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Research Letter

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Biomodification of acenocoumarol by bifidobacteria

Melisa Fragomeno[1], Sabrina Assad[1], Pablo Mobili[1], Pablo J. Peruzzo[3], Jessica Minnaard[1,2,*,†] and Pablo Fernando Pérez[1,2]

[1]Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA)-Universidad Nacional de La Plata (UNLP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET- CCT La Plata) and Consejo de Investigaciones Científicas de la Provincia de Buenos Aires (CICPBA). Calle 47 y 116, CP 1900, La Plata, Argentina, [2]Área Microbiología e Inmunología, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, UNLP. Calle 47 y 115, CP 1900, La Plata, 13, Argentina and [3]Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas - INIFTA (UNLP – CONICET CCT La Plata), Diag. 113 y 64, CC 16 Suc. 4 (B1904DPI) La Plata, Argentina

[*]**Corresponding author:** Centro de Investigación y Desarrollo en Criotecnología de Alimentos, Calle 47 y 166, CP1900, La Plata, Argentina. Tel: +54 221 4254853; E-mail: jm@biol.unlp.edu.ar

One sentence summary: Bifidobacterial isolates of human origin biomodified in vitro the essential structure of anticoagulant acenocoumarol.

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[†]Jessica Minnaard, https://orcid.org/0000-0003-3782-0187

ABSTRACT

The increased interest of consumers in probiotic foods requires a deeper knowledge on the possible interactions with drugs, because their pharmacological properties could be modified. In this context, these studies are relevant for drugs such as acenocoumarol, whose dosage must be controlled due to, among other factors, food-drug interactions. Acenocoumarol is an oral anticoagulant with a narrow therapeutic range. The aim of the present research is to evaluate, *in vitro*, the effect of bifidobacteria on acenocoumarol. The drug was incubated with *Bifidobacterium bifidum* CIDCA 5310 or *Bifidobacterium adolescentis* CIDCA 5317 in MRS broth at 37°C for 24 h in anaerobic conditions. The effect of incubation with sterilized spent culture supernatants (SSCS) was also evaluated. Analysis by RP-HPLC showed that both bifidobacterial strains reduced the area of the acenocoumarol peak and two new peaks were evidenced. In addition, a decrease in the intensity of the bands at 1650, 1390 and 1110/cm was observed in the FTIR spectroscopic determinations. Moreover, a new band appeared at 1720/cm. No effect on the drug was observed when incubation was performed with SSCS. The present study showed a significant change in the concentration of the anticoagulant after incubation with bifidobacteria and results are compatible with biomodification of the drug due to enzymatic activity of bifidobacteria.

Keywords: bifidobacteria; biomodification; enzymes; acenocoumarol; probiotic; anticoagulants

INTRODUCTION

Probiotic microorganisms have been extensively studied, evidencing many beneficial properties when administrated in appropriate doses to the host. To highlight some of them, antioxidant activity, enhanced immune response and prevention of diseases caused by pathogenic bacteria can be mentioned (Joung *et al.* 2021; Kim *et al.* 2018; Plaza-Diaz *et al.* 2019; Cruz *et al.* 2021; Xia *et al.* 2021).

It is worth noting that probiotics have shown the ability to modify drug pharmacokinetics (Kato *et al.* 2007; Lee *et al.* 2012; Matuskova *et al.* 2014; Stojančević *et al.* 2014; Kim *et al.* 2018). In an *in vivo* assay in rats, an increase in amiodarone bioavailability was observed in animals administered with *Escherichia*

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coli strain Nissle 1917 (Matuskova *et al.* 2014). Similar results were observed when nifedipine was administered concomitantly with *Lactobacillus casei* (Kato *et al.* 2007). On the other hand, the metabolic activity of probiotics on sulfasalazine was demonstrated, although this activity did not alter the blood concentrations compared to the control group of rats which did not receive the probiotic strains (Lee *et al.* 2012).

Probiotics could modify pharmacokinetics by affecting either the permeability of the intestinal epithelium or the activity of cytochromes, which are mainly responsible for the metabolism of many drugs (Matušková *et al.* 2011, 2014; Selwyn *et al.* 2016). Additionally, indirect effects of probiotic microorganisms, such as modification of intestinal microbiota, also contribute to the metabolization of many xenobiotics (Sousa *et al.* 2008; Stojančević *et al.* 2014; Jourova, Anzenbacher and Anzenbacherova 2016). Many studies describe the interaction with drugs of bacteria residing in the human gut, and how they can affect their pharmacodynamics and pharmacokinetics (Sousa *et al.* 2008; Jourova, Anzenbacher and Anzenbacherova 2016; Wilkinson, Ilhan and Herbst-Kralovetz 2018). It has been described, for example, that the action of bacterial reductases and hydrolases can generate nonpolar compounds (Sousa *et al.* 2008). The large number and variety of microorganisms in the intestine would place this organ at the same level as the liver in terms of drug metabolism (Stojančević *et al.* 2014).

Acenocoumarol is an anticoagulant derived from 4-hydroxicoumarin. It is structurally similar to warfarin but it presents a nitro group in the 4´ position (Thijssen, Baars and Reijnders 1983; Saraeva *et al.* 2007). Acenocoumarol is a vitamin K antagonist that inhibits the enzyme vitamin K epoxide reductase, thus interfering in the carboxylation of the coagulation factors (Militaru *et al.* 2015). It is one of the most widely used oral anticoagulants after warfarin (Gschwind *et al.* 2013; Militaru *et al.* 2015) and the commercial presentations are racemic mixtures of S (-) and R (+) enantiomers. The R form elicities most of the therapeutic effects due to its longer half-life, compared to the S form.

Metabolism of acenocoumarol is carried out mainly by cytochrome P4502C9 (CYP2C9) and in a lesser extent by cytochromes CYP1A2 and CYP2C19 (Tassies *et al.* 2002; Ufer 2005). In the first phase of metabolism, acenocoumarol is biotransformed to 6- and 7-hydroxy-acenocoumarol, amine and acetamide acenocoumarol, and two diastereomeric alcohols. In addition, 8-hydroxy-acenocoumarol has been reported as a metabolite of minor quantitative importance. This hydroxylation is CYP- dependent (Saraeva *et al.* 2007).

Acenocoumarol presents a narrow therapeutic range and thus, patients under anticoagulant therapy have to be monitored frequently for correct dosage (Trejo 2004; Keeling 2017). Several intra- and inter-individual factors like body weight, sex, age and polymorphisms in genes involved in the drug or vitamin K metabolism affect the response to anticoagulant treatment (Wadelius *et al.* 2004; Saraeva *et al.* 2007; Militaru *et al.* 2015; Cullell *et al.* 2020). The interaction of oral anticoagulants with foods and/or concomitant treatment with other medications has also been described (Vranckx, Valgimigli and Heidbuchel 2018). Nevertheless, there is no scientific evidence to support the interaction between oral anticoagulants and probiotic foods.

Microorganisms belonging to the genus *Bifidobacterium* early colonize the intestine of newborns and remain as part of the commensal protective microbiota in adults. Different species of this genus are frequently found in probiotic food formulations (Bottacini *et al.* 2014; O'Callaghan and van Sinderen 2016).

Intra- and extra-cellular enzymes such as hydrolases, reductases, epimerases and mutases have been described in *Bifidobacterium* (Nakamura *et al.* 2002; Manasian *et al.* 2020). These findings place this genus in a prominent position in the degradation of food ingredients and exogenous substances, thus contributing to the beneficial effects on human hosts (Pokusaeva, Fitzgerald and Van Sinderen 2011; Jung *et al.* 2020; Manasian *et al.* 2020; Modrackova *et al.* 2020; Dias de Queirós *et al.* 2020). However, there are no reports on the interaction of bifidobacteria with oral anticoagulants.

The aim of this work was to study, *in vitro,* the effect of bifidobacteria on acenocoumarol. Considering that nowadays lots of people are switching their dietary habits by increasing consumption of probiotic foods, the present study is relevant to understand the effect of those foods on consumers that are also under anticoagulant therapy.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A total of two bacterial strains from the CIDCA collection were selected: *Bifidobacterium bifidum* CIDCA 5310 and *Bifidobacterium adolescentis* CIDCA 5317 (Pérez, Minnaard Disalvo and De Antoni 1998).

Bacteria were stored at −80°C with 10% w/v glycerol as cryoprotectant. Frozen bacterial suspensions were thawed, inoculated in MRS broth (Biokar Diagnostics, Beauvais, France), supplemented with 0.05% w/v L-cysteine (final concentration) and incubated at 37°C for 48 h in anaerobiosis (AnaeroPackTM anaerobic system, Mitsubishi Gas Chemical America, Inc. New York, NY). For the experiments, bacteria were inoculated (1% v/v) in the same medium and incubated for 24 h in the conditions specified above.

Acenocoumarol

Stock (100x) solutions of acenocoumarol (Laboratories Bagó, La Plata, Argentina) were prepared by dissolving 16 mg of drug in 1 mL of dimethylformamide (DMF) (Laboratories Anedra, Buenos Aires, Argentina) and filtering the resulting solution through a 0.45 μm pore size membrane.

Effect of acenocoumarol on bacterial growth

Growth curves of both microorganisms were studied in the presence or not of 0.16 mg/mL acenocoumarol. Bacterial counts were assessed by plating appropriate dilutions on MRS agar and incubating for 48 h at 37°C in anaerobic conditions (AnaeroPackTM anaerobic system, Mitsubishi Gas Chemical America, Inc.).

Effect of bacteria on acenocoumarol

Bacteria were grown in the presence of the drug as previously described. After incubation for 0 and 24 h, samples were centrifuged for 5 minutes at 3000 *g*, filter sterilized (0.45 μm) and frozen at −20°C until analysis.

To assess the effect of spent culture supernatants, 24 h-old cultures of each strain were centrifuged (15000 g for 15 min) and collected supernatants were filter sterilized (0.45 μm). Then, supernatants were incubated with 0.16 mg/mL acenocoumarol for 24 h at 37°C and samples were stored at −20°C until analysis.

Chromatographic analysis-HPLC

Acenocoumarol concentration was determined by using reverse-phase high-performance liquid chromatography (RP-HPLC) by modification of a previously reported method (De Orsi *et al.* 1998). Hewlett Packard HP 1100 HPLC equipment with UV detection was used. Diode array detector was set at 280 nm. Chromatographic separations and subsequent quantifications were carried out at room temperature using a LiChrospher 100 RP-18 (4×250 mm, 5 μm) column. The mobile phase consisted of acetonitrile (Biopack, Zárate, Buenos Aires, Argentina)/phosphoric acid (Cicarelli, San Lorenzo, Santa Fe, Argentina) 60/40, prepared with MilliQ water, and the flow rate was adjusted to 1.2 mL/min at 25°C. Mobile phase was filtered before use (Nylon membranes, 0.45 μm,13 mm, Osmonics Inc, Fisher Scientific, Pittsburgh, PA) and samples were previously filtered through 0.22 μm filters (GVS ABLUO, Sanford, FL). The injection volume was 20 μL and each sample was analyzed in triplicate. Peak areas were used for quantitative analyses. Calibration curve of peak areas versus acenocoumarol concentration was made with different dilutions of a stock solution (1.6 mg/mL). Linear relationship was observed in the range of 0.001–0.050 mg/mL. Retention times (t_R) and peak areas were evaluated using PeakFit (Systat Software, Inc, San Jose, CA).

Spectroscopic analysis-FTIR

Approximately 4 μL of each sample were placed on the sample holder of an Attenuated Total Reflectance FTIR (ATR-FTIR) Thermo Nicolet iS10 spectrometer (Thermo Scientific, Waltham, MA). Spectra were registered in the 4000–600/cm range by co-adding 64 scans with 4/cm spectral resolution, using OMNIC software (version 8.3, Thermo Scientific). At least five spectra were recorded for each sample.

Whenever necessary, residual contributions due to atmospheric water vapor and CO_2 were eliminated by subtraction of the corresponding spectra from the registered sample spectra, in order to obtain a flat baseline.

Statistical analysis

Statistical analysis was performed by the Mann–Whitney U Test by using InfoStat v 2020 (Di Rienzo *et al.* 2020). Differences were considered as statistically significant when $P < 0.05$. Figures were made using Microsoft Office Excel or OMNIC software (v8.3, Thermo Scientific; for FTIR figures).

RESULTS

Effect of acenocoumarol on bacterial growth

Figure 1 represents the growth curves for strains CIDCA 5310 (A) and CIDCA 5317 (B) in the presence or not of acenocoumarol. Both strains were able to grow in the presence of the anticoagulant and no significant differences were found between growth kinetics ($P > 0.05$).

Effect of bacteria on acenocoumarol

When RP-HPLC chromatograms were analyzed, the characteristic peak of acenocoumarol at $t_R = 15.25 \pm 0.15$ min was observed (Figs 2 and 3, peak 1).

RP-HPLC chromatograms corresponding to samples from 0- and 24-hour-old cultures as well as the controls without drug are shown in Figs 2 and 3, and Figure S2 (Supporting Information). For both strains, at 24 h of incubation, the area of the acenocoumarol peak (peak 1) decreases and two new peaks are evident: peak 2 and peak 3. For both strains under study, these two new peaks are observed at t_R values ranging from 6.25 to 6.30 min for peak 3 and 10.60 to 10.74 min for peak 2 (Fig. 2B, C and 3B, C). It is important to note that the samples from cultures without the anticoagulant did not show peaks in the time range of interest (5–15 min) as shown in Figure S2 (Supporting Information) as an example.

The initial concentrations of acenocoumarol for peak 1 were 0.120 ± 0.017 mg/mL and 0.119 ± 0.016 mg/mL for the samples obtained from the incubation with strain CIDCA 5310 and strain CIDCA 5317, respectively. If these concentration values are compared with those obtained after 24 h of incubation a decrease is evident for both strains. Indeed, drug concentrations lowered down to 0.020 ± 0.008 mg/mL and 0.015 ± 0.001 mg/mL for samples corresponding to the strains CIDCA 5310 and CIDCA 5317, respectively.

Regarding the effect of the spent culture supernatants on acenocoumarol, after 24 h of incubation, there were no changes neither in the t_R nor in the area of the peaks (Figure S2, Supporting Information). No additional peaks were observed, and chromatograms resemble those for controls without bacteria (Figure S2, Supporting Information).

FTIR analysis

To gain further insight on the effect of bifidobacteria on acenocoumarol, we conducted FTIR analysis. As presented in Fig. 4A, the FTIR spectra of acenocoumarol in aqueous solution is characterized by a high intensity band at 1650/cm, corresponding to the stretching of the carbonyl bond in the lactone ring and two other medium intensity bands at 1390/cm and 1110/cm, ascribed to the -NO_2 symmetric stretching and to the stretching of the lactone C–O bond, respectively (Karci and Ertan 2005; Kostova and Nikolova 2006; Hubert Joe *et al.* 2009). The intensity of these bands correlated well with the concentration of the drug in MRS broth.

In the presence of *B. bifidum* CIDCA5310, the band at 1650/cm diminished its intensity during incubation. This band overlaps that of acenocoumarol at the beginning of the bacterial growth and decreased by around 50% after 24 h incubation (Fig. 4B).

In addition, the intensity of the bands at 1390/cm and in the 1110/cm region also decreased after incubation with bifidobacteria (Fig. 4B). At the same time, a new band, ascribed to the stretching of the C = O bond of carboxylic acid, appeared at 1720/cm (Fig. 4B).

DISCUSSION

As reports on the beneficial effects of probiotic microorganisms on the health of consumers increase, probiotic consumption has grown steadily in the population. However, the interaction of probiotic microorganisms with oral anticoagulants is seldom reported in the scientific literature (Lindh 2010). It is worth noting that people who are prescribed with acenocoumarol require dietary surveillance (e.g. restriction in foods with high vitamin K content; Holmes, Hunt and Shearer 2012). In this context, the presence of microorganisms in foods could modify the effectiveness of the anticoagulation therapy. In this study we assessed the effect on acenocoumarol of bacteria belonging to the genus *Bifidobacterium*. This is relevant since this genus is often included in the formulation of fermented foods and, in addition, is a main

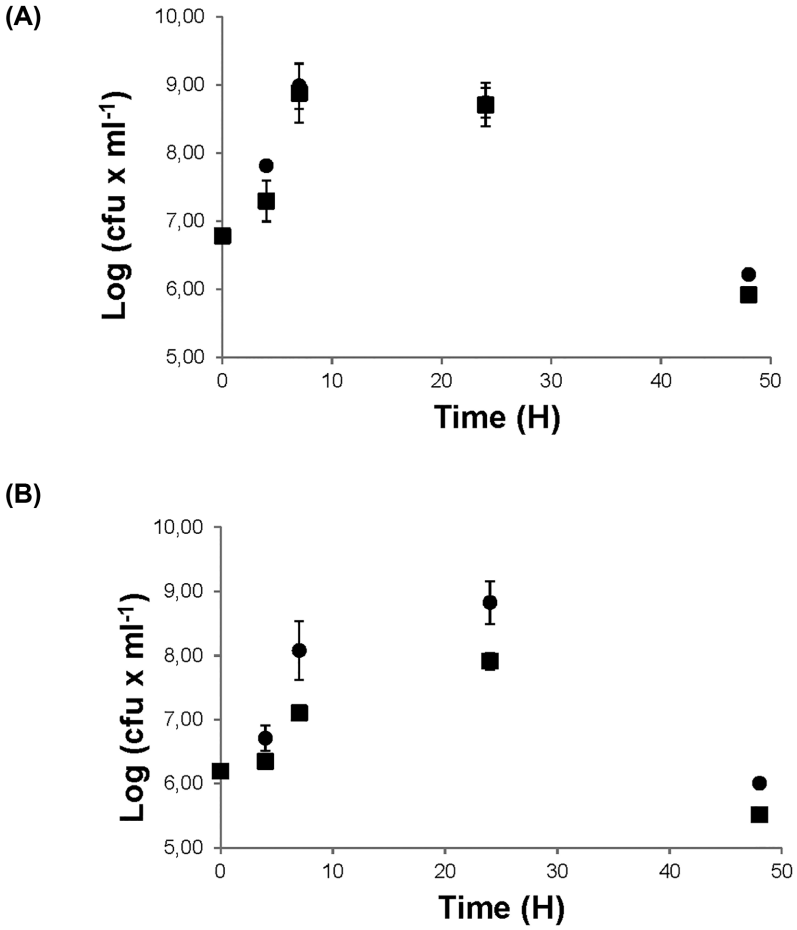

Figure 1. Acenocoumarol did not affect the growth kinetic of bifidobacteria. Growth curves of *B. bifidum* CIDCA 5310 **(A)** and *B. adolescentis* CIDCA 5317 **(B)** incubated in MRS broth with acenocoumarol (■) or control medium (•). Results show a representative experiment from three independent experiments. Bars show standard deviation.

member of the normal microbiota of children and adults (Turroni *et al.* 2009; Bottacini *et al.* 2014; Sun *et al.* 2015; Redondo-Useros *et al.* 2019).

The strains used in this work were isolated from the feces of healthy infants (Pérez *et al.* 1998) and they have demonstrated their potential as probiotic microorganisms (Trejo *et al.* 2006; Trejo, Pérez and De Antoni 2010; Trejo, De Antoni and Pérez 2013; Assad *et al.* 2020). Interestingly, some regulatory agencies consider that the species *B. bifidum* are representative of probiotic bifidobacteria (principle of "*shared benefits*") (Marco *et al.* 2021).

Since in the present study we demonstrated that bifidobacteria decreased the concentration of anticoagulant, HPLC and FTIR were used as complementary analytical approaches.

Analysis by HPLC showed that the area of the characteristic peak of acenocoumarol (peak 1) was decreased in the presence of bifidobacteria and new peaks were detected (Figs 2 and 3). These findings are compatible with a biotransformation of acenocoumarol into other products. Since the column used in the present study separates species according to their polarity (Ceresole *et al.* 2008) we can hypothesize that bifidobacteria modifies acenocoumarol leading to more polar species. This effect was observed with both strains under study. Of note, analysis of the UV-spectra of these peaks is compatible with the occurrence of new species (data not shown).

To rule out that the observed effects are due to the low pH produced by the fermentative metabolism of bifidobacteria, we tested the effect of an artificially acidified culture medium. In these conditions both the peak area and the t_R remained unchanged (Figure S1, Supporting Information) thus demonstrating that our findings are not related to the acidification of the culture medium by the microorganisms. Results obtained when anticoagulant was incubated with spent culture supernatants demonstrate that the observed effects are not due to extracellular factors released during bacterial growth (Figure S2, Supporting Information).

Results of FTIR analysis allowed us to gain further insight on the possible biomodifications due to the presence of bacteria. Firstly, at the 1650/cm region, corresponding to the stretching of the carbonyl of the lactone ring, the height of the peak correlates with acenocoumarol concentrations (Fig. 4A). This peak is then a good readout to assess biomodifications on acenocoumarol concentrations. Interestingly, after 24 h incubation, a new band at around 1720/cm was observed (Fig. 4B). This finding is compatible with an opening of the lactone ring that correlates with the decrease of the intensity observed in the band at 1110/cm, ascribed to the stretching of the lactone C-O bond. An opening of a lactone ring as the result of gut microbiota activity has been previously reported for lovastatin (Yoo *et al.* 2014).

Another change in FTIR spectra observed after the incubation of acenocoumarol with bifidobacteria was a decrease in the intensity of the peak at 1390/cm, ascribed to the nitro group, and

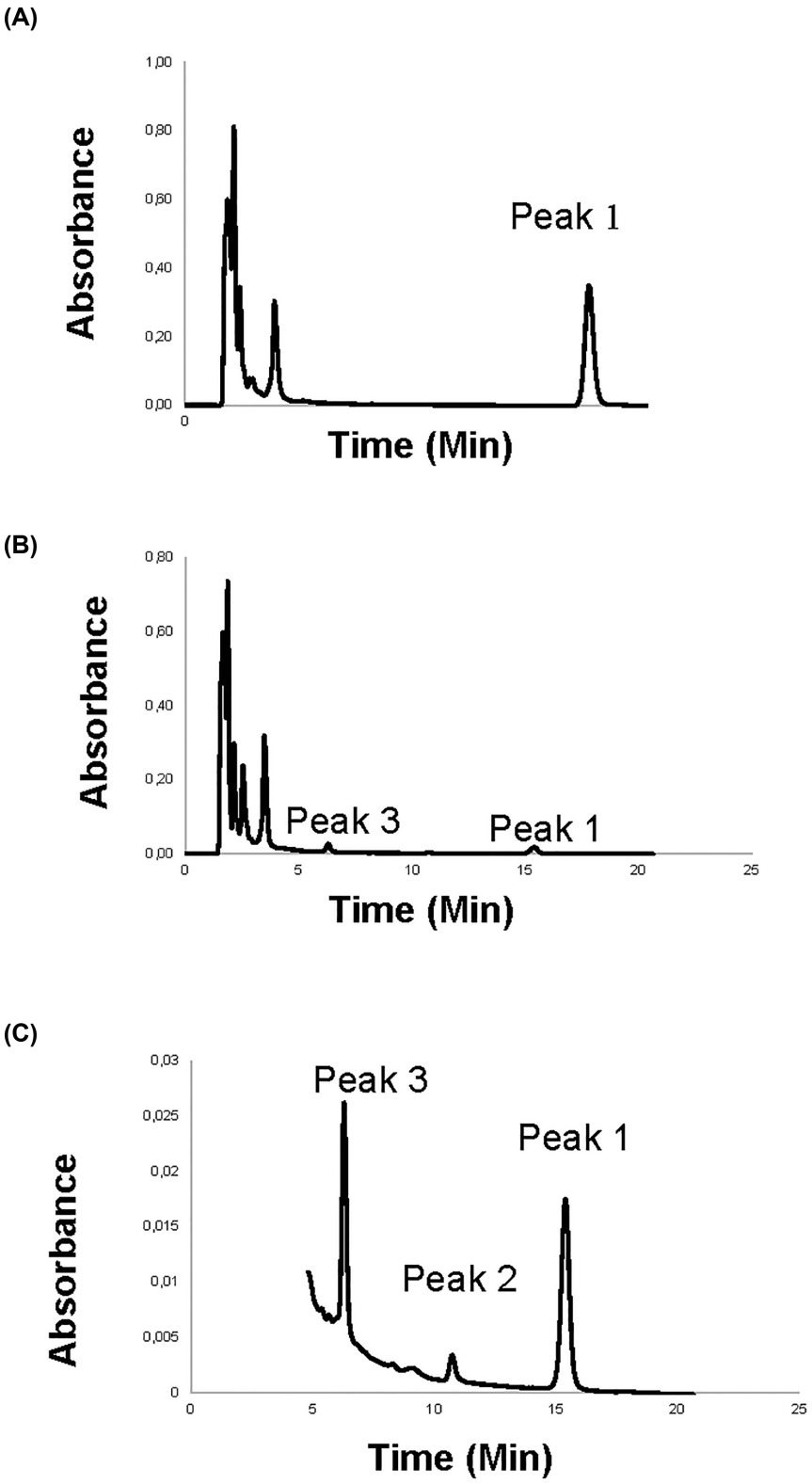

Figure 2. Acenocoumarol was biomodified by strain CIDCA 5310. RP-HPLC chromatograms of samples from cultures of the strain CIDCA 5310 in MRS with acenocoumarol (AC) incubated at 37°C for 0 h **(A)** and 24 h **(B)**. AC was observed in peak 1 at $t_R = 15{,}27$ min. Peaks 2 ($t_R = 10.60$ min) and 3 ($t_R = 6.25$ min) can be observed in a magnification of the chromatogram at 24 h **(C)**. Results show a representative experiment from three independent experiments.

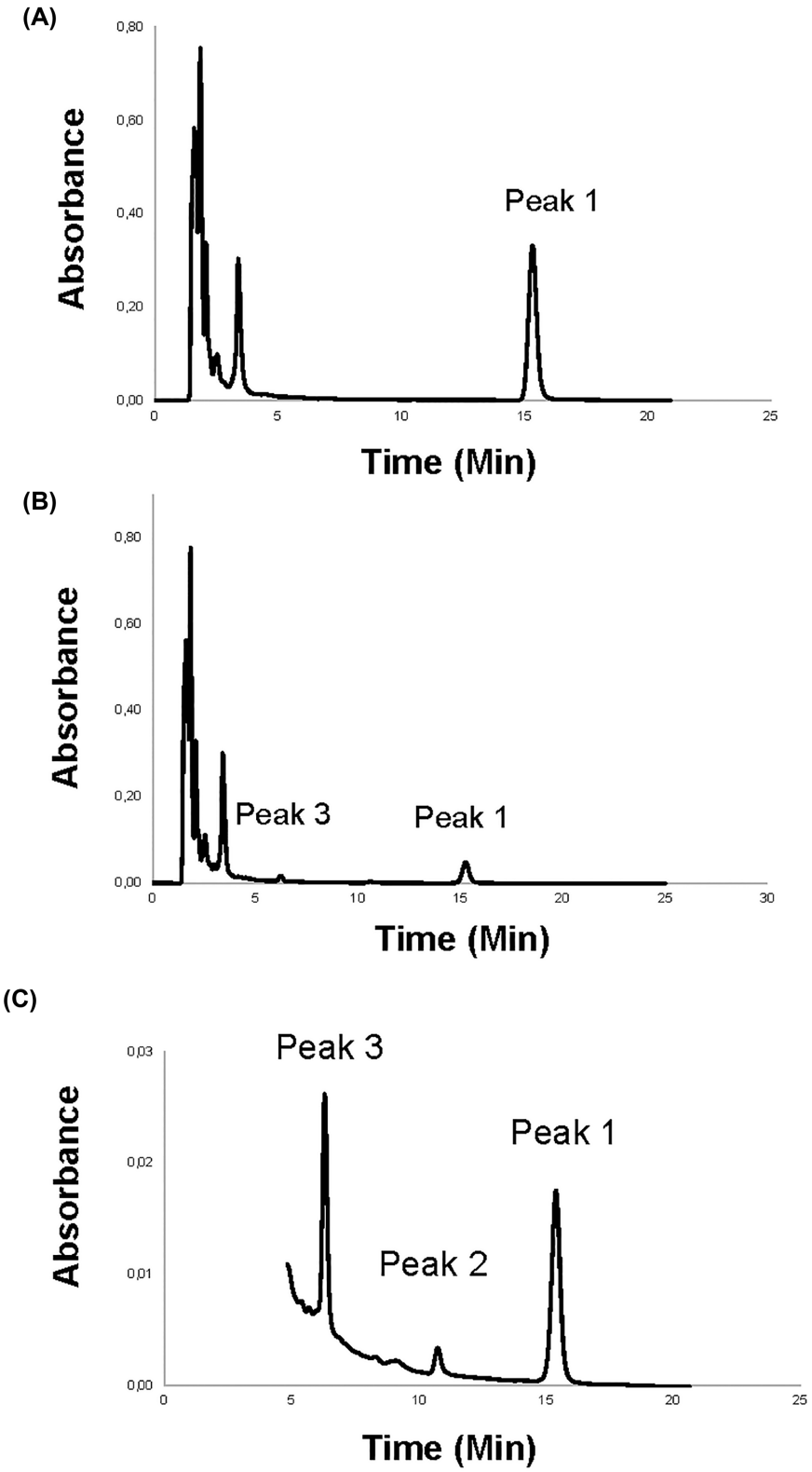

Figure 3. Acenocoumarol was biomodified by strain CIDCA 5317. RP-HPLC chromatograms of samples from cultures of the strain CIDCA 5317 in MRS with acenocoumarol (AC) incubated at 37°C for 0 h **(A)** and 24 h **(B)**. AC was observed in peak 1 at $t_R = 15{,}40$ min. Peaks 2 ($t_R = 10.74$ min) and 3 ($t_R = 6.30$ min) can be observed in a magnification of the chromatogram at 24 h **(C)**. Results show a representative experiment from three independent experiments.

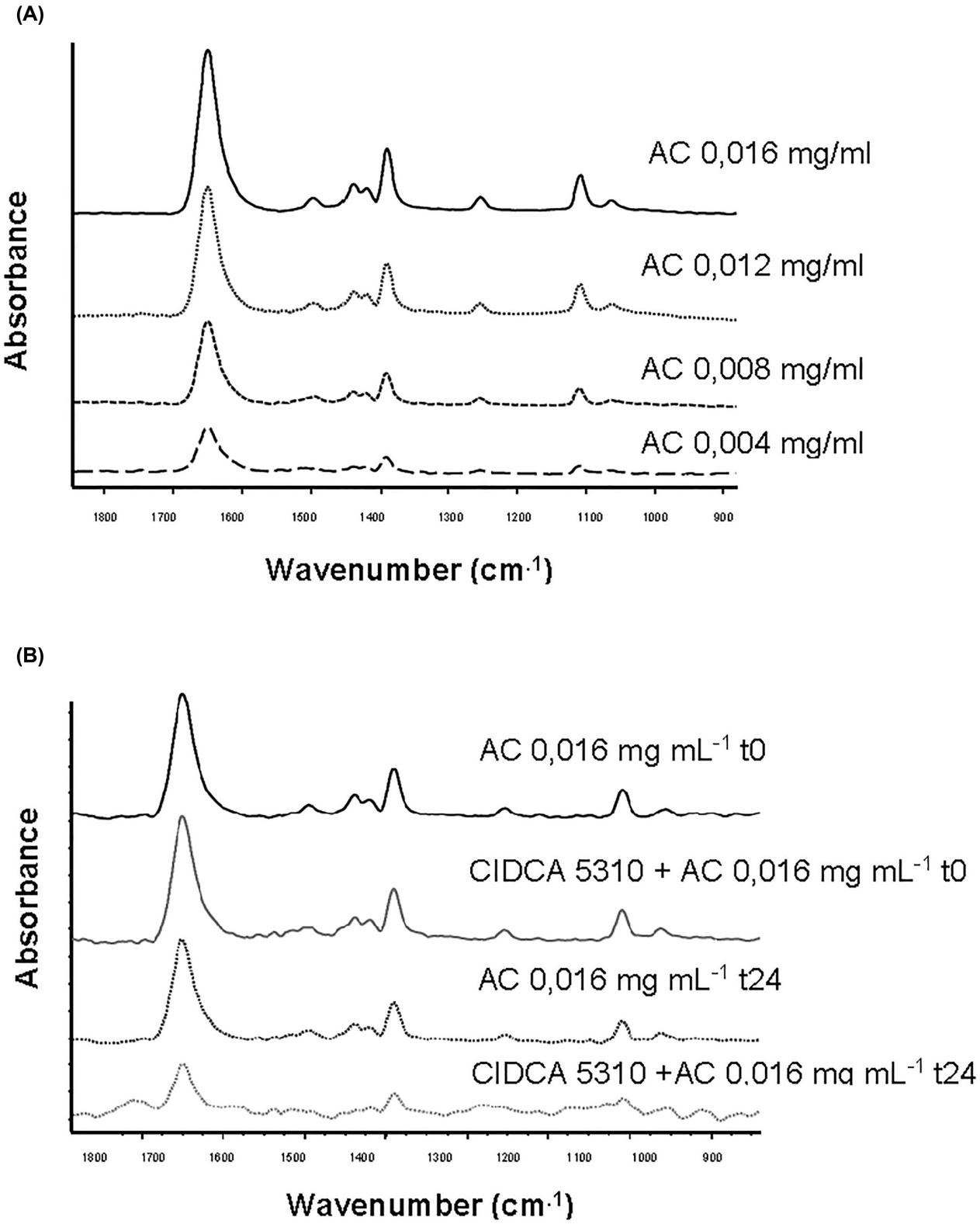

Figure 4. Acenocoumarol ATR-FTIR spectra was modified during incubation with strain CIDCA 5310. ATR-FTIR spectra, in the 900–1800/cm region, of different acenocoumarol (AC) concentrations in MRS broth **(A)**, and spectra of AC after incubation with strain CIDCA 5310 for 0 h () and 24 h (…) and the control conditions of AC alone incubated for 0 h () and 24 h (…) **(B)**. Results show a representative experiment from three independent experiments.

it is worth noting that the relation of the areas of this peak and the 1650/cm peak remained constant after 24 h incubation.

Taken together, our results are compatible with a modification of acenocoumarol structure affecting the lactone ring, due to bifidobacterial activity. As a consequence of this, acenocoumarol activity could be altered, considering that the lactone ring conforms the coumarinic core which confers its anticoagulant effect (Thijssen, Baars and Reijnders 1983; Kasperkiewicz *et al.* 2020).

Results of the present work are in agreement with previous studies that described the effect of bacterial enzymes on the activity of diverse compounds. In this context, reports showed that enzymes like reductases, esterases and dehydroxylases were found in different bifidobacteria and lactobacilli (McBain and Macfarlane 1998; Nakamura *et al.* 2002; Fritsch *et al.* 2017; Manasian *et al.* 2020).

If we analyze the acenocoumarol molecule in detail we can identify different functional groups such as –OH, -NO2, -C = O that could be targets for the above-mentioned enzymes. As it has already been described, the normal *in vivo* route of metabolization of acenocoumarol includes hepatic enzymes which carry out oxidations and reductions (Lopez de Luca *et al.* 2006; Kasperkiewicz *et al.* 2020). Reductions turn the nitro group to an amine or the keto group to a hydroxyl. Oxidation leads to 6- and 7- hydroxyl metabolites (Kasperkiewicz *et al.* 2020).

Furthermore, Leonart *et al.* (2017), using a UPLC-MS method, described new molecules in human urine after coumarin metabolization (Leonart *et al.* 2017). These new metabolites included those resulting from hydroxylation, glucuronidation, sulfation, methylation and conjugation with N-acetylcysteine of the original molecule, showing the proneness of the drug to be biomodified by different types of enzymes. These results correlate with our findings in HPLC, given the new molecules detected were more polar than the original one.

It is evident that changes on acenocoumarol related to bacterial enzymatic activity, could impact on the anticoagulant effect. However, it must be highlighted, that *in vivo*, the scenario involves additional variables. Indeed, interactions between the drug, the gut microbiota, foods and host cells lining the gastrointestinal tract (Bailey and Dresser 2004; Sousa *et al.* 2008; Russel 2010; Stojančević *et al.* 2014; Enright, Joyce and Gahan 2017; Koziolek *et al.* 2019; Vertzoni *et al.* 2019). It is known that intestinal microorganisms modify the expression of intestinal transporters thus impacting on the bioavailability of drugs (Matusková *et al.* 2011; Saksena *et al.* 2011; Stojančević *et al.* 2014). In addition, they modulate the expression of cytokines (Azad, Sarker and Wan 2018; Yousefi *et al.* 2019; Wang *et al.* 2020) that in turn change permeability, drug metabolism and transport (Bertilsson, Olsson and Magnusson 2001; Le Vee *et al.* 2009; Thagia *et al.* 2015). Of note, *Bifidobacterium* and *Lactobacillus* strains have demonstrated to increase the barrier function of the intestinal epithelium through different mechanism (Hyland, Quigley and Brint 2014; Nébot-Vivinus *et al.* 2014; Srutkova *et al.* 2015; Guo *et al.* 2017).

Besides, consumption of probiotic foods along with acenocoumarol treatment in addition to pathologies that delay absorption (e.g. ulcerative colitis, intestinal bowel disease and Crohn´s disease), could enhance the biotransformation of the drug by bifidobacteria by increasing residence time in the intestinal tract (Hatton *et al.* 2018).

Summarizing, our results show for the first time that bifidobacteria can modify acenocoumarol. This ability could lead to significant changes in the drug concentration thus adding a relevant aspect to be considered for the pharmacological effect of this drug.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants or animals performed by any of the authors.

AVAILABILITY OF DATA AND MATERIALS

The original data with the respective analysis corresponding to the results shown in this work are available up to reasonable requirements.

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Conflict of interest **.** None declared.

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