solids were washed several
116 times with 35 mL of distilled water under magnetic stirring at room temperature for 4 h followed by
117 centrifugation at 2115 ×g for 2 h. The solid was dried under vacuum at room temperature and the
118 supernatant analyzed for NaCl absence by [titration](#) with 0.1 M [silver nitrate](#) solution according to [U.S.](#)
119 [Pharmacopeia](#). The [activation process](#) was carried out in triplicate.

120 **2.3. Bt/GM preparation**

121 Gentamicin was adsorbed onto both activated and non-activated Bt at constant drug concentration
122 corresponding to about two times Bt CEC measured for activated Bt/GM (aBt/GM) and non-activated
123 Bt/GM (Bt/GM), respectively. Glass tubes filled with 20 mL GM water solution (1 mg/mL) and 100 mg of
124 milled Bt were horizontally shaken in the darkness for 24 h, a time suitable to fully saturate the
125 montmorillonite interlayer with GM. The dispersions were centrifuged (2115 ×g, 20 min) and solids washed
126 twice with 35 mL [deionized water](#) under magnetic stirring for 15 min. The obtained organo-modified clays
127 were dried under vacuum at room temperature and stored in the darkness. Three batches were prepared
128 for each sample.

129 **2.4. Gentamicin adsorption measurements**

130 In this paper, the term “adsorption” was used to generally refer to the [immobilization](#) of GM onto Bt thus
131 without distinguish between [intercalation](#) in the interlayer of montmorillonite and adsorption on the outer
132 surface of montmorillonite and illite-kaolinite. However, when dealing with each single mineral phase the
133 term adsorption and intercalation will be suitably used.

134 The amount of GM adsorbed onto Bt in both aBt/GM and Bt/GM was calculated as the difference between
135 the initial GM concentration and that in the supernatants obtained during organo-modified clay
136 preparation. GM was derivatized by reaction with *o*-phtaldialdehyde of an aliquot of 1 mL from the
137 supernatants according to Sampath and Robinson method ([Sampath and Robinson, 1990](#)) and determined
138 spectrophotometrically (Lambda 3B, Perkin-Elmer, Norwalk, CT, USA) at 274 nm wavelength. The GM
139 concentrations were expressed as drug/clay weight percentage as well as yield (actual/theoretical drug)
140 percentage on three determinations from three different batches.

141 **2.5. Size, surface charge, and pH value**

142 Bt/GM particle size, [Polydispersity Index](#) (PDI), and Z-potential were determined on 10 mg/mL organo-
143 modified [clay water](#) dispersion by using Photon Correlation [Spectroscopy](#) (PCS) (Zetasizer version 6.12,
144 Malvern Instruments Ltd) equipped with a 4 mW He—Ne laser (633 nm) and a DTS software (Version 5.0)
145 and compared with those of Bt. pH value of 2% Bt/GM water dispersion was measured by [potentiometry](#)
146 immediately after the preparation of the dispersion and after 1 h; obtained data were compared with the
147 value of Bt dispersion, according to U.S. Pharmacopeia monograph for bentonite, and with 0.1% (w/v) GM
148 water solution. The reported values were averaged on three determinations from three different batches.

149

150 **2.6. CHN elemental microanalysis**

151 CHN elemental [microanalysis](#) (Elemental analyzer, mod. 1106, Carlo Erba, Milan, Italy) was performed on
152 Bt/GM in comparison with bulk Bt and GM. The analysis was carried out in triplicate.

153 2.7. Elemental composition by EDX analysis

154 Clay elemental composition was determined by Energy Dispersive X-ray (EDX, Oxford INCA-350, FEI
155 Company-Oxford Instruments, Oregon, USA) analysis coupled with an Environmental Scanning Electron
156 Microscopy (ESEM, Quanta 200 Fei Company-Oxford Instruments). Elements can be identified qualitatively
157 and semi-quantitatively in function of the X-ray energy emitted by their electrons transferring from a higher
158 energy shell to a lower energy one. X-ray emission from $K\alpha$ or $K\beta$ levels of the atoms calcium, potassium,
159 oxygen, sodium, magnesium, aluminum, silicon, and other elements with atomic numbers from 4 were
160 recorded by the selected area method related to whole clay particles from samples mounted without a
161 [conductive coating](#) on carbon stubs with the following experimental settings: low vacuum (0.70 Torr),
162 [accelerating voltage](#) 12 kV, spot size 3, element detection limit ~ 0.05 wt%, spatial resolution $0.1 \mu\text{m}$, total
163 spectrum counts $>250,000$, accuracy within $\pm 5\%$ relative errors by reference to standards. [EDX spectra](#)
164 representing the plots of X-ray counts (intensity) vs. energy peak (keV) of each element were acquired and
165 semi-quantitative compositions, obtained by a standardless method of acquisition and expressed as
166 relative weight percentage of each element, were calculated. The carbon peak at low energy level, related
167 to the hydrocarbon contamination growing the carbon stub signal, was not considered ([Rolland et al.,](#)
168 [2004](#)). The reported data were averaged on three determinations for each sample.

169 2.8. FT-IR measurements

170 FT-IR measurements were performed using a Perkin-Elmer FT-IR 1600 (abscissa accuracy of 0.01 cm^{-1} using
171 HeNe laser reference; resolution from 2 to 16 cm^{-1} ; lithium [tantalite](#) temperature-stabilized detector) on
172 bulk GM, bulk Bt, Bt/GM, and Bt/GM physical mixture at GM content corresponding to that of the organo-
173 modified clay. The samples were maintained in a drier, dispersed in a Nujol mull (typically 2% w/w) and
174 measured. Spectra were collected in air in the MID Infra-Red (mid-IR) region. The analyses were performed
175 in triplicate.

176 2.9. X-ray powder diffraction

177 X-ray powder diffraction (XRD) was employed mainly to detect basal periodicity variation in
178 montmorillonite before and after treating Bt with GM. The XRD patterns were recorded from (00 l) oriented
179 mounts, in the temperature range from 25 to $500 \text{ }^\circ\text{C}$ with a [heating rate of](#) $10 \text{ }^\circ\text{C}/\text{min}$ using a PANalytical
180 X'Pert PRO [diffractometer](#) equipped with X'Celerator detector. Before measurements all the samples were
181 simultaneously equilibrated at the same environmental conditions. Experimental conditions were: Incident
182 beam: monochromatic $\text{Cu } K\alpha_1$ radiation (1.54060 \AA), 40 kV and 40 mA; filter, nickel; Soller slits, 0.04 rad ;
183 anti-scatter mask, 20 mm; anti-scatter slit, $1/4^\circ$; divergence slit, $1/4^\circ$. [Diffracted beam](#): X-ray detector,
184 X'Celerator (Position Sensitive Detector, PSD); anti-scatter mask, 5.0 mm; Soller slits, 0.04 rad ; integration
185 time, 20 s in continuous scanning (PSD length of $2.12^\circ 2\theta$ corresponding to a step size of $0.0170^\circ 2\theta$).
186 [Diffraction patterns](#) were recorded from 3 to $75^\circ (2\theta)$ at room temperature, and from 3 to $20^\circ (2\theta)$ when
187 measuring in non-ambient temperature conditions. NIST corundum was used as calibrating standard.

188 2.10. Thermogravimetric measurements coupled with evolved gas mass spectrometry

189 Thermogravimetric analyses were performed with a Seiko SSC 5200 thermal analyzer equipped with a
190 [quadrupole mass spectrometer](#) (ESS, GeneSys Quadstar 422) to analyze gases evolved during [thermal](#)
191 [reactions](#) (MSEGA). This device samples gases *via* an inert, fused silicon capillary system, heated to prevent
192 gas condensation. Analyses of evolved gas phases were carried out in multiple ion detection mode (MID),
193 which allows the qualitative determination of evolved masses vs. temperature or time. MID analyses were
194 carried out measuring the m/z ratios 17 and 18 for H_2O , 28 and 44 for CO_2 , 30 for NO and NO_2 , 34 for H_2S ,
195 46 for NO_2 , and 48, 64, 66 for SO_2 , where m/z is the ratio between the mass number and the charge of an
196 ion; SEM (Secondary Electron Multiplier) and FARADAY detectors operating at 900 V were employed with
197 1 s of integration time on each measured mass.

198 Measurements were performed on Bt, Bt/GM, and bulk GM air-dried samples at the following experimental
199 conditions: heating rate: 10 °C/min for Bt and Bt/GM, and 20 °C/min for pure GM; heating range: 25–
200 1000 °C (25–900 °C for pure GM); data measurement: every 0.5 s; purging gas: ultrapure helium, at a flow
201 rate of 100 µL/min.

202

203 **2.11. Differential scanning calorimetry**

204 Bt/GM was subjected to thermal analysis on a Differential Scanning [Calorimeter](#) (DSC-4, Perkin-Elmer) and
205 compared with GM and Bt/GM physical mixture at GM content corresponding to that of the organo-
206 modified clay. The samples (6–7 mg) were accurately weighed in crimped aluminum pans and heated from
207 30 °C to 280 °C at a scanning rate of 10 °C/min under dry nitrogen flow (30 mL/min). All the thermograms
208 were obtained in triplicate.

209 **2.12. Molecular dynamics simulations**

210 A $X_{0.75}[\text{Si}_{7.75}\text{Al}_{0.25}][\text{Al}_{3.5}\text{Mg}_{0.5}]\text{O}_{20}(\text{OH})_4$ (X = monovalent cation) ideal montmorillonite model was used for the
211 present study. This model shows $-0.75e$ per unit cell, leading to a CEC of 101.9 meq/100 g, a value close to
212 the experimental one. The simulation box consists of one layer made of 64 unit-cells corresponding to a
213 basal surface of 4.14 nm × 7.17 nm. In order to mimic the partial cation exchange process obtained in the
214 experiments, we consider the total negative charge of the layer to be counterbalanced by ~79.2% by C-
215 1(GM) molecules and ~20.8% of sodium ions (see Supporting information for details). A certain amount of
216 water molecules was also inserted in the interlayer (corresponding to the 2% wt.). In order to
217 accommodate the organic cations, the [interlayer distance](#) was pre-expanded to 2.5 nm and the C-1 and
218 water molecules and Na ions were randomly inserted. An initial [energy minimization](#) and MD simulation in
219 the NPT-ensemble of 1 ns (298 K, 1 atm, time step 1 fs) have been used to optimize the interlayer region
220 reaching an equilibrium density. Different initial starting configurations have been tested (in terms of
221 organic, water, and sodium ions placement) and in all cases for a given system they converge to similar final
222 arrangements and layer-to-layer distances. Then, the ones with the lowest energy have been further
223 simulated for 1 ns in the NVT-ensemble. The last 0.5 ns have been used for the data analyses in terms of
224 density profiles, pair correlation functions, ion coordinations. In all the simulations, both the atoms of the
225 montmorillonite layer as well as the atoms confined in the interlayer were allowed to move and the
226 periodic boundary conditions were applied. MD simulations were performed using the Discover module of
227 the Materials Studio package (v. 5.0, Accelrys Inc.).

228 **2.13. Antimicrobial activity**

229 A microbiological agar well diffusion method was performed ([Giamarellou et al., 1975](#)) on Bt/GM in
230 comparison with the respective physical mixture as well as pure GM and Bt. Tryptic soy agar (15 mL) and
231 [Staphylococcus aureus](#) (*S. aureus*) strain ATCC 6538 or [Pseudomonas aeruginosa](#) (*P. aeruginosa*) strain ATCC
232 27853 (10^5 CFU/mL) were used as growth medium and indicator [microorganisms](#), respectively. The wells in
233 [agar plates](#) were filled with the samples in water dispersions (5 µg/mL, 100 µL). The glass plates were
234 incubated at 37 °C overnight and zone inhibition diameters determined and related to GM concentration of
235 standard water solutions. The analyses were made in triplicate.

236 **2.14. In vitro gentamicin desorption**

237 Gentamicin dissolution and desorption from the organo-modified clay were examined under sink
238 conditions using the flow-through cell, USP Apparatus 4 (Disotest CE-1, Sotax, Basel, Switzerland) on
239 exactly weighed samples in 100 mL of phosphate buffer solutions (pH 5.4 or 7.4 according to the European
240 Pharmacopeia) at a temperature of 37.0 ± 0.5 °C under a flow rate of 25 mL/min. The dissolved or desorbed
241 concentration of drug was determined spectrophotometrically (Lambda 3B, Perkin-Elmer) following

242 derivatization according to Sampath and Robinson ([Sampath and Robinson, 1990](#)) at fixed time intervals for
243 3 h. The reported data were averaged on three determinations.

244 **2.15. *In vivo* skin permeation**

245 *In vivo* skin [permeation](#) study was carried out following the application of Bt/GM, Bt, and GM, mechanically
246 mixed into petroleum jelly immediately before skin application, on both the two shaved volar forearms and
247 forehead of 3 healthy Caucasian volunteers (2 females and 1 male, aged 20–62), free of any dermatological
248 disorder after obtaining informed consent for the experimentation following the recommended guidelines
249 as set out in the Declaration of Helsinki. A delineated area of 2 × 5 cm received 200 mg of each formulation
250 containing pure GM (4 mg), Bt/GM (65 mg) both corresponding to GM dose of 0.4 mg/cm², or pure Bt
251 (65 mg). The samples were homogeneously distributed by means of rubber gloves. After an application
252 time of 60 min, which had been found to have a predictive value for penetration resulting from longer
253 times of application ([Howes et al., 1996](#)), the *stratum corneum* (SC) was stripped twelve times by using an
254 [adhesive tape](#) (Scotch Film Tape 600-3M). This number of stripped tapes is considered proper by Food and
255 Drug Administration bioequivalence guidelines ([Shah et al., 1998](#)). The tapes were applied to the skin with a
256 constant pressure by a 500 g roller. The first stripped tape was not considered in the penetration study
257 because it represents unabsorbed materials. Twelve tapes stripped from SC that has received pure
258 petrolatum jelly as well as from untreated SC were also assayed as controls. The tapes were combined into
259 2 groups (group 1: tapes 2–6; group 2: tapes 7–12) in order to increase determination sensitivity and
260 subjected to an extraction procedure by [isopropyl alcohol](#) to determine GM according to the method
261 described above. Data were expressed in penetrated GM percentage of the applied dose. A further tape-
262 stripping test was conducted on the volunteers under the same conditions after a resting period of 14 days.
263 Tapes n. 2, 6, and 12 were assayed by Energy Dispersive X-ray (EDX) analysis coupled with an Environmental
264 Scanning Electron Microscopy (ESEM) using the selected area method. An area of 1.25 cm² of each tape
265 was cut from the center of the tape, mounted without conductive coating on a carbon stub. X-ray emission
266 from K α and K β levels of the atoms carbon, oxygen, aluminum, silicon, and sulfur were registered under the
267 experimental setting described above. EDX spectra representing the plots of X-ray counts vs. elements and
268 semi-quantitative results expressed as relative weight percentage of the elements present in the specimen
269 were obtained. The reported data were averaged on the results obtained from the volunteers.

270 **2.16. Statistical analysis**

271 Data obtained were evaluated from a statistical point of view using ANOVA one-way. Differences at *p*-
272 values < .05 were considered significant.

273 **3. Results and discussion**

274 Adsorption of GM onto [Bt](#) is essentially driven by the CEC of the clay that is related to isomorphous
275 substitution in octahedral and tetrahedral sheets of [montmorillonite](#). To modulate drug [bioavailability](#)
276 through interactions with bentonite (Bt), the cationic gentamicin (GM) was selected. Each of the three
277 major components of GM complex (C1, C1a, C2) contains five basic amino functions exhibiting change in
278 [protonation](#) state of the amino groups as function of pH. At the acidic pH value of Bt/GM preparation, GM
279 molecules carry almost fully protonated charges (+5 and +4) ([Lesniak et al., 2003](#)) that are appropriate for
280 efficacious cation exchange on montmorillonite. The Bt selected to develop Bt/GM organo-modified clay
281 was previously characterized providing information on mineralogical and physico-chemical features
282 ([Iannuccelli et al., 2016](#)). Gentamicin capacity to be intercalated in a commercial montmorillonite to be
283 used as general drug carrier has been demonstrated by [Rapacz-Kmita et al. \(2015, 2017\)](#). In the present
284 work, more extensive knowledge about the specific interactions between GM and Bt was supplied
285 providing information on the arrangement of the guest molecule in the montmorillonite [interlayer](#) as well
286 as the potentiality of a topical application.

287 **3.1. Interlayer structure**

288 The first step of the research was to verify the usefulness of Bt activation procedure. Clay activation did not
 289 offer advantages in terms of GM adsorption extent (aBt/GM = 4.49 ± 0.56%, yield% = 25.13 ± 0.62;
 290 Bt/GM = 7.16 ± 0.91%, yield% = 35.29 ± 0.51). Therefore, the study was performed only on the non-
 291 activated Bt/GM.

292 Bentonite treatment with GM generated an increase of clay average particle size ($p > .05$) (Table 1) though
 293 remaining in the size range considered proper for several dermatological and cosmetic purposes (Lein and
 294 Oussoren, 2015). Both Bt and Bt/GM exhibited negative surface charge with a greater magnitude in Bt
 295 compared to Bt/GM ($p < .05$) (Table 1). Bentonite net surface charge is mainly due to montmorillonite pH-
 296 independent permanent structural charges accounting for 90–95% of the total charges (Au and Leong,
 297 2016; Pecini and Avena, 2013). Furthermore, montmorillonite is characterized by a surface charge due to
 298 the hydrolysis of Si—O and Al—OH bonds on the external surfaces of tetrahedral sheets as well as along
 299 the edges. Consequently, the lower Bt/GM Z-potential in absolute value is probably ascribable to GM
 300 interactions with the edges of clay particles and/or acidic pH medium of GM water solution (Delgado et al.,
 301 1986; Furukawa et al., 2009). Upon contact with water, Bt provided alkaline dispersions (pH of about 9,
 302 Table 1), in agreement with the values required by both U.S. and European Pharmacopeias. Alkalinity, that
 303 remained unchanged after 1 h, is generated by quick diffusion from the interlayer surfaces of exchangeable
 304 Na⁺ ions retained by electrostatic attraction. Conversely, Bt/GM water dispersions exhibited pH values
 305 consistent with those of pure GM suggesting the occurred exchange process between montmorillonite
 306 interlayer cations and GM.

Table 1
 Size, polydispersity index (PDI), surface charge, and pH values of Bt, GM, and Bt/GM. Mean values ± SD.

Sample	Z-average (µm) ± SD	PDI	Z-potential (mV) ± SD	pH value
GM	—	—	—	4.52 ± 0.05
Bt	3.64 ± 0.98	0.42 ± 0.15	-28.40 ± 0.98	9.43 ± 0.48 (time = 0) 9.30 ± 0.07 (time = 1 h)
Bt/GM	6.82 ± 0.75	0.62 ± 0.12	-17.60 ± 1.30	4.66 ± 0.13 (time = 0) 4.91 ± 0.10 (time = 1 h)

307

308 In order to define the arrangement of GM molecules within montmorillonite lattice and elucidate the
 309 interaction mechanism occurring in Bt/GM, a suite of analyses was performed.

310 Elemental CHN analysis carried out on Bt/GM showed a C/N ratio in nice agreement ($p < .05$) with that of
 311 GM alone (Table 2) and therefore evidenced that the drug was present in the organo-modified clay.

Table 2
 CHN microanalysis and C/N ratios of Bt, GM, and Bt/GM. Mean values ± SD.

Sample	C%	H%	N%	C/N
Bt	—	0.90 ± 0.21	—	—
GM	51.80 ± 1.05	8.91 ± 0.52	15.12 ± 0.83	3.43 ± 0.11
Bt/GM	2.28 ± 0.20	1.26 ± 0.23	0.63 ± 0.005	3.62 ± 0.30

312

313 The occurrence of a possible exchange process between GM and the exchangeable cations of
 314 montmorillonite as a preliminary evidence of GM intercalation was provided by Energy Dispersive X-ray
 315 (EDX) analysis carried out on Bt/GM compared with Bt. The identification and relative quantification
 316 analysis (Table 3, see Fig. S1) showed the Si and Al elements of tetrahedral and octahedral sheets,
 317 respectively, as well as Mg due to Al partial isomorphous substitution. Isomorphous substitutions in the
 318 octahedral sheets create an excess of negative structural charge within the lattice that is balanced by
 319 inorganic cations (mainly Na⁺ and Ca²⁺). A significant ($p < .05$) less abundance of Ca²⁺ ions in Bt/GM
 320 compared with those of Bt suggests the almost complete substitution of Ca²⁺ ions by the cationic form of
 321 GM and, consequently, a possible drug arrangement within montmorillonite interlayers. The detection of a

322 minimal content of S in the mixture suggests, however, a non-negligible intercalation of GM without losing
323 the [sulfate](#) group.

Table 3

EDX semi-quantitative analysis of the elements present in Bt and Bt/GM samples. All data are expressed as relative percentage of the elements > 0.5%. Mean values \pm SD.

	Bt	Bt/GM
O	32.25 \pm 3.24	59.48 \pm 0.63
Na	1.13 \pm 0.73	0.90 \pm 0.25
Mg	0.79 \pm 0.59	0.71 \pm 0.20
Al	11.01 \pm 0.76	7.32 \pm 0.23
Si	46.92 \pm 2.42	27.99 \pm 0.45
S	–	0.26 \pm 0.20
K	2.38 \pm 0.60	1.47 \pm 0.17
Ca	2.30 \pm 0.59	0.44 \pm 0.16
Fe	3.22 \pm 0.86	1.43 \pm 0.27

324

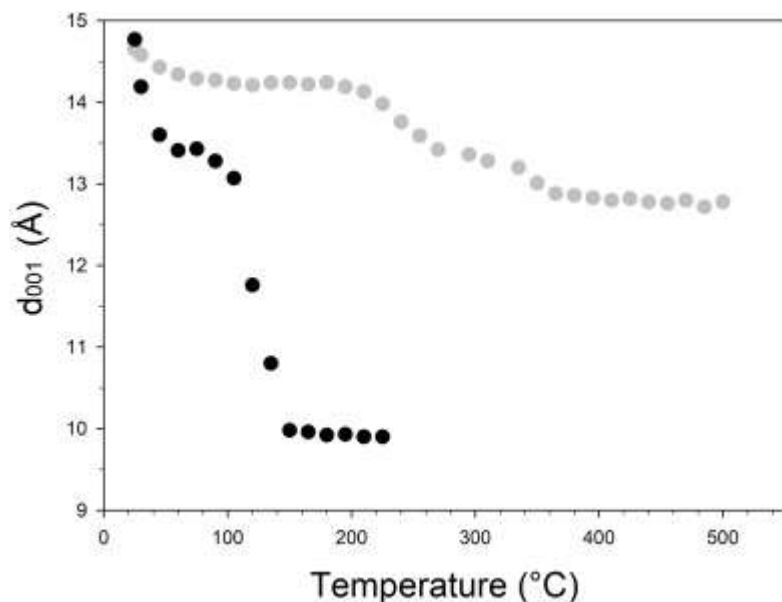
325 Nonetheless, it has to be considered that GM could interact also with additional negative polar sites at the
326 broken edges as well as by exposed hydroxyl end-groups on the terminated planes.

327 Another preliminary evidence was provided by mid-IR spectroscopy (see Fig. S2). The most relevant feature
328 of the mid-IR spectra is the band at about 3400 cm^{-1} , related to the overlapping of asymmetric ν_3 and
329 symmetric $\nu_1(\text{H}\text{---}\text{O}\text{---}\text{H})$ stretching vibrations of water bound by [hydrogen bonds](#) in the interlayer
([Farmer, 1974](#)). Unlike in Bt and Bt/GM physical mixture, the band position in the Bt/GM shift to a lower
330 value, suggesting a decrease of the coordinated water amount induced by the substitution of the
331 exchangeable cations of montmorillonite by GM. This conclusion is supported by the shift of the shoulder at
332 about 3250 cm^{-1} related to the [overtone](#) ($2\nu_2$) of the bending mode of the solvated water ([Farmer, 1974](#))
333 and by the positions of the [absorption bands](#) at about 1635 cm^{-1} attributable to $\nu_2(\text{H}\text{---}\text{O}\text{---}\text{H})$ [bending](#)
334 [vibration](#). The position of this band is the same in Bt and Bt/GM physical mixture ($\sim 1637 \text{ cm}^{-1}$), it is instead
335 moved to a lower value ($\sim 1628 \text{ cm}^{-1}$) in the Bt/GM suggesting a decrease of the coordinated water amount
336 as demonstrated also by [Madejová et al. \(2002\)](#) in samples where the amount of water has been
337 decreased. Other features of the mid-IR spectra can be related both to montmorillonite and to other
338 mineralogical phases as well ([Iannuccelli et al., 2016](#)). More in detail we refer to the bands at about
339 ([Madejová and Komadel, 2001](#)): i) 3692 and 3620 cm^{-1} related to OH stretching of structural hydroxyl
340 groups of kaolinite and montmorillonite, respectively; ii) 916 cm^{-1} , a band related to (AlAlOH) bending in
341 montmorillonite; iii) 1100, 795 and 690 cm^{-1} related to the Si–O vibration of quartz; iv) 1030 cm^{-1} related
342 to Si–O stretching vibration in montmorillonite. These features, however do not significantly change in Bt,
343 Bt/GM and Bt/GM physical mixture suggesting that they are unaffected by the presence of the antibiotic.

345 The main technique for detecting structural variations such as changes in the basal periodicity of
346 montmorillonite, is obtained by [XRD](#). The comparison between XRD pattern of Bt and Bt/GM did not
347 highlight considerable differences except for a minor reduction of d_{001} value in Bt/GM montmorillonite
348 ($d_{001} = 14.19 \text{ \AA}$) respect to Bt montmorillonite ($d_{001} = 14.95 \text{ \AA}$). Hence, temperature controlled XRD analysis
349 was performed to acquire more information about GM arrangement within montmorillonite framework. In
350 fact, in montmorillonite the thickness of the interlayer depends greatly on the type of occupying molecules
351 (e.g., cations, organic molecules, etc.) and on the amount of solvating water. Conversely, by measuring the
352 variation of the distances between the stacked layers along the c axis (*i.e.*, the d values of the (001)
353 reflections) as a function of temperature, information can be acquired on the thermal stability of the
354 molecules occupying the interlayer.

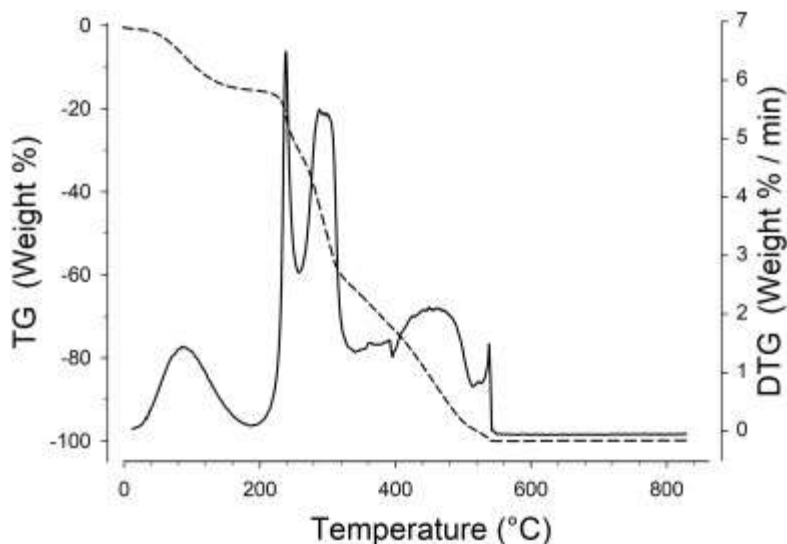
355 [Fig. 2](#) compares the trends of d_{001} reflections of montmorillonite in Bt and Bt/GM samples. The most
356 significant data of the comparison is the persistence of the periodicity of Bt/GM montmorillonite despite

357 the rapid decrease observed for Bt montmorillonite. In fact, at 150 °C Bt montmorillonite interlayer
358 exhibited the typical value of the completely dehydrated interlayer ($d_{001} = \sim 10 \text{ \AA}$), and this value did not
359 change until the occurring of framework collapse at about 600 °C. Conversely, the reduction of the
360 interlayer spacing in Bt/GM montmorillonite only starts after 210 °C. Thermal analyses here after reported,
361 in nice agreement with these finding, will better highlight that at about this temperature begins the
362 [thermal decomposition](#) of the antibiotic.



363
364 Fig. 2. Plot of d_{001} values for [Bt](#) (black circle) and Bt/GM (gray circle) [montmorillonite](#) as a function of
365 temperature. The position of each (001) peak has been determined at the mid-height of the reflection,
366 through the use of the software X-Pert High Score Plus. The error of the measurement falls within the
367 dimensions of the used symbol.

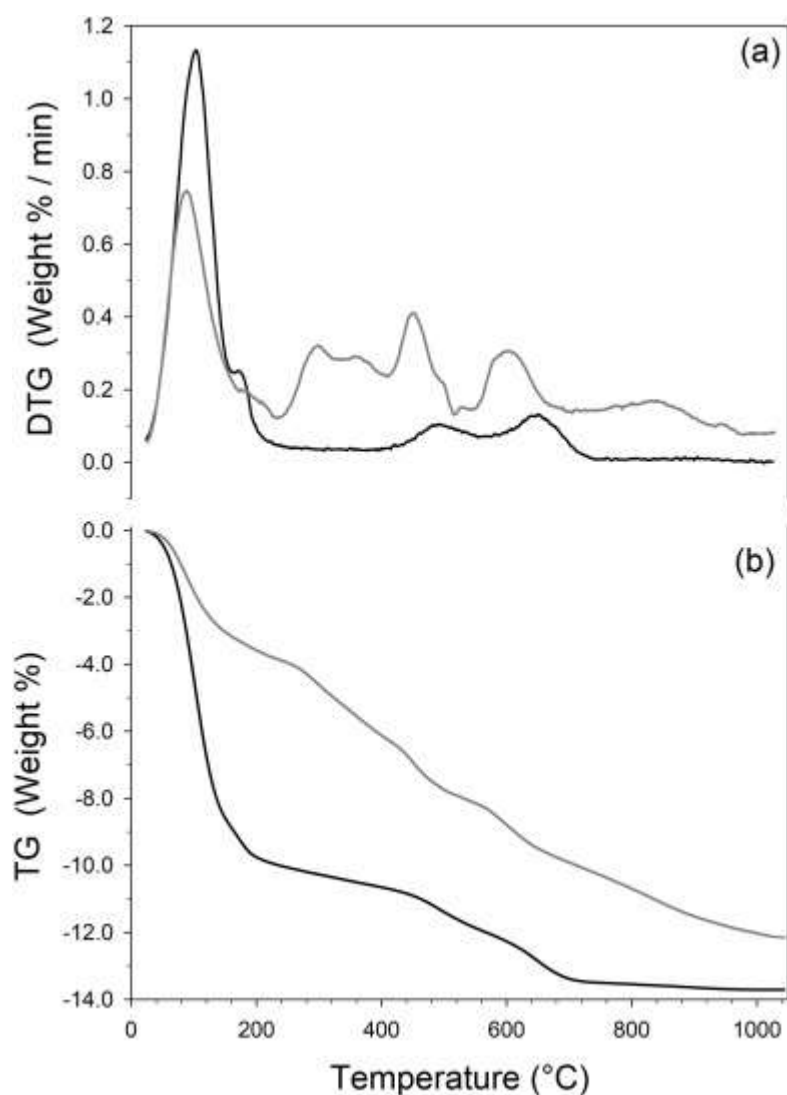
368 Thermogravimetric (TG) and its first derivative (DTG) curves for GM are shown in [Fig. 3](#). Five main reactions,
369 with maxima at 80, 260, 317, 500, and 582 °C (DTG curve), lead to a nearly complete thermal
370 decomposition of the antibiotic. The weight loss with maximum at 80 °C (mass loss of 14.7%, TG curve) is
371 related to the removal of free water molecules, whereas reactions at higher temperature are related to the
372 thermal decomposition of both the organic fraction and the [sulfate group](#). The asymmetry and/or band-like
373 shape of the DTG peaks, in particular for the thermal event that occurs at $T > 200 \text{ °C}$, maybe related to the
374 different forms (C1, C1a, and C2) of GM that thermally decomposed at significantly different temperature
375 values.



376

377 Fig. 3. TG (dashed line) and DTG curves (solid line) of GM.

378 The [thermal behaviors](#) of Bt and Bt/GM are compared in [Fig. 4](#). The DTG curve ([Fig. 4a](#)) of Bt shows four
 379 [thermal reactions](#) with maxima at 103, 165, 485 and 645 °C. The first two reactions occurred between 25
 380 and 210 °C and are attributed to the dehydration of montmorillonite with the removal of two water layers
 381 differently bound to the interlayer cation (maxima at 103 and 165 °C with mass losses of 9.0 and 0.84%,
 382 respectively - [Fig. 4b](#)). This finding is in agreement with the presence of [divalent cations](#) (Ca^{2+}) as major
 383 interlayer species ([Iannuccelli et al., 2016](#); [Mackenzie, 1970](#)) and with XRD data ([Fig. 2](#)). The two reactions
 384 at higher temperature are ascribable to the dehydroxilation of the octahedral sheets of kaolinite (maximum
 385 at 485 °C, mass loss 1.2%) and montmorillonite (maximum at 645 °C, mass loss 1.9%). Differential thermal
 386 analyses (not reported) additionally evidenced an [endothermic reaction](#) with maximum at 573 °C related to
 387 the transition from the trigonal α to the hexagonal β form of quartz ([Iannuccelli et al., 2016](#)).



388

389 Fig. 4. DTG (a) and TG (b) curves of [Bt](#) (black line) and Bt/GM (gray line).

390 The TG and DTG curves of Bt/GM nearly parallel those of Bt, but with some major differences, arising from
 391 the presence of GM, that are: i) a drastic reduction of the mass loss related to the removal of hydration
 392 water (reactions between 25 and 235 °C, mass loss 3.85%); ii) two additional thermal events with maxima
 393 at 298 and 360 °C (reactions between 220 and 405 °C, mass loss 2.30%); iii) a shift toward lower
 394 temperature values of the two dehydroxilation reactions; iv) an additional thermal events with maximum at
 395 about 830 °C.

396 The lower amount of water in Bt/GM compared to Bt may explain the small difference in layer periodicity
 397 of montmorillonite measured by XRD at room temperature. The two reactions between 220 and 405 °C
 398 occur nearly in the same temperature of those observed for pure GM, however with a lower decomposition
 399 rate (DTG curve). As already pointed out, it is not negligible that the reaction at temperature at which starts
 400 the first of the two reactions (220 °C) is about the same at which begins the reduction of layer periodicity
 401 ([Fig. 2](#)). This finding may be thus definitively attributed to the beginning of the thermal decomposition of
 402 the intercalated antibiotic, as moreover suggested by MS-EGA curves.

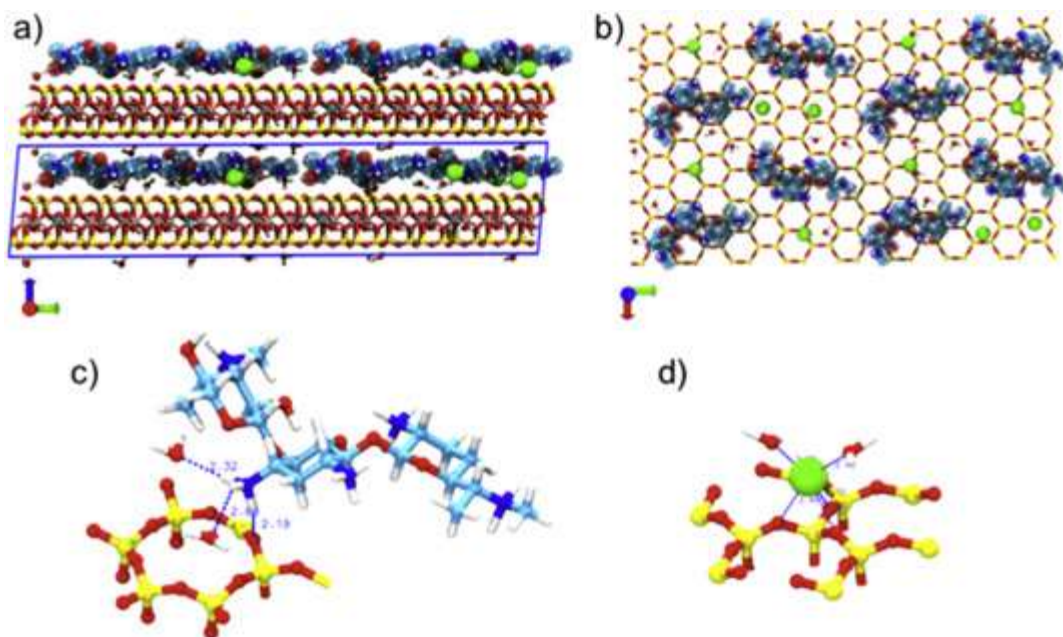
403 The thermal decomposition of pure GM is complete at about 600 °C ([Fig. 3](#)); however, TG curves indicated
 404 that the intercalated antibiotic follows a different thermal path. More in detail, MS-EGA curves (see [Fig. S3](#))
 405 indicated that the two just mentioned reactions lead to the release of H₂O (m/z = 18), NO (m/z = 30), and
 406 CO₂ ([Fig. S3](#)), further supporting the intercalation of GM. The detection of a MS-EGA signal for SO₂

407 highlights, however, the presence of marginal content of GM in its sulfate form, in accordance with the
408 elemental analysis. XRD measurements showed that the layer periodicity of Bt/GM montmorillonite after
409 the reaction with maximum at 360 °C was about 13 Å, higher than that in Bt montmorillonite (*i.e.*, 9.9 Å). It
410 may be concluded that after the two thermal reactions occurring between 220 and 405 °C, a pillar-like
411 residue, that thermally decomposes at higher temperature, forms in the interlayer of montmorillonite.
412 However, as above mentioned, all these high temperature reactions (included the modification of the
413 temperature at which occurred dehydroxylation) have not been taken into account in the present work, as
414 they are related to the [thermal evolution](#) of the already decomposed antibiotic and mineral. Nevertheless,
415 they well support the hypothesis of intercalation.

416 Further evidence supporting the intercalation of gentamicin is provided by [DSC](#) measurements that showed
417 the absence of GM endothermic reaction in Bt/GM. On the opposite, this reaction is visible when DSC
418 curves are collected for pure GM and physical mixture of Bt and GM.

419 Comparison of experimental measurements with MD simulations provided a more detailed understanding
420 of the arrangement and bonding promoted by the GM molecules intercalated in montmorillonite. A
421 computational approach attempted to simulate the interactions in the inter-structure of an ideal model of
422 montmorillonite ($X_{0.75}[Si_{7.75}Al_{0.25}][Al_{3.5}Mg_{0.5}]O_{20}(OH)_4$ which corresponds to a CEC of 101.9 meq/100 g),
423 gentamicin molecules (using C1 as a molecule model), sodium ions and [solvent molecules](#) (H₂O).
424 Gentamicin components present both hydroxyl and amino groups and this affects the total positive charge
425 (from 1 to 5 possible positive charge) of each molecule depending on the pH of the solution ([Lesniak et al.,](#)
426 [2003](#)). In this study the system was simulated considering a pH ~ 5 [aqueous environment](#) corresponding to
427 the preparation pH value at which GM molecules carry almost fully protonated charges (~85% of +5 and
428 ~15% of +4), taking into account the distribution diagram of the different protonated species of component
429 C1. This was achieved by inserting into the interlayer ~78.9% of molecules with +5 and ~21.1% molecules
430 with 4+ charges and a certain content of water molecules (~2 wt%). Plus, in order to mimic the partial
431 exchange process obtained experimentally a certain amount of sodium ions were also inserted. The
432 simulated interlayer is made of ~79.2% of GM and ~20.8% of sodium ions with respect to the theoretical
433 CEC. In this situation, the content of GM molecules useful to balance the negative charge of
434 montmorillonite corresponds to an organic content of ~7.7% by weight.

435 Modeling results clearly show that Bt/GM is characterized by a monolayer arrangement of the GM (C1)
436 molecules with a resulting layering periodicity of 14.2 ± 0.3 Å, a value in fair agreement with XRD finding
437 (14.19 Å). The side and top views of a typical configuration of Bt/GM hybrid system optimized by simulation
438 are shown in [Fig. 5a](#) and b. The analyses of density profiles show clear peaks for all the intercalated species.
439 On the basis of [radial distribution functions](#), the interatomic distance between the individual components
440 of the sub-networks was further assessed. In particular, the ammonium H atoms of the GM molecules
441 resulted located within the 2.1–2.9 Å range from the oxygen atoms of the montmorillonite surfaces, a
442 distance slightly larger than those found in alkyl ammonium ions-based [organoclays](#) (1.8–2.5 Å) ([Liu et al.,](#)
443 [2007](#)). This difference can be ascribed to the larger molecular flexibility of the alkyl [ammonium cation](#) due
444 to its alkyl chain with respect to the more rigid and sterically hindered nature of the cyclic GM (C1)
445 molecules. Both Na ions and the ammonium H atoms promote strong interactions with the oxygen atoms
446 of the tetrahedral sheets ([Fig. 5c](#) and d), being mostly located above the surface [siloxane](#) rings (*i.e.* six-
447 member rings). Water molecules partially affect such interactions by competing with the oxygen atoms of
448 the tetrahedral sheets in promoting H-bonding with GM (C1) ammonium H atoms and hydroxyl groups and
449 solvating the inner- and outer-sphere Na complexes ([Brigatti et al., 2011](#)).



450

451 Fig. 5. a) Side view of [montmorillonite](#) in the Bt/GM hybrid system optimized by MD modeling. The
 452 simulated [supercell](#) is shown within the blue line and it is replicated twice along the z direction for
 453 clearness. b) Top view of the local arrangement of GM and water molecules and Na ions within the
 454 montmorillonite [interlayer](#). c) Local environment around the GM ammonium group where the interaction
 455 between its [hydrogen atoms](#) with the oxygen of the tetrahedral sheets and the oxygen of water molecules
 456 are highlighted with a blue dashed line. d) Local coordination environment of a Na ion located on top of
 457 tetrahedral Al (Si substitution, charged site). For clearness, only the bottom [siloxane](#) sheet surface is shown.
 458 Color legend: Si atoms, yellow; O atoms, red; H atoms, white; C atoms, cyan; N atoms, blue; Na ions, green.
 459 (For interpretation of the references to color in this figure legend, the reader is referred to the web version
 460 of this article.)

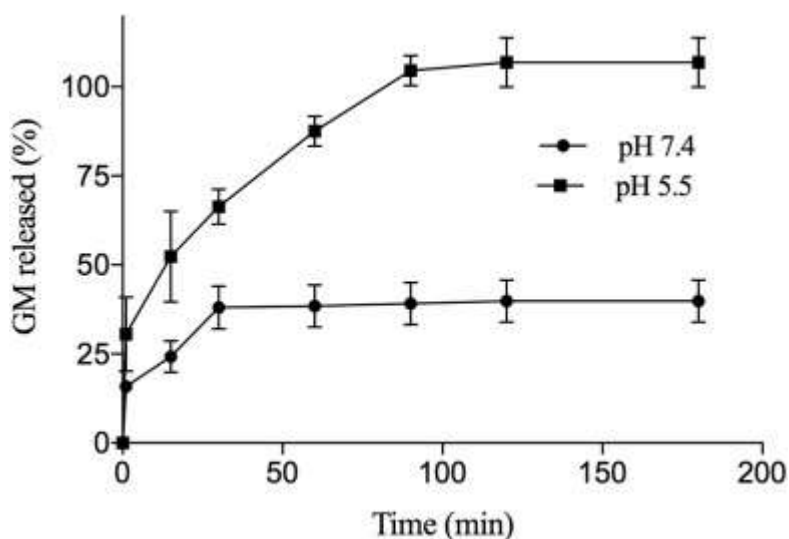
461

462 3.2. Antibacterial activity and *in vivo* skin permeation

463 In a perspective of Bt/GM use as cutaneous drug delivery system for local or systemic control of infections,
 464 the study involved the evaluation of GM biological activity within the organo-modified Bt, *in vitro*
 465 desorption, and *in vivo* skin [permeation](#) on human beings. The biological activity of GM within Bt was
 466 assessed to point out a possible synergistic clay action. In fact, clay minerals, in particular smectites, have
 467 [antimicrobial activity](#) due to their high absorbing/adsorbing power with respect to water forming
 468 unfavorable hydrophobic conditions for the growth of [microorganisms](#) (Kim et al., 2016; Williams and
 469 Haydel, 2010). Antimicrobial activity of Bt/GM was determined on *S. aureus* or *P. aeruginosa* and compared
 470 with that of pure GM, pure Bt and the physical mixture of the antibiotic with the clay in the same ratio as
 471 Bt/GM.

472 The results obtained highlighted the absence of antimicrobial activity of the pure Bt and an almost
 473 complete activity of the physical mixture compared with pure GM ($96.59 \pm 5.68\%$ against *S. aureus*,
 474 $88.01 \pm 2.90\%$ against *P. aeruginosa*). On the other hand, Bt/GM exhibited an activity against both the
 475 strains less than those obtained for GM solutions having the same antibiotic concentration ($50.37 \pm 1.59\%$
 476 against *S. aureus*, $50.07 \pm 1.88\%$ against *P. aeruginosa*). It is reasonable therefore to suppose that GM,
 477 though maintaining antibacterial activity against both Gram-positive and Gram-negative bacteria, desorbs
 478 from Bt/GM within the culture media of the [agar plate](#) incompletely indicating that drug intercalation
 479 between montmorillonite interlayer reduces drug availability (Ambrogi et al., 2017).

480 Nevertheless, complete GM desorption from Bt/GM was obtained at pH 5.5 buffer solution mimicking the
481 acidic environment of skin surface (Fig. 6). Unlike the pure GM dissolving within 1 min regardless of the pH
482 value, drug desorption profile involved a burst phase corresponding to about 40% of GM adsorbed onto Bt
483 followed by a sustained phase reaching 100% drug delivered in about 2 h. Conversely, following a burst
484 phase in which about 15% GM diffused into the medium, drug desorption at pH 7.4 was incomplete
485 reaching a plateau corresponding to about 50% of GM payload after about 30 min. Burst phases could be
486 related to GM fraction deposited or weakly linked to Bt particle surface whereas the subsequent phases to
487 an exchange process between the intercalated GM and the cations present in the media reaching
488 equilibrium according to the available cations (Joshi et al., 2009).



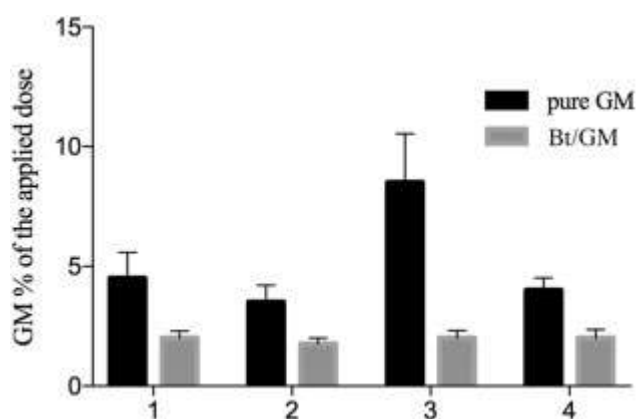
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490 Fig. 6. Profiles of gentamicin desorption from Bt/GM in pH 5.5 and pH 7.4 media.

491 The higher GM percentage amounts desorbed at pH 5.5 with respect to pH 7.4 may reasonably arise from a
492 competition by H⁺ ions present in the [assay medium](#) on the same interaction sites of GM (Iannuccelli et al.,
493 2015; Joshi et al., 2009). In biological fluids, physiological counter-ions can displace differently the drug
494 from the substrate and deliver it into the body (Aguzzi et al., 2007).

495 The understanding of the mechanism by which insoluble particles can cross the *stratum corneum* (SC) is
496 relevant to both prevent any possible local side effects or systemic exposure and properly exploit their
497 potential benefits such as the reservoir role inside the [hair follicles](#) (Wosicka and Cal, 2010) or the drug
498 transport modulation (Scalia et al., 2013). Since the passive transport through intact skin is considered
499 highly unlikely (SCCP, 2007), particle penetration is most likely along the intercellular route following the
500 lipid channels between the [corneocytes](#) and the appendage route along the hair follicles. Unlike the open
501 question concerning [nanoparticles](#), there is agreement that [microparticles](#) up to 10 μm can enter into the
502 follicle orifices that can act as an efficient long-term drug reservoir (Lademann et al., 2007) from which
503 soluble compounds could also diffuse into the viable epidermis (Borm et al., 2006). In relation to this, the
504 density and size of hair follicles as well as the lipophilicity of the material applied on the skin have been
505 assumed to contribute to differences in penetration rates (Feldmann and Maibach, 1967; Knorr et al., 2009;
506 Otberg et al., 2004). To assess the mechanism by which the organo-modified bentonite enters the SC, the
507 present study has considered the *in vivo* skin [penetration profile](#) of Bt/GM in comparison with that of pure
508 GM and pure Bt applying the samples, incorporated in petroleum jelly, on human skin regions having two
509 different hair follicle densities, the volar forearm (18 follicles/cm² corresponding to 0.09% skin surface,
510 78 μm diameter of hair follicle orifice) and the forehead (292 follicles/cm² corresponding to 1.28% skin
511 surface, 66 μm diameter of hair follicle orifice) (Otberg et al., 2004).

512 The investigation was performed using EDX analysis on twelve repetitive stripped tapes containing the
 513 outermost layers of SC, generally the *stratum disjunctum* from the 2nd to the 5th tape, the *stratum*
 514 *compactum* from the 6th to the 12th tape (Jacobi et al., 2005) by both GM extraction from combined tapes and
 515 Bt detection on each tape. Skin exposure to pure GM at the level of the forehead region provided drug
 516 concentrations higher than those obtained on the volar forearm region ($p < .05$ concerning the first tape
 517 group) suggesting the involvement of the trans-follicular route pathway across SC. This assumption is
 518 consistent with the results from other Authors arguing that skin appendage route gained renewed interest
 519 for hydrophilic drugs representing a significant access also for gentamicin (Barry, 2002; Fadli et al., 2015;
 520 Ogiso et al., 2002). Moreover, GM levels are inclined to decrease with the increase of SC depth. The
 521 interaction of GM with Bt decreased antibiotic permeation extent compared with pure GM permeation
 522 ($p < .05$) leading to a constant GM concentration (about 2% of the applied dose), regardless of SC depth
 523 (Fig. 7).



524

525

526 Fig. 7. GM permeated dose (% of the applied dose) across SC from pure GM and Bt/GM: (1) volar forearm,
 527 tape group 1 (tapes 2–6); (2) volar forearm, tape group 2 (tapes 7–12); (3) forehead, tape group 1 (tapes 2–
 528 6); (4) forehead, tape group 2 (tapes 7–12).

529 It follows a hampering effect of GM permeation provided by its reaction with Bt and the irrelevance of the
 530 application region, *i.e.* follicle density indicating presumably a different pathway. To monitor the possible
 531 translocation and distribution of Bt/GM across SC, each stripped tape (tapes 2, 6, and 12) was assayed by
 532 EDX analysis. All EDX spectra obtained from both pure Bt and Bt/GM skin exposure exhibited peaks from
 533 carbon, oxygen, aluminum, silicon, and sulfur atoms. The presence of sulfur in all tapes removed from
 534 untreated skin, attributable to the emission from SC keratin, prevented assessing the permeation of GM in
 535 its sulfate form. The elements Si and Al that are not naturally occurring elements in SC entail the presence
 536 of Bt (Cullander et al., 2000; Moretto et al., 1999). Accordingly, the presence of S atoms and the absence of
 537 Si and Al atoms were pointed out in all the control tapes (untreated skin and skin treated with only
 538 petroleum jelly). By measuring the intensity of characteristic X-rays spectra, the relative weight fraction of
 539 Si and Al can be calculated (Table 4).

Table 4
 EDX analysis: relative element weight percentage detected on tapes 2, 6, and 12 following skin exposure to pure Bt and Bt/GM. Mean values \pm SD.

Sample	Volar forearm		Forehead	
	Relative silicon weight	Relative aluminum weight	Relative silicon weight	Relative aluminum weight
pure Bt tape 2	29.3 \pm 0.7	7.3 \pm 0.3	13.7 \pm 0.6	4.1 \pm 0.5
pure Bt tape 6	3.9 \pm 0.6	1.8 \pm 0.6	5.5 \pm 0.3	1.6 \pm 0.3
pure Bt tape 12	5.3 \pm 0.4	1.6 \pm 0.4	5.3 \pm 0.2	2.3 \pm 0.2
Bt/GM tape 2	28.9 \pm 0.4	7.0 \pm 0.2	51.4 \pm 0.9	10.8 \pm 0.3
Bt/GM tape 6	17.6 \pm 0.4	4.3 \pm 0.3	18.0 \pm 0.5	4.9 \pm 0.3
Bt/GM tape 12	7.8 \pm 0.5	2.4 \pm 0.4	1.0 \pm 0.5	1.6 \pm 0.5

540

541 The detection of Si and Al atoms in all the tapes reveals the ability of clay particles to translocate across SC
542 until the *stratum compactum* though Si and Al extents decreased with the increase of SC depth. Differences
543 in quantitative distribution of Si and Al atoms between volar forearm and forehead application of both Bt
544 and Bt/GM among the tapes were considered pointless to be argued. The limited influence of the sample
545 exposure region is consistent with the results obtained by GM extraction from the tapes as the evidence of
546 drug/clay association at the time of selecting SC pathway, plausibly different from the trans-follicular one.
547 Besides skin appendages, SC is interrupted by inter-cluster corneocyte regions up to 100 µm in width, made
548 of unsteady lipid packing generating openings having a low resistance to hydrophilic compounds ([Cevc and](#)
549 [Vierl, 2010](#); [Dayan, 2005](#); [Iannuccelli et al., 2013](#)). Taking into account the size together with the [hydrophilic](#)
550 [nature](#) of clayey samples, their motion along this trans-epidermal pathway could be considered as the
551 favorite pathway. Moreover, Bt could increase SC hydration by means of the occlusive effect decreasing
552 corneocyte packing and improving the clay transport. Such a route could also bypass the hindering effect
553 on hydrophilic drug permeation provided by sebum along the trans-follicular route ([Verma et al., 2016](#)).
554 Even if restricted to the superficial SC layers, it follows that a long-term Bt/GM reservoir for gradual GM
555 release may be expected to perform leading to sustained antibacterial activity and minimized drug side
556 effects.

557 **4. Conclusions**

558 The approach consisting of new carriers redeveloping already-approved drugs and excipients was
559 addressed to modulate gentamicin release and skin [permeation](#) by exploiting drug arrangement in the
560 [interlayer](#) of [montmorillonite](#). Comprehensive examination of the organo-modified clay combined with a
561 computational approach elucidated the mechanism of drug interaction with montmorillonite
562 demonstrating the occurred [intercalation](#). From that, gentamicin sustained desorption and the possible
563 pathway across inter-cluster [corneocyte](#) regions of the *stratum corneum* may be ensued in the perspective
564 of contribution to a novel antibiotic material offering a potential more effective anti-infective therapy.

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