Antimicrobial drug susceptibility tests involving multiple time-consuming steps are still used as reference methods. Today, there is a need for the development of new automated instruments that can provide faster results and reduce operating time, reagent costs, and labor requirements. Nuclear magnetic resonance (NMR) spectroscopy meets those requirements. The metabolism and antimicrobial susceptibility of *Escherichia coli* ATCC 25922 in the presence of gentamicin have been analyzed using NMR and compared with a reference method. Direct incubation of the bacteria (with and without gentamicin) into the NMR tube has also been performed, and differences in the NMR spectra were obtained. The MIC, determined by the reference method found in this study, would correspond with the termination of the bacterial metabolism observed with NMR. Experiments carried out directly into the NMR tube enabled the development of antimicrobial drug susceptibility tests to assess the effectiveness of the antibiotic. NMR is an objective and reproducible method for showing the effects of a drug on the subject bacterium and can emerge as an excellent tool for studying bacterial activity in the presence of different antibiotic concentrations.
Escherichia coli is one of the main causes of nosocomial infections in humans, and the selection of antibiotic resistance mechanisms in its pathogenic and nonpathogenic isolates are associated with the widespread use of antibiotics (20). This work focuses on the use of $^1$H NMR spectroscopy compared with a broth macrodilution reference method to study the antimicrobial susceptibility of E. coli ATCC 25922 (21).

**MATERIALS AND METHODS**

**Organism.** The bacterial strain used for the current study was E. coli ATCC 25922.

**Antimicrobial agent.** A stock solution of gentamicin pure powder (Sigma-Aldrich Co., St. Louis, MO) was made up in sterile water. Serial dilutions of the drug (0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml) were prepared.

**Broth macrodilution MICs.** The isolation of bacteria was carried out on brain heart infusion (BHI) agar (Panrec Quimica S.L.U., Barcelona, Spain) to check bacterial viability at 37°C for 24 h. After that, resiolation was performed to ensure a pure culture in the same conditions. The inoculum of bacteria was prepared to obtain a suspension with a turbidity of a 0.5 McFarland standard, using the direct suspension method. For this, the bacterial suspension was prepared in 5 ml of sterile saline solution (NaCl 0.9%) by matching its turbidity to a 0.5 McFarland standard resulting in a viable bacterial count of $10^6$ CFU/ml. MICs were determined by the reference macrodilution method of the Clinical and Laboratory Standards Institute (22). Samples and control solutions were prepared in a total volume of 2 ml containing bacterial suspension, antimicrobial gentamicin, and Mueller-Hinton medium (Becton Dickinson Microbiology Systems, Sparks, MD). Serial dilutions of gentamicin were tested. A concentration of 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, or 64 μg/ml was added into each tube. Control I was the Mueller-Hinton medium with an inoculum of bacteria prepared under similar conditions as the samples but without an antibiotic, and control II was only Mueller-Hinton medium. All samples and controls were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of the drug that completely inhibited visible bacterial growth. For the viable cell count, culture plates were prepared with BHI agar medium. From samples and controls, different dilutions were prepared into sterile water and uniformly spread with sterile spreaders on the plates. All plates were incubated at 37°C for 24 h, and after that time, bacterial colonies were counted manually. The total numbers of bacteria in each sample and control I were determined from the average colony count obtained from different dilutions.

**Broth macrodilution samples for NMR spectroscopy.** After incubation for 24 h, specimens of 0.540 ml from each sample and control were transferred into 5-mm NMR tubes (5-mm high-precision NMR sample tubes, Norell Inc., Mayslanding, NJ). Furthermore, 60 μl of trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) (sodium salt; Sigma-Aldrich Co., St. Louis, MO) diluted in deuterated water (D$_2$O; deuterium oxide, 2,2,3,3-tetadeuteropropionic acid, sodium salt; Sigma-Aldrich Corp., St. Louis, MO) was added to each tube. Control I was the Mueller-Hinton medium with an inoculum of bacteria prepared under similar conditions as the samples but without antibiotic, and control II was only Mueller-Hinton medium. All samples and controls were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of the drug that completely inhibited visible bacterial growth. For the viable cell count, culture plates were prepared with BHI agar medium. From samples and controls, different dilutions were prepared into sterile water and uniformly spread with sterile spreaders on the plates. All plates were incubated at 37°C for 24 h, and after that time, bacterial colonies were counted manually. The total numbers of bacteria in each sample and control I were determined from the average colony count obtained from different dilutions.

**$^1$H NMR experiments.** The equipment used for the acquisition of the spectra was a Bruker Avance 400 spectrometer UltraShield (Avance II 400 MHz, Bruker Corporation, Regensburg, Germany) equipped with a BBI H-BB Z-GRD probe, specifically for proper acquisition of the proton. Spectra acquisition was carried out with the program TOPSPIN version 2.1 (Bruker Corporation, Regensburg, Germany). Processing of spectra was carried out under the program MestReNova version 8.1 (Mestrelab Research, Santiago de Compostela, Spain). Before spectra acquisition, the magnetic field was adjusted for the mix H$_2$O-D$_2$O, and all experiments were performed at 25°C. $^1$H NMR spectra were recorded with a pulse sequence signal presaturation of water located at 1,875 Hz using the pulses zgpr program. For a large and quantitative experiment, we used a relaxation time (d1) of 10 s and a number of scans (ns) of 64 to give a total acquisition time of 20 min. For a short experiment, we used a d1 of 2 s and an ns of 8 to give a total acquisition time of 3 min.

**RESULTS**

Stack plots of one-dimensional spectra of incubated bacterial media of E. coli ATCC 25922 with gentamicin and spectra of control media (control II) and control of the incubated media without an antibiotic in the presence of bacteria (control I) are shown in Fig. 1.

The comparison of the two control $^1$H NMR spectra showed different signals in the range of 1.0 to 2.5 ppm. Succinic acid, acetic acid, and ethanol were only detected in the control I spectra and threonine was only detected in the control II medium. These signals were assigned through comparison of their chemical shifts with those reported in the literature (16) and by comprising the spiking with the corresponding compounds in the control samples.

When we registered antibiotic spectra at different concentrations, we detected the presence of succinic acid, acetic acid, and ethanol only in samples with concentrations of gentamicin lower than 0.5 μg/ml. Moreover, when the concentration of gentamicin was greater than 0.5 μg/ml, we detected the presence of threonine (Fig. 1).

According to the results obtained by visual turbidity, the lowest concentration of drug that completely inhibited visible growth (MIC) was 0.5 μg/ml (MIC, 0.25 to 1 μg/ml) (22). These data suggested that the results obtained by $^1$H NMR spectroscopy were in agreement with those obtained by visual turbidity. A viable count of culture plates showed no bacterial growth on plates with gentamicin concentrations from 64 to 1 μg/ml. However, the plate containing 0.5 μg/ml of gentamicin showed a viable bacterial number in the range of $10^6$ CFU/ml. The remaining samples (0.25 to 0.06 μg/ml, including control I) demonstrated a viable bacterial count in the range of $10^3$ to $10^5$ CFU/ml. Data extracted from the counts of viable bacterial growth on the agar plates allowed us to obtain the minimal bactericidal concentration (MBC), that is, the lowest concentration of gentamicin that kills the bacteria. In this case, MBC was observed at 1 μg/ml.

Figure 2 shows the time course evolution of peak areas corresponding to metabolites (succinic acid, acetic acid, and ethanol) or nutrient (threonine) as a function of antimicrobial gentamicin concentration for the E. coli strain used. The results obtained from spectra showed that E. coli is able to metabolize components of the medium to produce succinic acid, acetic acid, and ethanol. The intensity of these signals varied according to the viable bacteria (CFU/ml) present in the sample, indicating that the intensity was higher in the samples with concentrations of gentamicin lower than the MIC (0.5 μg/ml) in the rest of the samples. Furthermore, threonine only appeared in the spectra of those samples with gentamicin concentrations of ≥0.5 μg/ml.

We were also able to optimize the acquisition time of the NMR experiments. We spent only 3 min on each experiment, conse-
quetly decreasing the analysis time considerably without information loss.

To evaluate the potential of this tool, we carried out the same biological experiments but, in this case, using an NMR tube as the incubation reactor. We introduced into the NMR tube the infected culture medium, previously prepared, and registered NMR experiments at 37°C every 20 min. We observed that the ethanol signal appeared at 3 h 40 min while the disappearance of the threonine signal occurred at 5 h 20 min (Fig. 3A). From this data, we can confidently establish that bacterial activity occurred effectively within the NMR tube, and the metabolic process started around 3 h 20 min and ended at 6 h.

Thus, we introduced the infected culture media supplemented by several gentamicin concentrations, previously prepared, into the NMR tubes. Stacked plots of the one-dimensional spectra of incubated bacteria in the NMR tube corresponding to concentrations of 0.06 and 0.125 μg/ml are shown in Fig. 3B and C, respectively. The ethanol signal appeared later using 0.06 μg/ml of gentamicin (4 h 40 min) compared with the experiments performed in the absence of the antibiotic (3 h 20 min) and much later (8 h 40 min) when the gentamicin concentration used was close to MIC (0.125 μg/ml). Similarly, threonine consumption by bacteria was delayed when the concentration of antibiotic in the medium was higher.

**DISCUSSION**

*E. coli* is a facultative aerobic bacterium able to ferment sugars into a mixture of different compounds, such as acetic and succinic acids, among others, and ethanol by a mixed-acid type fermentation (Fig. 1) (23, 24). Differences in peak intensities for these metabolites observed in spectra allowed us to determine the MIC of gentamicin using NMR spectroscopy. Consumption of the amino acid threonine, present in the culture medium, was interrupted when MIC was achieved. It is well-known that threonine is involved not only in the metabolism of *E. coli* but also in its regulation, and it is one of the amino acids that bacteria quickly catabolize (25, 26). Therefore, we assume that succinic acid, acetic acid, and ethanol are metabolites produced by bacteria and threonine is an amino acid consumed by *E. coli*. These data indicate that microbial activity ceases when the concentration of antibiotic is 0.5 μg/ml.

Although the time taken to perform each experiment (NMR spectroscopy and reference method) was the same, the use of NMR allowed us to observe the bacterial metabolism, ensuring the inviability of the bacteria when MIC was reached. Furthermore, the use of NMR offered us the possibility to determine the viable bacterial count faster than using the reference method. With our results, we can firmly conclude that NMR is able to detect MIC.
correctly. Although the viable count of the plates showed bacteria in the 0.5 μg/ml agar plate (range of 10^6 CFU/ml), 1H NMR did not detect bacterial metabolism at this range or at MBC. This result suggests that the range of 10^6 CFU/ml may be the limit of detection for the NMR spectrometer used or the limit in which the population of bacteria activates the metabolism.

The experiments carried out using the NMR tube as an incubation reactor enabled the development of the AST experiments directly in the NMR tube to assess the effectiveness of the antibiotic. This NMR technique can become a useful tool for studying bacteria that do not grow on habitual media, whose metabolism is not well known, and for those bacteria that standardized antimicrobial susceptibility tests are not available.

There is a complex relationship between concentrations of antibiotic and growth and death rates of bacteria. This functional relationship, which is called pharmacodynamics, is an important parameter in the rational design of effective antibiotic treatment protocols (27). The bacterial behavior throughout several concentrations of antibiotic can usually be examined by killing or growth curves, which enables the attainment of additional information regarding antimicrobial properties of antibiotics in vitro, particularly with regard to their initial bactericidal activity against different pathogens (28). Killing or growth curves can be used to study anti-infective effects in in vitro models, with the advantage of providing more detailed information about the time course of antibacterial effect (29). As we have shown in the previous experiment, NMR is an outstanding technique to study the time course evolution of a biological process.

Additionally, the introduction of the infected culture media in the presence of several concentrations of the antibiotic in NMR tubes allowed us to evaluate the kinetic behavior of bacteria in the presence of gentamicin. As we can see in Fig. 3, it is very easy to follow the bacterial growth using NMR spectroscopy. Taking into account that studies using kill or growth kinetics are expensive in terms of time and cost and that they are not likely to be performed in a routine laboratory, this novel approach using the NMR technique might become a powerful tool for studying antimicrobial drugs.

NMR spectroscopy is a suitable tool to be considered an alternative to those methods that show difficulties in accuracy and reproducibility (30). Moreover, recently Gupta et al., using the 1H NMR metabolic approach, identified and quantified E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Proteus mirabilis isolates in urine samples from patients with urinary tract infections (UTIs) faster than using culture methods. This NMR method of metabolic profile exploration in urine provided an alternative approach for screening and identification of UTI (31–34). These perspectives, along with the study reported in this paper, suggest the great potential of 1H NMR for antimicrobial analysis. In the field of fungi infections, drug-induced inhibition of fungal growth is used in the diagnostic laboratory to predict the therapeutic efficacies of antifungal agents. NMR spectroscopy has emerged as a modern and powerful tool for detecting subtle effects on fungal metabolism. Specifically, in the NMR spectra, inhibition of fungal growth through metabolite changes, such as ethanol, glucose, succinate, or acetate, may be detected (35–37). Our team has initiated more complex studies using NMR with fastidious microorganisms such as Rickettsia spp. with promising results (38).

Future application of this NMR tool in microbiology requires
large studies involving metabolite identification, obtaining metabolic profile information, and correlating the data to find predictive models for validation and standardization. Costs of this technique (hardware and software installation) are relatively high, but in the long term they are diminished due to the reduced operating time, cost of reagents, low-maintenance, and the small quantities necessary per sample compared with conventional methods or with methods based on molecular biology. The use of few and inexpensive reagents allow for this technique to be used in clinical laboratories, in a similar way to how it is used for the analysis of food in reference centers. Moreover, the new technology of low-field magnets that can be in the benches of laboratories can provide a great opportunity to introduce the methodology in the daily routine. Further antimicrobial susceptibility studies directly applied to clinical samples can show differences in the results compared with in vitro studies where the stability and the composition of the culture medium is usually well known and differs from the organism composition.

In conclusion, the results obtained from this study on the use of 1H NMR in antimicrobial susceptibility analyses indicate that NMR is an objective and reproducible method to show the drug effect on the bacteria studied. MIC determined by the macrodilution method (CLSI) would correspond with the end of the bacterial metabolism observed by NMR, which also predicts the MIC. In addition, we demonstrated that the NMR technique can emerge as an excellent tool for studying the bacterial behavior in the presence of different concentrations of antibiotic. As we have demonstrated, further studies and the automatization of the technique can provide a rapid and reproducible method for studying antimicrobial drug susceptibility and bacterial identification.

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REFERENCES

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