

Antibacterial activity of Na-clinoptilolite against Helicobacter pylori: in-vitro tests, synergistic effect with amoxicillin and stability of the antibiotic formulated with the zeolite

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Highlights

Clinoptilolite is selective towards NH_4^+ , which is crucial for *H. pylori* survival

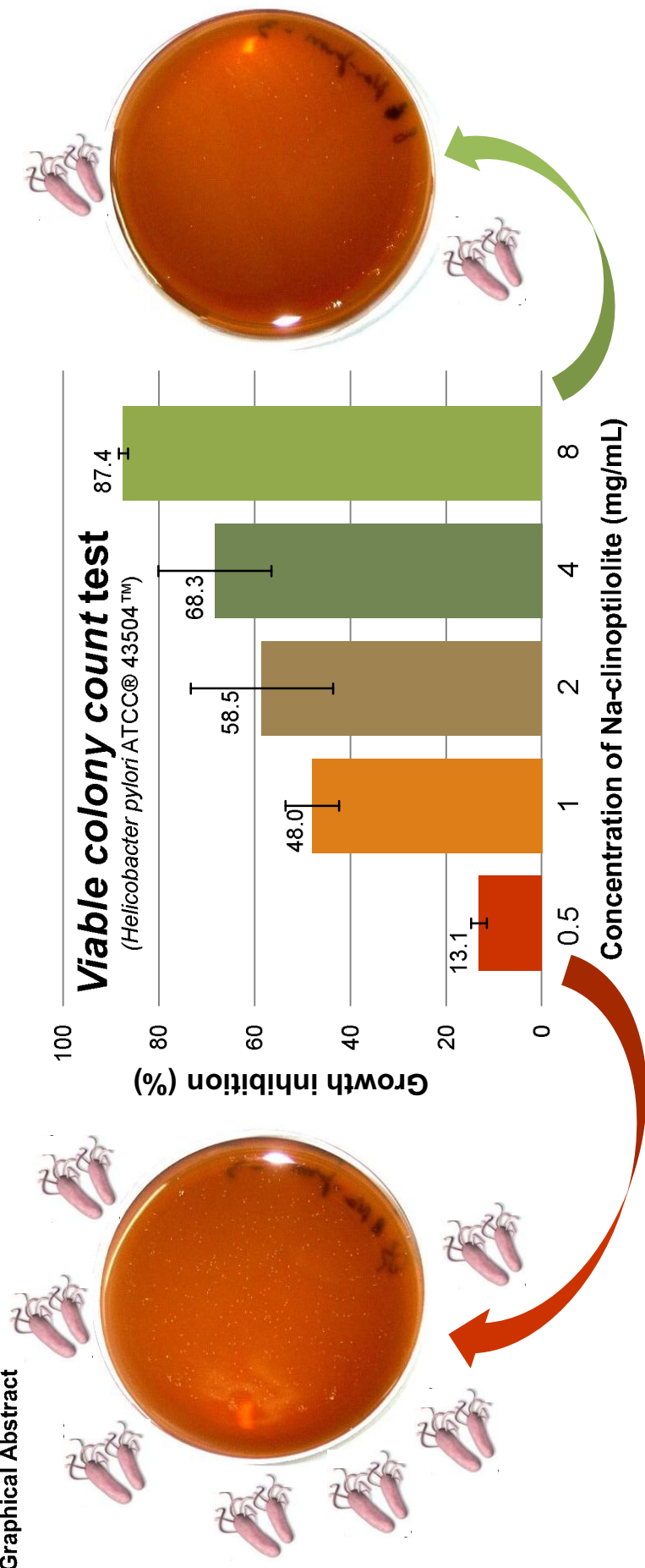
Na-clinoptilolite shows antibacterial activity, conversely to NH_4 -clinoptilolite

The antibacterial activity of Na-clinoptilolite depends on its ability to remove NH_4^+

Na-clinoptilolite evidences a synergy with amoxicillin against *H. pylori* growth

Na-clinoptilolite does not affect the solid state stability of the antibiotic

*Graphical Abstract



Test in media with NH_4^- or Na-clinoptilolite

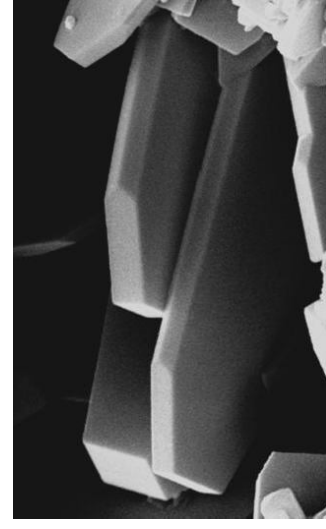


growth



NH_4^- -form
30 mg/mL

NH₄-zeolite cannot subtract NH_4^+



Na-form
30 mg/mL

inhibition



Na-zeolite can subtract NH_4^+

Antibacterial activity of Na-clinoptilolite against *Helicobacter pylori*: in-vitro tests, synergistic effect with amoxicillin and stability of the antibiotic formulated with the zeolite

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Abstract

Helicobacter pylori is a bacterium whose pathogenic strains cause severe gastroduodenal diseases. Ammonium plays a crucial role in the survival of *H. pylori*, and potentiates the effect of a toxin produced by the bacterium. This research has evaluated the possibility to exploit, against *H. pylori* growth, the selectivity of clinoptilolite towards ammonium. A specifically-prepared material containing 90% Na-clinoptilolite and *H. pylori* reference strain ATCC® 43504™ have been used to perform in-vitro tests. The *viable colony count test* has evidenced, compared to the zeolite-free

control, a decrease in bacterial growth from 13 to 87% for Na-clinoptilolite concentrations from 0.5 to 8 mg/mL. *H. pylori* growth has been inhibited in media containing 30 mg/mL of Na-clinoptilolite, whereas the same concentration of NH₄-clinoptilolite, prepared through exchange from the Na-form, has allowed bacteria proliferation. The *disc diffusion test* revealed the existence of a synergy between amoxicillin trihydrate and Na-clinoptilolite, as the diameter of inhibition halo caused by the antibiotic has increased by 24% in growth media containing 0.125 mg/mL of Na-clinoptilolite, and by at least 70% for a zeolite concentration of 0.250 mg/mL. Conversely, NH₄-clinoptilolite has not affected inhibition halo of amoxicillin. The results lead to correlate the antibacterial activity of Na-clinoptilolite with its ability to remove ammonium by cation exchange. Na-clinoptilolite does not affect the solid state stability of amoxicillin trihydrate, as determined by XRD and HPLC analyses performed on a physical mixture stored for 18 months at 20 °C and relative humidity of 20, 50, and 80%.

Keywords: *Helicobacter pylori* ATCC[®] 43504[™]; Zeolite; Ammonium; Amoxicillin; Cation exchange.

1. Introduction

Helicobacter pylori (*H. pylori*) is a spiral shaped, gram-negative, microaerophilic, flagellated human pathogen that successfully colonizes gastric mucosa of majority of individuals [1]. A recent review concerning the epidemiology of *H. pylori* infection reports that the highest prevalence of *H. pylori* was found in Africa (79.1%), Latin America and the Caribbean (63.4%), and Asia (54.7%), and the lowest prevalence in Northern America (37.1%) and Oceania (24.4%) [2]. In Europe, the lowest prevalence of the bacterium was reported in Northern countries, while the highest was in Eastern and Southern Europe with up to 84% in Portugal and Poland [2]. The global prevalence ranges from 60.3 to 44.3%, depending on the study considered, however the prevalence of *H. pylori* infection shows a declining trend, particularly in Europe [2]. The presence of the bacterium in the stomach of infected individuals is linked to the development of several gastric diseases, such as chronic gastritis [3]. Luckily, only a small percentage of infected patients develop severe pathologies, such as ulcers (10%-15%) and stomach adenocarcinomas (less than 1%) [3]. On the other hand, the key role played by *H. pylori* in development of gastric cancer is recognized [1]. Indeed, the International Agency for Cancer Research established that chronic infection with *Helicobacter pylori* is carcinogenic to humans [4,5], and 78% of all gastric cancer cases are now estimated to be attributable to this chronic infection [6]. *H. pylori* is able to colonize gastric mucosa, predominantly the antrum and pyloric canal, directly interacting with gastric epithelial cells [7]. The bacterium possesses a urease enzyme which converts host's urea to ammonia and carbon dioxide, thus buffering gastric acid in its vicinity and facilitating its survival in the acidic gastric environment because, due to H^+ presence, NH_3 is converted to ammonium ions that lead to a local rise in pH [8]. The most recommended

first-line treatment in *H. pylori* eradication is a standard triple therapy which includes a proton pump inhibitor and two antibiotics such as clarithromycin, amoxicillin, metronidazole, levofloxacin or tetracycline [9,10]. On the other hand, antibiotic resistance is among the major factors causing decreasing eradication rates of *H. pylori* [11,12], an issue of particular relevance with regard to metronidazole and clarithromycin [10,12-15]. The emergence of antibiotic resistant human pathogens has accelerated inquiries into alternative antibacterial compounds, and mineral-based therapies against bacterial infections have gained attention [16-18]. Researches on possible applications of clays and zeolites in biomedical sector are steadily increasing and, as concerns *H. pylori* infection, a smectite has recently been evaluated as tetracycline-deliver able to distribute homogeneously the antibiotic on the gastric mucosa due to its mucoadhesive properties [19]. Considering the role played by the ammonium ions in the survival of *H. pylori* (see above), their subtraction in the microenvironment of the bacterium might give some advantage in therapies for *H. pylori* infection. Another factor worth considering is the mutual interaction between the vacuolating toxin (VacA) produced by *H. pylori* and ammonia, in fact, in human gastric cells the toxin does not form large vacuoles unless combined with protonated ammonia [20,21]. Clinoptilolite is a natural zeolite that combines cation exchange capacity, high selectivity toward ammonium cation and good stability in acid media [22]. It has been used in animal nutrition, and found to be efficacious in reducing ammonia concentration in the gastrointestinal tract [23]. *In-vivo* experiments with rats evidenced that clinoptilolite is able to bind NH_4^+ in the upper gastrointestinal tract [24]. As concerns biomedical applications on humans, clinoptilolite is the most studied among natural zeolites; just to cite some examples, it has been evaluated as anti-diarrheic, adjuvant in

anticancer therapy, antimicrobial agent, drug carrier, haemostatic and wound-healing accelerator, histamine regulator, antiphlogistic agent, and in the treatment of dyslipidemia [25-34]. In oral administrations, acid resistance of zeolite is an imperative pre-requisite, and this is the main reason for the preference of zeolites with high Si/Al ratio, such as clinoptilolite (Si/Al > 4), compared to the more aluminous phillipsite and chabazite [29]. In assessing a natural zeolite for pharmaceutical purposes, it is essential to perform the experiments using a reproducible material which maximizes the zeolite properties and minimizes any type of interference (e.g.: associated minerals; presence of toxic elements and/or of microbial species), because i) the material should be compatible with the planned application, and ii) this allows to relate unambiguously the results to the zeolite [35].

The present research has been addressed to investigate if clinoptilolite, by virtue of its selectivity toward NH_4^+ , is able to develop, *in-vitro*, an antagonistic activity against *H. pylori* growth. In particular, by using a reference strain of *H. pylori*, and a material based on Na-clinoptilolite specifically prepared to develop oral formulations [35], the following aspects have been explored: i) the growth of *H. pylori* in presence of Na-clinoptilolite; ii) the growth of *H. pylori* in presence of NH_4 -clinoptilolite, used as "control"; iii) the possible existence of a synergistic action between Na-clinoptilolite and antibiotics - amoxicillin, levofloxacin, and metronidazole - widely used in *H. pylori* eradication therapy; iv) the stability of amoxicillin when combined with Na-clinoptilolite, since zeolites are hydrate minerals and water content is important for the solid state stability of all penicillins [36].

2. Experimental

2.1. Material

2.1.1 Na-clinoptilolite

The material used for the experiments is part of the lot realized and characterized by Cerri et al. [35] for the development of pharmaceuticals. Briefly, a clinoptilolite-rich rock collected in Sardinia island (Italy) has been subjected to a beneficiation process, obtaining a powder containing (by weight) $90.2\pm 2.0\%$ of clinoptilolite, $0.4\pm 0.10\%$ of quartz, $1.2\pm 0.2\%$ of biotite, $3.2\pm 0.3\%$ of feldspars, $1.2\pm 0.2\%$ of opal-CT and $3.8\pm 1.0\%$ of amorphous. Subsequently the material has been Na-exchanged (sample FA-Na in Ref. 35), reaching the chemical composition reported in Table 1. FA-Na shows unimodal size distribution curve, with particles ranging from 0.4 to 80 μm and modal diameter of $10.83\pm 0.35 \mu\text{m}$ [35].

2.1.2 NH_4 -clinoptilolite

Part of the material in sodium form (hereafter, FA-Na) has been used to prepare the ammonium form (hereafter, FA-NH₄). FA-Na has been subjected to cation exchange using a 0.5 M NH₄Cl solution (Sigma Aldrich salt; purity 99.5%), performing a sequence of five exchange cycles of 2 h each, executed in batch at 65 °C under continuous stirring and with a solid/liquid ratio of 30 g/L. FA-NH₄ has been thoroughly washed with deionized water until complete removal of chloride solution (test performed on elutes with AgNO₃). The powder has been dried at 35 °C overnight, then rehydrated for 24 h at 22 °C at about 50% of relative humidity.

2.2 Chemical analyses

Chemical analysis of FA-NH₄ has been performed at the Activation Laboratories Ltd (Actlabs - Ancaster, ON, Canada). Major elements have been determined, after lithium metaborate/tetraborate fusion, through Inductive Coupled Mass Atomic Emission Spectrometry (ICP-AES) performed with a Varian Vista 735 ICP (Varian, Inc., Palo Alto, CA, USA). NH₄ content has been calculated on the basis of the Total N determined through the Total Kjeldahl Nitrogen (TKN) method [37]. The Loss on Ignition (LoI) of the material has been determined in our laboratory, by calcination for 2 h at 1000 °C. The H₂O content in FA-NH₄ has been calculated as the difference between the LoI and the (NH₄)₂O content [38].

2.3 Evaluation of inhibitory ability of FA-Na against H. pylori growth: Viable Colony Count test

The antibacterial activity of Na-clinoptilolite has been tested *in-vitro* using a reference strain of *Helicobacter pylori* (ATCC[®] 43504[™]). In particular, the Viable Colony Count method has been chosen to evaluate whether FA-Na was capable to inhibit or reduce the bacterial growth; this method, albeit time consuming, provides statistically accurate and repeatable results. The zeolite has been sterilized by heating for 3 hours at 160 °C, an effective procedure that does not compromise the structure of Na-clinoptilolite [35]. For the *H. pylori* culture, the recommendations of Megraud & Lehours [39] have been followed. A growth medium has been prepared using Mueller Hinton Broth (Oxoid) supplemented with 5% of defibrinated horse blood (Oxoid). In five test tubes, each containing 10 mL of the growth medium, FA-Na has been added to realize the following concentrations: 0.5, 1, 2, 4, and 8 mg/mL. A further tube, containing only the growth medium, has been used to make the control. A bacterial suspension with a

turbidity equivalent to a 4.0 McFarland standard has been prepared using sterile saline (NaCl 9 g/L), and 100 μ L of the suspension have been added to all test tubes; the latter have been incubated two days at 37 °C in microaerophilic conditions (85% N₂, 5% O₂, and 10% CO₂ - gas mixture obtained with CampyGen™ sachets, Oxoid). Afterwards, the contents of the six tubes have been subjected to serial dilutions (10⁻² - 10⁻⁶), and 100 μ L of each dilution have been dispersed in plates containing 20 mL of Mueller Hinton Agar (Oxoid) supplemented with 5% of defibrinated horse blood (Oxoid). After the solidification of the growth medium the plates have been incubated, upside down, at 37 °C for three days in microaerophilic conditions (CampyGen™ sachets, Oxoid). The number of viable bacteria has been evaluated by counting the colonies on the plates and taking into account the dilution factors. The test has been performed in duplicate. The results have been expressed as percentage of inhibition (I%) of bacterial growth with respect to the control, according to the formula

$$I\%_x = 100 \cdot (n_c - n_x) / n_c \quad (1)$$

where:

I%_x is the percentage of inhibition determined by FA-Na at the concentration x;

n_c is the number of colonies in the control;

n_x is the number of colonies grown in presence of FA-Na at the concentration x.

2.4 Comparison of FA-Na and FA-NH₄ activities against *H. pylori* growth

This test has been performed to compare the growth of *H. pylori* in presence of Na-clinoptilolite, which is potentially capable to subtract ammonium from the microenvironment by cation exchange, with the growth of the bacterium in presence of the same material, but in NH₄-form, thus unable to remove ammonium because already

"saturated" with this cation. The test has been performed in duplicate. FA-Na has been carefully dispersed in plates containing 20 mL of growth medium (Mueller Hinton Agar supplemented with 5% of defibrinated horse blood, hereafter MHAS) still liquid, until a concentration of 30 mg/mL has been reached. The plates containing FA-NH₄ have been prepared in the same way. A third pair of plates, zeolite-free, has been prepared as control. Once the growth media have solidified, two spots (3 µL each) of a bacterial suspension (prepared as described at 2.3 paragraph) have been deposited on the surface of all six plates. After three days of incubation at 37 °C in microaerophilic conditions (see paragraph 2.3) the plates have been visually checked for bacterial growth.

*2.5 Study on the existence of a synergy between FA-Na and antibiotics used in *H. pylori* eradication*

To evaluate if, in inhibiting bacterial growth, Na-clinoptilolite develops a synergy with antibiotics currently used in the eradication therapy of *H. pylori*, the disk diffusion test has been used [40]. Commercial disks of amoxicillin (2 µg, Oxoid), levofloxacin (5 µg, Oxoid), and metronidazole (5 µg, Oxoid) have been employed. As far amoxicillin, also disks with a drug content of 0.5 µg have been used, prepared by impregnating sterile paper disks (9 mm in diameter; Sigma-Aldrich) with a solution of the antibiotic (amoxicillin trihydrate, Sigma-Aldrich, **Ph. Eur. quality**). A sterile cotton-tipped swab has been dipped into the bacterial suspension (see paragraph 2.3) and streaked across plates (90 mm in diameter) containing 25 mL of MHAS. The plates have been dried for 2-3 min, then the disks containing the antibiotics have been placed on the agar surface (one disk per plate). Each type of antibiotic disk has been tested in duplicate.

Furthermore, a pair of plates without antibiotic disks has been prepared as control. All

plates have been incubated three days in the same conditions described at the paragraph 2.3; afterwards, the diameters of the inhibition halos have been measured with a caliper. Another set of plates has been prepared by dispersing FA-Na in MHAS at concentrations of 0.125 and 0.250 mg/mL, and the same has been done for FA-NH₄; next steps have been performed as above described but, as regard amoxicillin, only the disks with a drug content of 0.5 µg have been used. Also these experiments have been executed in duplicate. For each antibiotic, the inhibition halo measured in the plates containing also the zeolite has been compared to the diameter of the inhibition zone recorded in the plates containing only the antibiotic.

2.6 Study of the stability of amoxicillin in a binary formulation with Na-clinoptilolite

The investigation has been conducted at 20±2 °C for 18 months, and considering values of relative humidity (hereafter, RH%) of about 20, 50 and 80%. A physical mixture composed, in weight, by 2/3 of FA-Na and 1/3 of amoxicillin trihydrate (Sigma-Aldrich, Ph. Eur. quality) has been prepared by thoroughly mixing the components in a mortar until a homogenous mixture has been obtained. Samples of the physical mixture (hereafter, PhM) and of amoxicillin trihydrate (hereafter, AMX) have been placed in three desiccators containing saturated solutions of, respectively, potassium acetate, calcium nitrate tetrahydrate, and ammonium chloride (Sigma-Aldrich chemicals; purity ≥ 99%). At 20 °C and in small closed spaces, these solutions are suitable for obtaining the desired values of RH% [41]. The three desiccators have been kept in a room (located in the basement) equipped with an independent air conditioning system whose temperature has been set at 20 °C. During the study, the humidity inside each desiccator has been monitored using three data loggers (EBRO EBI-TH1), which demonstrated

that RH% values have been maintained in the following ranges: $20\pm 3\%$, $52\pm 3\%$, and $80\pm 5\%$. The same data loggers have recorded the temperature, which remained in a range of 20 ± 2 °C. The stability of the amoxicillin in the physical mixture with Nalclinoptilolite has been compared, by means of XRD and HPLC analyses (see next subparagraphs), with that of pure amoxicillin stored under the same conditions.

2.6.1 X-Ray diffraction (XRD) analyses

XRD analyses have been performed with a Siemens D5000 diffractometer, equipped with a Cu-tube and a graphite monochromator on the diffracted beam, set as follows: 40 kV; 30 mA; 2θ range 2–70°; step size 0.020° (2θ); time per step 2 s. The alignment of the instrument has been preliminary calibrated using a reference material (NIST 1976b). Six sample holders have been used to accommodate three aliquots of PhM and three of AMX, then the materials have been equilibrated for 24 h at the different RH% (by placing a pair of sample holders in each desiccators), and subsequently analyzed. After the measurement, the sample holders have been put again into the desiccators. XRD analyses have been repeated after 3 days, 1 week, 4 weeks, 2, 4, 6, 8, 12, and 18 months, always keeping the powders inside the same sample holders; this procedure avoids loss of material and, above all, prevents the occurrence of differences due to sample preparation, a key aspect in comparing diffractograms of the same sample. The X-ray patterns have been evaluated using the software Bruker EVA v 14.2 (Bruker AXS, 2008) and the PDF-2 database.

2.6.2 High-Performance Liquid Chromatography (HPLC) analyses

Aliquots of PhM and AMX (other than those used for XRD measurements) stored within the three desiccators at different RH% for 18 months have been analyzed, in duplicate, to determine the content of amoxicillin and, by this, to evaluate if Na-clinoptilolite affects the degradation process of the antibiotic. For a given sample, if the final content of amoxicillin is lower than the initial one (33% in PhM; 100% in AMX), a degradation has occurred. To measure the content of antibiotic in the three AMX samples, 10 mg of each sample have been dissolved in 10 mL of phosphate buffer (pH 6.8); afterwards, each solution has been diluted twenty-five fold using again the phosphate buffer (final concentration of AMX = 40 $\mu\text{g}/\text{mL}$), and subjected to HPLC analysis. As concerns the PhM samples, 30 mg of each sample have been suspended in 10 mL of phosphate buffer (pH 6.8); later, the suspensions have been diluted twenty-five times with the phosphate buffer (final concentration of PhM = 120 $\mu\text{g}/\text{mL}$), filtered using 0.45 μm cellulose acetate membranes (Sartorius), and analyzed by HPLC. For the quantitative determination of amoxicillin, a simple and accurate HPLC method has been applied [42]. Analyses have been performed with a Varian ProStar 210, equipped with a PDA photodiode array detector and an AutoSampler 410 (Varian Inc. Scientific Instruments). The chromatographic separation has been performed on a C18 column (Thermo Scientific Hypersil BDS, 150 \times 4.6 mm I.D. and 5 μm of particle size). The analyses have been conducted at room temperature with a flow rate of 1.0 mL/min and UV detection at 210 nm. The volume of the injected samples has been 20 μL and the analysis time has been 4 min per sample. The mobile phase consisted of a binary mixture (5:95 v/v) composed by acetonitrile and a 30 mM solution of KH_2PO_4 adjusted to pH 4 by 1 M phosphoric acid. Before analysis, the mobile phase has been filtered (cellulose acetate membrane, 0.45 μm , Sartorius). To calculate the concentrations of

amoxicillin by interpolation, a calibration curve has been preliminary realized with solutions of the antibiotic in MilliQ water having the following concentrations: 5, 10, 25, 50, and 100 mg/L. All measurements have been collected in triplicate and processed using Varian Star Chromatography Workstation v. 6.20 (Varian, Inc. Cary).

3. Results and discussion

3.1 Study of Na- and NH₄-clinoptilolite activity against H. pylori

The results of the *Viable Colony Count test* are summarized in Fig. 1. Na-clinoptilolite is able to reduce *H. pylori* growth, and the efficiency of inhibition increases with zeolite concentration. Compared to the control, bacterial growth has been progressively reduced, from 13 to 87%, using FA-Na in concentrations from 0.5 to 8 mg/mL (Fig. 1). The inhibition should not have been determined by sodium released by clinoptilolite. In fact, in carrying out tests with *H. pylori* the preparation of a bacterial suspension in physiologic solution (NaCl 9 mg/mL) represents a routine step (see paragraph 2.3 and Ref. 39), and even the highest concentration of FA-Na used would provide, if the zeolite released all the sodium, an amount of Na⁺ ten times lower than the sodium contained in physiologic solution (i.e., 0.36 vs. 3.54 mg/mL). On the other hand, the results of the *Viable Colony Count test*, taken alone, are not sufficient to establish that the inhibition of bacterial growth has been determined by an ammonium subtraction caused by the Na-clinoptilolite; furthermore, at least in principle, it can not be excluded that the mineralogical and/or chemical composition of FA-Na is toxic to *H. pylori*. The experiments performed with FA-NH₄ have allowed to clarify these aspects. Table 1 shows the chemical composition of FA-Na (taken from Ref. 35) and FA-NH₄, the

material based on ammonium clinoptilolite prepared through cation-exchange from FA-Na. The growth of *H. pylori* has been tested in media containing Na- or NH₄-clinoptilolite, and compared with the bacterial growth obtained in media free of zeolite (see paragraph 2.4); the result is summarized by the images in Fig. 2. A concentration of 30 mg/mL of FA-Na has not allowed *H. pylori* growth. Conversely, the same concentration, but of the material in ammonium form, has not determined any inhibition of bacterial growth, which seemed always even greater than in the control plates (Fig. 2). These findings demonstrate that clinoptilolite, by itself, does not inhibit *H. pylori* growth (hence the material is not toxic to the bacterium), whereas inhibition should be related to the removal of ammonium resulting from the exchange $\text{Na}^+ \leftrightarrow \text{NH}_4^+$. It could be speculated, once again, that the inhibition might depend on the sodium released by clinoptilolite, rather than on the subtraction of ammonium; this hypothesis is not convincing because, even if sodium had been completely released during the test, it would have determined a concentration of Na⁺ in the growth medium 2.6 times lower than a physiologic solution (in which, as recalled above, the bacteria are usually suspended). On the other hand, the inhibition effect induced by ammonium removal is weak and requires relatively high concentrations of zeolite. Regarding the cation exchange process, however, an important aspect must be emphasized. In the *Viable Colony Count test*, clinoptilolite and bacteria have had the chance to interplay in a liquid growth medium (a broth) whereas, in the other test, *H. pylori* has been placed on the surface of a solid growth medium (a gel) containing the zeolite (which settled, reaching the bottom of the plate), a less favorable situation for the development of an ion exchange. This to underline that the two tests are very different, and their results can not be compared quantitatively. Given the relationship between ammonium removal and

inhibition of *H. pylori* growth, it is important that clinoptilolite is prepared in a cation-form capable of maximizing the subtraction of NH_4^+ by exchange. At the same time, the cation(s) released by the zeolite must not be harmful for human health [35], nor determine undesired interactions with the drugs administered to eradicate the bacterium. Basically, there is a need to manage mineralogical and biomedical aspects, and the use of a mono-cationic form of clinoptilolite simplifies this task. In addition, a pretreatment with sodium is a well established practice that improves the exploitation of the cation exchange capacity (CEC) of clinoptilolite in the subsequent exchanges [27,38,43-46]. The sodium cation is easily adsorbed and released by clinoptilolite, which exhibits very high preference for NH_4^+ over Na^+ [22,47]; furthermore, being an essential element for the human organism, sodium is well tolerated [48]. Among alkaline and alkaline-earth elements, also K, Ca and Mg are indispensable for the human health [48], but the use of a clinoptilolite prepared in one of these cation-forms would present some drawbacks. In fact, clinoptilolite is more selective towards K^+ than Na^+ [22,47], therefore, in the absorption of NH_4^+ , a better performance should be ensured by the zeolite in the Na-form, more inclined to release its cation; moreover, some authors report that clinoptilolite prefers K^+ over NH_4^+ [49,50], a feature that can limit ammonium removal. As concerns Ca^{2+} and Mg^{2+} , due to the well known "concentration-valency effect" [51], their exchange with a monovalent cation as NH_4^+ would be strongly affected by variations of the total concentration of the aqueous solution, with a concomitant change of zeolite's selectivity [47]; on this basis, a constant behavior of the zeolite in terms of cation exchange within the stomach can not be expected. Moreover, the difficulty to achieve a full exchange with Mg^{2+} is known [52-54]. The release of Ca^{2+} and Mg^{2+} in the stomach might present another drawback, because it has been hypothesized that these

ions play a role in attachment of urease enzyme to the surface of *H. pylori* cell [55]. Finally, in gastric environment Ca^{2+} and Mg^{2+} may decrease the absorption of tetracycline [56], an antibiotic used in some therapies for the treatment of *H. pylori* infections [13,57].

3.2 Study on the existence of a synergy between Na-clinoptilolite and three antibiotics

Table 2 reports the results of disk diffusion tests performed with the antibiotics in zeolite-free growth media. The reference strain of *H. pylori* used in the experiments turned out resistant to metronidazole, sensitive to levofloxacin and extremely sensitive to amoxicillin, in fact no inhibition halo has developed in plates containing the first antibiotic, whereas bacterial growth has been completely inhibited in the dishes containing the discs impregnated with 2 μg of amoxicillin (Table 2). As regard the sensitivity of the bacterium to levofloxacin and amoxicillin, it can be noted that inhibition halos almost identical in diameter have been obtained by using discs containing, respectively, 5 μg and 0.5 μg of antibiotic (Table 2). Compared to the experiments performed without zeolite, the tests performed with metronidazole and levofloxacin in plates containing Na- or NH_4 -clinoptilolite have not evidenced differences of inhibition halos, whatever the concentration of the zeolite in the growth media (0.125 or 0.250 mg/mL). Such results lead to conclude that the two cation-forms of clinoptilolite do not develop a synergistic nor antagonist action with metronidazole and levofloxacin against the *H. pylori* (ATCC® 43504™) reference strain. Conversely, when amoxicillin has been tested in growth media containing Na-clinoptilolite, inhibition halos have evidenced an increase in diameter directly related with zeolite concentration (Table 3). In detail, the diameter of inhibition halo determined by

amoxicillin alone (53 mm - Table 2) has increased by 24% in plates containing 0.125 mg/mL of Na-clinoptilolite (Table 3), and by at least 70% for a zeolite concentration of 0.250 mg/mL (inhibition halos reached the edge of the plates - Table 3). It should be remarked that, in the tests carried out with amoxicillin in growth media containing NH₄-clinoptilolite, the zeolite has not affected the diameter of inhibition halos, whose dimensions resulted as in the test performed using only the antibiotic. The synergistic action of Na-clinoptilolite with amoxicillin in inhibiting the growth of the reference strain of *H. pylori* should be attributed to the subtraction of ammonium by cation exchange, since NH₄-clinoptilolite has not determined any effect.

3.3 Stability study of amoxicillin in a formulation with Na-clinoptilolite

The results above led to evaluate the stability of amoxicillin when combined with Na-clinoptilolite, because zeolites are hydrate minerals, and the solid state stability of amoxicillin depends on temperature and relative humidity (RH%) [36]; in particular, at a given temperature, degradation kinetic of the antibiotic increases with RH% [36,58]. Amoxicillin trihydrate (C₁₆H₁₉N₃O₅S·3H₂O) is a semisynthetic penicillin used in oral products, effective against a wide variety of organisms, both gram-negative (like *H. pylori*) and gram-positive [36]. Amoxicillin trihydrate (hereafter, AMX) crystallizes in the orthorhombic system with space group P2₁2₁2₁ and four molecules in a unit cell of dimensions $a = 15.62$, $b = 18.78$ and $c = 6.64$ Å [59]; Fig. 3 shows the X-ray pattern of the drug used. The formulation employed to study the stability of the antibiotic when combined with Na-clinoptilolite has been prepared as physical mixture (hereafter, PhM); the correspondent X-ray pattern is reported in Fig. 4. The content of antibiotic in PhM (33% by weight) ensures the possibility of a drug-clinoptilolite interaction

(important to verify if a zeolite-induced transformation of the antibiotic occurs), and a good detection of the peaks of the drug during the analyses in XRD and HPLC. As already reported for other antibiotics [23,27], also AMX molecule is too big to gain full access to the channel system of clinoptilolite, whose widest opening, formed by a compressed ten-membered ring, is $3.0 \times 7.6 \text{ \AA}$ [60]. A previous study [61], has not evidenced differences among the release profiles of pure AMX, AMX loaded onto the surface of Na-clinoptilolite by solvent evaporation or by sealing-heating, and AMX physically mixed with Na-clinoptilolite. Moreover, the release of AMX always resulted fast and complete [61]. Hence, even if a weak interaction between drug and zeolite would exist, it is not able to affect the release rate of the antibiotic, a situation that - in this context - can be regarded favorably, meaning that clinoptilolite should not determine a partial and/or delayed release of amoxicillin in the stomach, as instead occurs with other drugs [62]. In the present research, using a physical mixture reaches the purpose and is easier and cheaper compared to time-consuming procedures employed to load drugs onto zeolites, such as the solvent evaporation and the sealing-heating, or to methods that introduce a third component (such as a surfactant) which remains in the final formulation [29,63]. The XRD analyses have not evidenced variations of the pattern of amoxicillin trihydrate related to relative humidity and/or zeolite presence. In fact, regardless of RH% value, the diffractograms of AMX collected after 18 months overlap with those taken at the beginning of the stability study, and PhM behaved in the same way (Fig. S1 - Supplementary Data). It has been reported that amoxicillin trihydrate may reversibly change its X-ray pattern by dehydration and rehydration [36], however such phenomenon has not been observed in AMX and PhM. HPLC analyses have demonstrated that, after 18 months at 20 °C, the stability of the

antibiotic has not been significantly affected by the presence of Na-clinoptilolite, whatever the value of RH% in the range 20-80% (Fig. 5). The values of drug degradation (= 100 - drug content) determined at 20, 50, and 80% of RH% are 1.4, 2.9, and 2.2% for AMX, and 3.7, 3.9, and 2.2% for PhM. Taking into account the variation range of degradation values (see error bars in Fig. 5), a distinction between AMX and PhM based on the slight differences in stability observed at 20 and 50% of RH% would be questionable; moreover, in AMX and PhM the degradation of the drug at the highest relative humidity has resulted identical. It can be concluded that, at 20 °C and RH% from 20 to 80%, Na-clinoptilolite does not affect the stability of amoxicillin trihydrate. After 18 months, the degradation of the antibiotic contained in PhM has always been less than 4%, a value consistent with the literature data (10% after 3 years [64]) and much lower than that of some products marketed [65].

In eradicating *H. pylori*, a formulation containing both amoxicillin trihydrate and Na-clinoptilolite would have the advantage to provide, with one product, the components necessary to develop the synergistic action, a solution always preferable compared to multiple administrations.

4. Final Remarks and Conclusions

The *viable colony count test* has shown that Na-clinoptilolite is capable to reduce the growth of *H. pylori* reference strain (ATCC® 43504™) with an efficiency directly related to zeolite concentration. Compared to a zeolite-free control, a decrease in bacterial growth from 13 to 87% has been determined by Na-clinoptilolite in concentrations from 0.5 to 8 mg/mL. *In-vitro*, the growth of *H. pylori* has been

completely inhibited in media containing 30 mg/mL of Na- clinoptilolite, on the contrary, the same concentration of NH₄-clinoptilolite has allowed bacteria proliferation. Such results lead to relate the antibacterial activity of Na-clinoptilolite to its ability to remove ammonium through Na⁺ ↔ NH₄⁺ exchange. Sodium cations released by zeolite should not be responsible for inhibition as, in carrying out tests with *H. pylori*, suspending the bacteria in physiological solution (NaCl 9 mg/mL) is a standard procedure. On the other hand, the antibacterial activity of Na-clinoptilolite is weak, and an eradication therapy of *H. pylori* based solely on the use of Na-clinoptilolite can not be hypothesized. From this point of view, the observed synergistic action between Na-clinoptilolite and amoxicillin trihydrate is interesting, since occurred in growth media containing zeolite concentrations relatively low (0.125 - 0.250 mg/mL). Vice versa, the same concentrations of Na-clinoptilolite have not evidenced a synergy with metronidazole or levofloxacin against the tested *H. pylori* strain. Finally, at 20 °C, and for RH% between 20 and 80%, Na-clinoptilolite does not affect the solid state stability of amoxicillin trihydrate, thus formulations containing these two components do not present particular concerns as far the degradation of the antibiotic.

This research opens up interesting scenarios, worthy of further studies. Ammonium removal may be a good strategy to: i) weaken the protective "cloud" that the bacteria create around themselves, and ii) avoid the increase in vacuolization generated by the mutual interaction between ammonium and VacA toxin produced by *H. pylori* [20,21]. The evaluation of clinical strains of *H. pylori* would be very important, because they are usually more resistant than reference ones. Moreover, studies on the possible development of synergistic actions drug-zeolite should be widened, taking into consideration also other antibiotics, since there is a great variety of clinical *H. pylori*

strains, each characterized by different sensitivity (often by resistance) to the antibiotics currently used in eradication therapies [10].

Finally, it would be interesting to verify if clinoptilolite exchanged with Ag^+ or Zn^{2+} is more effective than Na-clinoptilolite against *H. pylori*. Indeed, the antimicrobial properties of silver and zinc ions are widely recognized and these metals have been tested, for example, in the field of dental and bone applications [66,67], but also in the eradication of *H. pylori* [12]. In this perspective clinoptilolite would act not only as a subtractor of ammonium, but also as a supplier of an antimicrobial cation.

Acknowledgments

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References

- [1] J. Khaton, R.P. Rai, K.N. Prasad, World J. Gastrointest. Oncol. 8 (2016) 147-158.
- [2] O. Sjomina, J. Pavlova, Y. Niv, M. Leja, Helicobacter 23 (2018) e12514.
- [3] D. Bravo, A. Hoare, C. Soto, M.A. Valenzuela, A.F.G. Quest, World J. Gastroenterol. 24 (2018): 3071-3089.

- [4] IARC, Schistosomes, liver flukes and *Helicobacter pylori*, in: Monogr. Eval. Carcinog. Risks Hum., vol. 61, International Agency for Research on Cancer Scientific Publications, Lyon, France, 1994, pp. 1–241.
- [5] IARC, *Helicobacter pylori*, in: Monogr. Eval. Carcinog. Risks Hum., vol. 100B, International Agency for Research on Cancer Scientific Publications, Lyon, France, 2012, pp. 385–435.
- [6] IARC, *Helicobacter pylori* Eradication as a Strategy for Preventing Gastric Cancer, in: International Agency for Research on Cancer, *Helicobacter pylori* Working Group Reports, No. 8. Lyon, France, 2014
(<http://www.iarc.fr/en/publications/pdfonline/wrk/wrk8/index.php>).
- [7] A. De Luca, G. Iaquinto, Cancer Lett. 213 (2004) 1–10.
- [8] D.J. Stewart, R. Ackroyd, Surgery 29 (2011) 568-574.
- [9] J. Bien, M. Bozko, N.P. Malek, P. Bozko, Curr. Pharm. Des. 20 (2014) 4487-4488.
- [10] S. Shiota, Y. Yamaoka, Curr. Pharm. Des. 20 (2014) 4489-4500.
- [11] D.J. Graham, L. Fischbach, Gut 59 (2010) 1143-1153.
- [12] D. Lopes, C. Nunes, M.C.L. Martins, B. Sarmiento, S. Reis, J. Control. Release 189 (2014) 169–186.
- [13] Z. Samra, H. Shmuely, Y. Niv, G. Dinari, D.J. Passaro, A. Geler, E. Gal, M. Fishman, J. Bachor, J. Yahav, J. Antimicrob. Chemother. 49 (2002) 1023-1026.
- [14] T.N. Phan, A. Santona, V.H. Tran, T.N.H. Tran, V.A. Le, P. Cappuccinelli, S. Rubino, B. Paglietti, Int. J. Antimicrob. Ag. 45 (2015) 244–248.
- [15] V.H. Tran, T.M.T. Ha, P.T.Q. Le, V.N. Nguyen, T.N. Phan, B. Paglietti, J. Infect. Dev. Countr. 12 (2018) 526-532.
- [16] M.I. Carretero, M. Pozo, Appl. Clay Sci. 46 (2009) 73-80.

- [17] J. Cervini-Silva, A. Nieto-Camacho, M.T. Ramírez-Apan, V. Gómez-Vidales, E. Palacios, A. Montoya, E. Ronquillo de Jesús, *Colloids Surf. B Biointerfaces* 129 (2015) 1–6.
- [18] K.D. Morrison, R. Misra, L.B. Williams, *Sci. Rep.* 6 (2016) 19043 doi: 10.1038/srep19043.
- [19] V. Iannuccelli, E. Maretti, M. Montorsi, C. Rustichelli, F. Sacchetti, E. Leo, *Int. J. Pharm.* 493 (2015) 295-304.
- [20] V. Ricci, P. Sommi, R. Fiocca, M. Romano, E. Solcia, U. Ventura, *J. Pathol.* 183 (1997) 453-459.
- [21] C. Montecucco, E. Papini, M. de Bernard, M. Zoratti, *FEBS Letters* 452 (1999) 16-21.
- [22] T. Armbruster, *Stud. Surf. Sci. Catal.* 135 (2001) 13-27.
- [23] D. Papaioannou, P.D. Katsoulos, N. Panousis, H. Karatzias, *Micropor. Mesopor. Mat.* 84 (2005) 161-170.
- [24] W. G. Pond, J. T. Yen, D. A. Hill, *Proc. Soc. Exper. Biol. Med.* 166 (1981) 369-373.
- [25] G. Rodríguez-Fuentes, M.A. Barrios, A. Iraizoz, I. Perdomo, B. Cedré, *Zeolites* 19 (1997) 441-448.
- [26] K. Pavelić, M. Hadžija, in: S.M. Ayerbach, K.A. Carrado, P.K. Dutta, (Eds), *Handbook of Zeolite Science and Technology*, Markel Dekker Inc., New York, 2003, 1128-1159.
- [27] G. Cerri, M. de' Gennaro, M.C. Bonferoni, C. Caramella, *Appl. Clay Sci.* 27 (2004) 141-150.

- [28] M.C. Bonferoni, G. Cerri, M. de' Gennaro, C. Juliano, C. Caramella, *Appl. Clay Sci.* 36 (2007) 95-102.
- [29] J. Milić, A. Daković, D. Krajišnik, G.E. Rottinghaus, in: A. Tiwari (Ed), *Advanced Healthcare Materials*, John Wiley & Sons, Inc., Hoboken, 2014, 361-403.
- [30] T. Selvam, W. Schwieger, W. Dathe, *Clay Miner.* 49 (2014) 501-512.
- [31] T. Selvam, W. Schwieger, W. Dathe, *Environ. Geochem. Health* 40 (2018) 2657-2665.
- [32] C. Laurino, B. Palmieri, *Nutr. Hosp.* 32 (2015) 573-581.
- [33] J. Cervini-Silva, A. Nieto-Camacho, S. Kaufhold, K. Ufer, E. Palacios, A. Montoya, W. Dathe, *Micropor. Mesopor. Mat.* 228 (2016) 207-214.
- [34] M. Cutovic, M. Lazovic, V. Vukovic-Dejanovic, D. Nikolic, I. Petronic-Markovic, D. Cirovic, *J. Altern. Complement. Med.* 23 (2017) 9.
- [35] G. Cerri, M. Farina, A. Brundu, A. Daković, P. Giunchedi, E. Gavini, G. Rasso, *Micropor. Mesopor. Mat.* 223 (2016) 58-67.
- [36] A.E. Bird, Amoxicillin, in: H.G. Brittain (Ed), *Analytical Profile of Drug Substances and Excipients*, vol. 23, Academic Press, San Diego, California, 1994, pp. 1-52.
- [37] [U.S. Environmental Protection Agency, Method 1687, \(2001\) EPA-821-R-01-004.](#)
- [38] A. Brundu, G. Cerri, E. Sale, *Minerals* 7 (2017) 11.
- [39] F. Megraud, P. Lehours, *Clin. Microbiol. Rev.* 20 (2007) 280–322.
- [40] A.L. Barry, M.B. Coyle, C. Thornsberry, E.H. Gerlach, R.W. Hawkinson, *J. Clin. Microbiol.* 10 (1979) 885-889.
- [41] P.W. Winston, D.H. Bates, *Ecology* 41 (1960) 232-237.

- [42] Q. Pei, GP. Yang, ZL. Li, XD. Peng, JH. Fan, ZQ. Liu, J. Chromatogr. B 879 (2011) 2000-2004.
- [43] M.J. Semmens, M. Seyfarth, in: L.B. Sand, F.A. Mumpton (Eds), Natural Zeolites: Occurrence, Properties, Use, Pergamon Press, New York, 1978, 517-526.
- [44] M. Sprynskyy, M. Lebedynets, A.P. Terzyk, P. Kowalczyk, J. Namieśnik, B. Buszewski, J. Colloid Interface Sci. 284 (2005) 408–415.
- [45] A. Brundu, G. Cerri, Micropor. Mesopor. Mat. 208 (2015) 44–49.
- [46] A. Brundu, G. Cerri, Int. J. Environ. Sci. Technol. 14 (2017) 223–232.
- [47] R. Pabalan, F.P. Bertetti, in: D.L. Bish, D.W. Ming (Eds.), Natural Zeolites: Occurrence, Properties, Applications, Reviews in Mineralogy and Geochemistry, Mineralogical Society of America, Washington, vol. 45, 2001, pp. 453-518.
- [48] C. Gomes, J. Silva, Appl. Clay Sci. 36 (2007) 4-21.
- [49] M.A. Jama, H. Yücel, Sep. Sci. Technol., 24 (1989) 1393-1416.
- [50] H.T. Dryden, L.R. Weatherley, Aquacult. Eng. 6 (1987) 51-68.
- [51] R.M. Barrer, J. Klinowski, J. Chem. Soc., Faraday Trans. 1 70 (1974) 2080-2091.
- [52] A. Arcoya, J.A. González, G. Llabre, X.L. Seoane, N. Travieso, Microporous Mater. 7 (1996) 1-13.
- [53] G. Cerri, A. Langella, M. Pansini, P. Cappelletti, Clays Clay Miner. 50 (2002) 127-135.
- [54] A. Jayaraman, A.J. Hernandez-Maldonado, R.T. Yang, D. Chinn, C.L. Munson, D.H. Mohr, Chem. Eng. Sci. 59 (2004) 2407 – 2417.
- [55] G. Pérez-Pérez, C.B. Gower, M.J. Blaser, Infect. Immun. 62 (1994) 299-302.
- [56] P.J. Neuvonen, Drugs 11 (1976) 45–54.

- [57] M.M. Gerrits, M. Berning, A.H.M. Van Vliet, E.J. Kuipers, J.G. Kusters, *Antimicrob. Agents Chemother.* 47 (2003) 2984-2986.
- [58] J. Xue, K. Zhu, X. Chong, Y. Wang, C. Hu, *Chin. Pharm. J.* 3 (2016) 224-229.
- [59] M.O. Boles, R.J. Girven, P.A.C. Gane, *Acta Cryst.* B34 (1978) 461-466.
- [60] T. Armbruster, M.E. Gunter, in: D.L. Bish, D.W. Ming (Eds.), *Natural Zeolites: Occurrence, Properties, Applications, Reviews in Mineralogy and Geochemistry*, Mineralogical Society of America, Washington, vol. 45, 2001, pp. 1-67.
- [61] M. Farina, *Zeoliti naturali in formulazioni farmaceutiche per uso orale: preparazione, valutazione e possibile impiego di un materiale a base di clinoptilolite*, Ph.D. Thesis, Università degli Studi di Sassari, Italy, 2016.
- [62] D. Krajišnik, A. Daković, A. Malenović, L. Djekić, M. Kragović, V. Dobričić, J. Milić, *Micropor. Mesopor. Mat.* 167 (2013) 94-101.
- [63] B. de Gennaro, A. Fenti, S. Salvestrini, *Adv. Sci. Lett.* 23 (2017) 5944-5947.
- [64] R. Mendez, M.T. Alemany, C. Jurado, J. Martin, *Drug Dev. Ind. Pharm.*, 15 (1989) 1263-1274.
- [65] H. Arshad, S. Bashir, S. Nazir, H.M.M. Asjad, N. Irshad, A. Razzaque, A. Malik, *Int. J. Adv. Pharm. Res.* 4 (2013) 2275-2282.
- [66] O.-M. Goudouri, E. Kontonasaki, U. Lohbauer, A.R. Boccacini, *Acta Biomaterialia* 10 (2014) 3795-3810.
- [67] A. De Mori, M. Hafidh, N. Mele, R. Yusuf, G. Cerri, E. Gavini, G. Tozzi, E. Barbu, M. Conconi, R. Draheim, M. Roldo, *Pharmaceutics* 11 (2019) 116.

Figure captions

Fig. 1. *Viable Colony Count test*: inhibition (I%) of *H. pylori* growth with FA-Na concentration of 0.5 (A), 1 (B), 2 (C), 4 (D), and 8 (E) mg/mL.

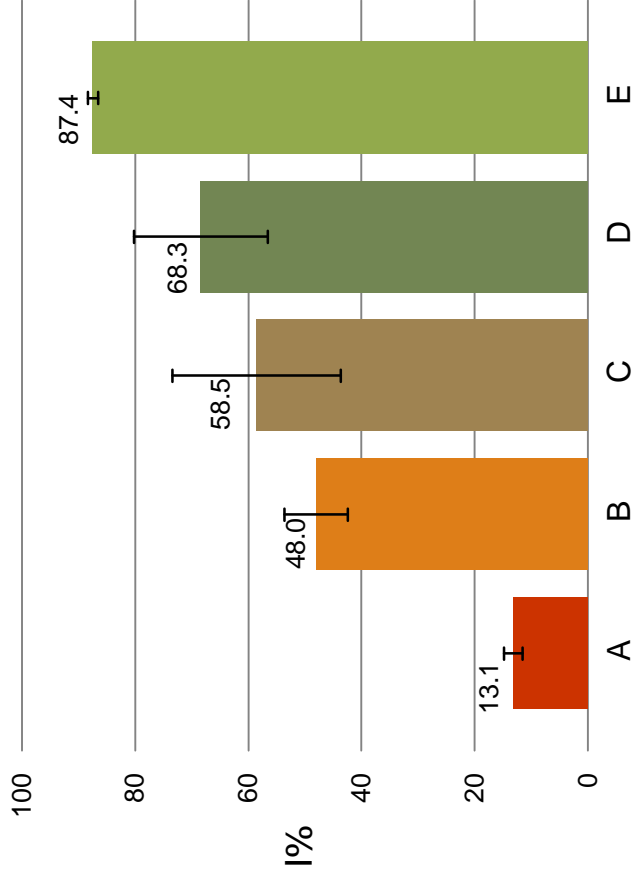
Fig. 2. Comparison of *H. pylori* growth in zeolite-free media (center), containing Na-clinoptilolite (left), or NH₄-clinoptilolite (right). **In the latter plate colonies look even more developed compared to the control, whereas in the dish with Na-clinoptilolite only two small whitish stains can be detected (see arrows), that is the residue of NaCl left by the bacterial suspension.**

Fig. 3. XRD pattern of amoxicillin trihydrate (AMX).

Fig. 4. XRD pattern of the physical mixture (PhM = $\frac{2}{3}$ FA-Na + $\frac{1}{3}$ of amoxicillin trihydrate). Black bars: clinoptilolite (PDF N. 80-0464). Fuchsia bars: amoxicillin trihydrate (PDF N. 39-1832).

Fig. 5. Drug content, referred to the initial one, measured after a storage of 18 months at 20±2 °C and at the RH% indicated (HPLC determinations).

Figure(s)

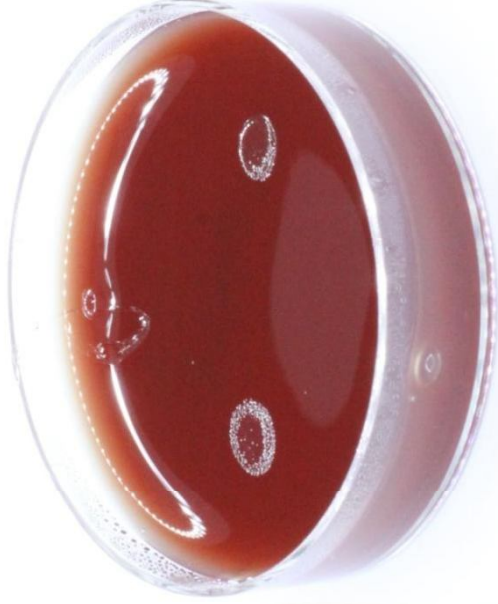


Figure(s)

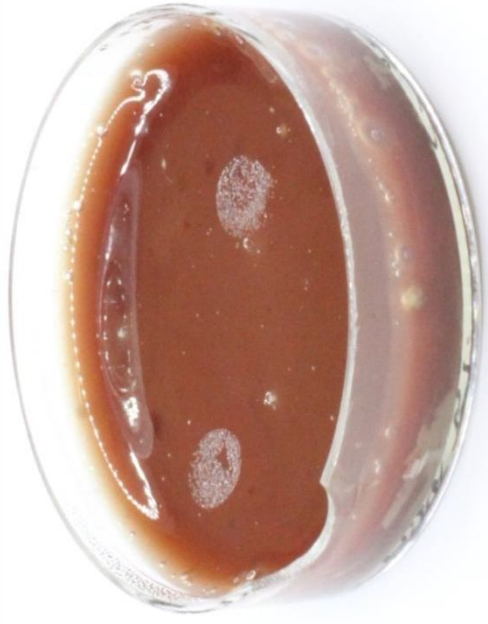
FA-Na 30 mg/mL



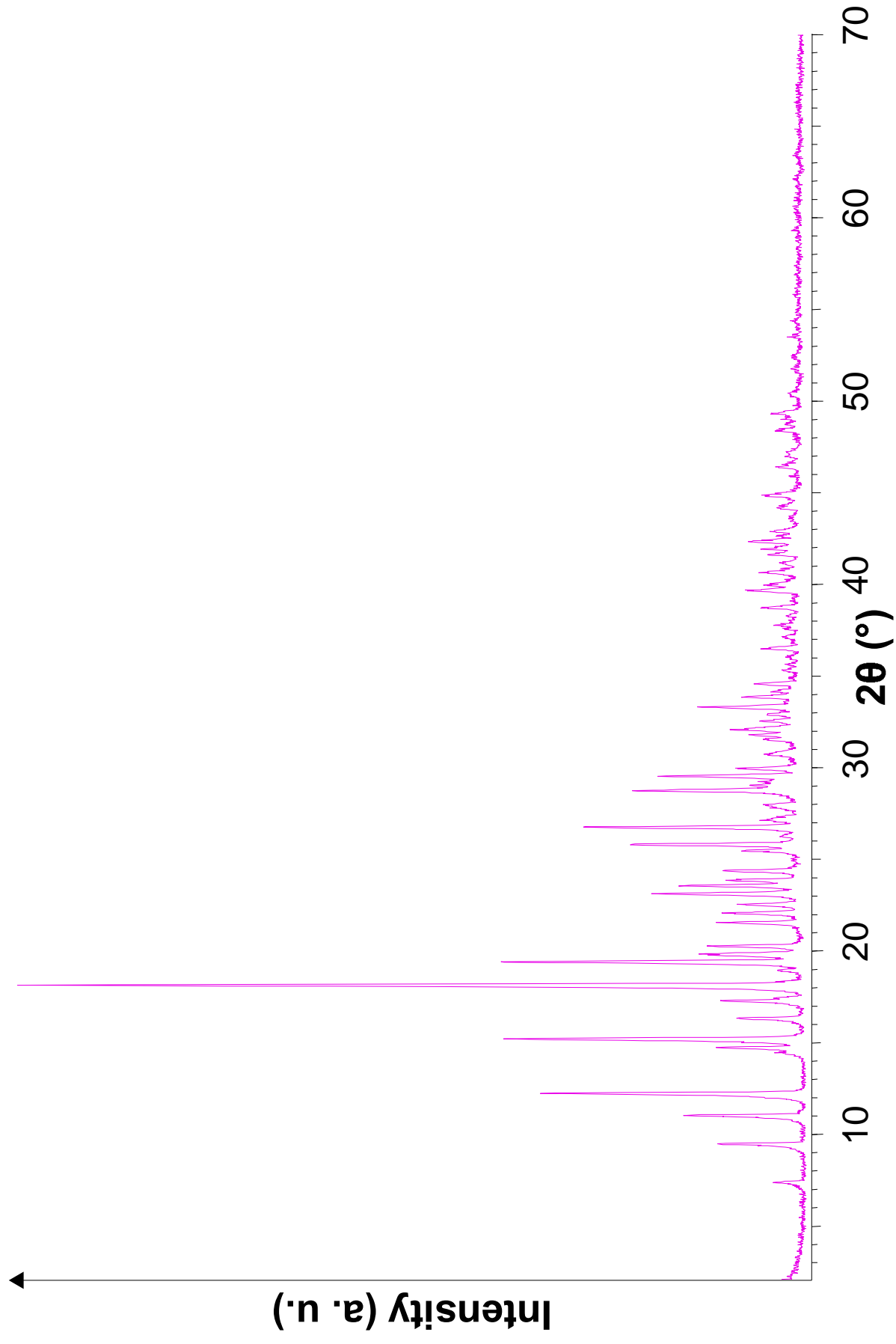
Control (no zeolite)



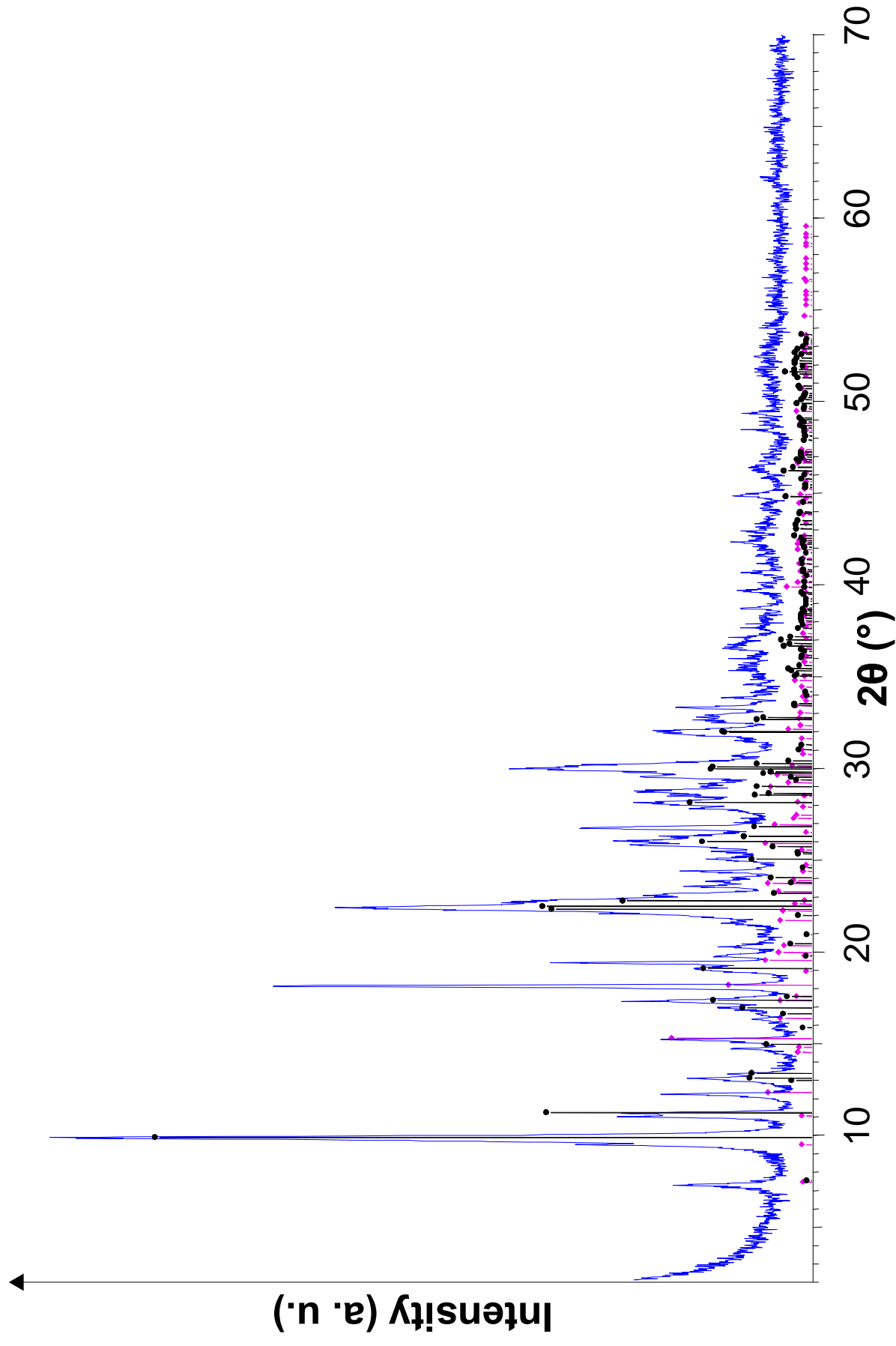
FA-NH₄ 30 mg/mL



Figure(s)



Figure(s)



Figure(s)

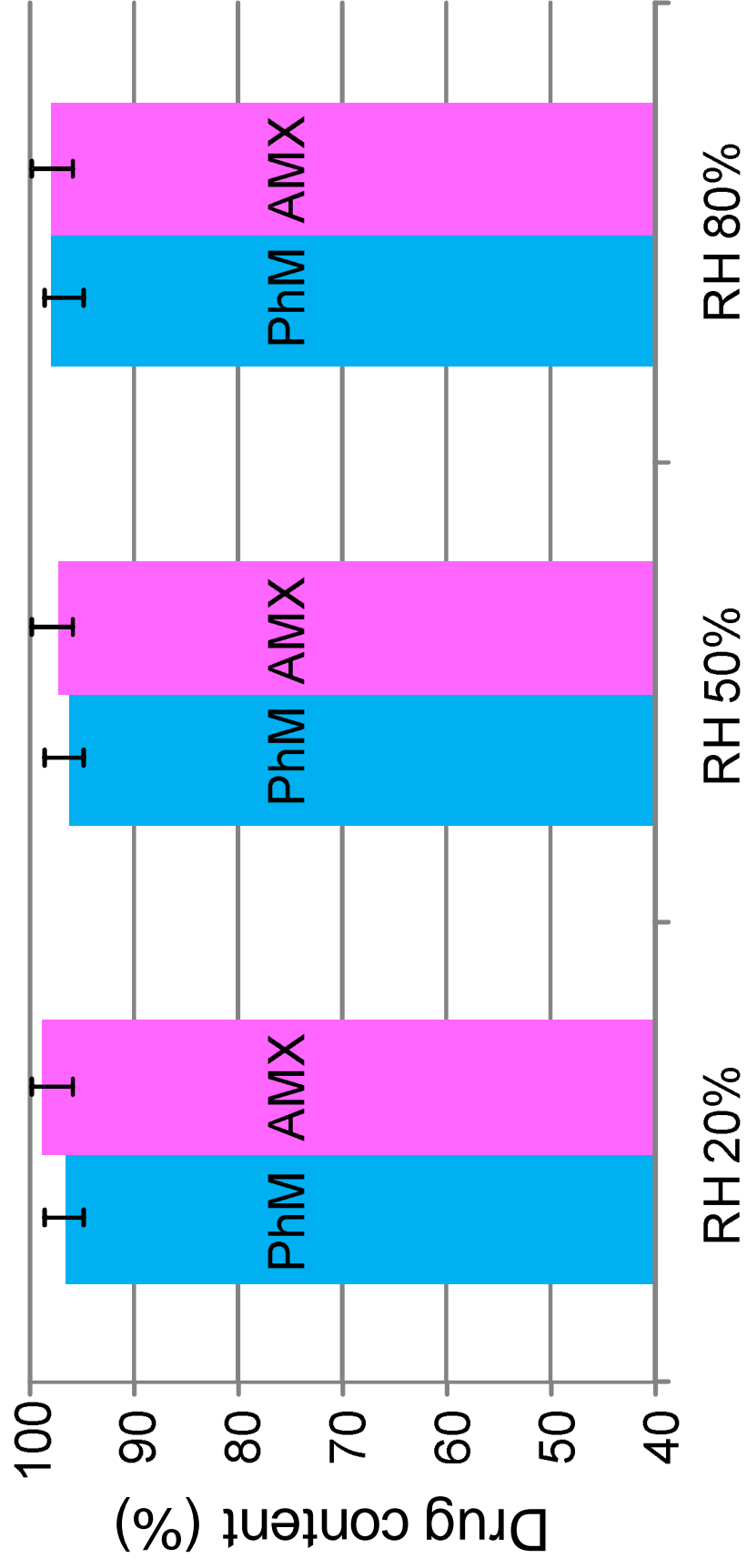


Table 1Chemical composition (in wt.%) of FA-NH₄ and FA-Na (*taken from Ref. 35).

	SiO ₂	Al ₂ O ₃	Fe ₂ O ₃	MnO	MgO	CaO	Na ₂ O	K ₂ O	TiO ₂	P ₂ O ₅	H ₂ O	(NH ₄) ₂ O	TOT
FA-NH ₄	67.93	12.94	0.72	0.01	0.26	0.27	0.16	0.36	0.21	0.03	11.46	5.75	100.10
*FA-Na	65.26	12.11	0.50	<0.01	0.51	0.35	5.85	0.43	0.18	0.05	14.76	---	100.01

Table 2

Disk diffusion test: results obtained with antibiotics in zeolite-free growth media.

	Metronidazole (5 µg)	Levofloxacin (5 µg)	Amoxicillin (2 µg)	Amoxicillin (0.5 µg)
Diam. inhibition	no inhibition	55	≥ 90	53
halo (mm)	no inhibition	55	≥ 90	53

Table 3

Disk diffusion test: results obtained with amoxicillin in growth media containing Na-clinoptilolite.

	Fa-Na	Diam. inhib. halo (mm)	Increase diam, inhib. halo
Amoxicillin (0.5 µg)	0.25 mg/mL	≥ 90	≥ 70%
		≥ 90	
	0.125 mg/mL	66	24%
		65	