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Development of an HPLC-DAD Method for the Extraction and Quantification of 5-Fluorouracil, Uracil, and 5-Fluorodeoxyuridin Monophosphate in Cells and Culture Media of *Lactococcus lactis*

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Abstract: The drug 5-fluorouracil (5-FU) is a common cancer chemotherapeutic, presenting toxicity. Mild toxicity is treated with administration of probiotics. The interaction of these probiotics with the drug may have a crucial effect on its therapeutic efficacy. In the present work, a method for the quantification of uracil, 5-FU, and its active metabolite 5-fluorodeoxyuridin monophosphate in cells and culture medium of the probiotic *L. lactis* is presented. Extraction using H_2O containing 0.05% v/v formic acid (1:5 v/v) was followed by ammonium sulphate protein precipitation and SPE. Analysis was conducted in a Nucleosil column using a gradient of water, formic acid, and acetonitrile. Calibration curves were constructed for 5-FU (5–100 μg/mL), uracil (5–20 μg/mL), and 5-fluorodeoxyuridin monophosphate (5–20 μg/mL) using 5-bromouracil as the internal standard ($R^2 \geq 0.999$). The photodegradation of 5-FU amounted to 36.2% at 96 h. An administration experiment in the dark revealed a decline in 5-FU concentration in the culture media (88.3%) and uptake by the cells, while the uracil and FdUMP levels increased in the cells. The inactive metabolite 5,6 dihydrofluorouracil was detected in the medium. Our results demonstrate that uptake and metabolism of 5-FU in *L. lactis* cells leads to a decline in the drug levels and in the formation of both the active and the inactive metabolites of the drug.

Keywords: HPLC-DAD; 5-Fluorouracil; Uracil; 5-fluorodeoxyuridin-monophosphate; probiotics

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1. Introduction

Chemotherapy, in combination with radiation and surgery, has been the treatment of choice against cancer for over sixty years [1]. The drug 5-fluorouracil (5-FU) was developed to target increased uracil uptake and use [2,3]. The chemotherapeutic 5-FU is currently used in the treatment of various types of cancers, including breast, head, neck, and digestive tract cancers including colorectal cancer [4,5].

Following its administration, 5-FU is transformed in the cells through anabolism to 5-fluorodeoxyuridin monophosphate (FdUMP) [4], which exerts inhibitory action on thymidylate synthase (TS), resulting in a reduced formation of thymidine precursors for DNA synthesis. Hence, the administration of 5-FU results in the depletion of such precursors and "thymineless cell death" [6]. However, a major part of the administered drug is subjected to catabolism and is converted to 5,6-dihydro-5-fluorouracil (5-FUH2), an inactive metabolite, through a reaction catalyzed by dihydropyrimidine dehydrogenase (DPD) [7]. The activity of this enzyme is highly variable, and its absence results in the life-threatening

toxicity of 5-FU [8,9]. As a diagnostic tool for DPD deficiency, uracil or thymine determination in plasma or urine has been proposed [10]. Therefore, the analysis of 5-FU, uracil, and 5-FU metabolites following administration of a test dose is considered essential to achieve both increased efficacy and safety of the drug [11]. The toxicity of 5-FU induces vomiting and diarrhea, which have been associated with an imbalance in the gut microbiome [12]. Such side effects are commonly treated with probiotics, mainly of the genera *Lactobacillus* or *Bifidobacteria* [13–15], increasing the apoptotic action of chemotherapeutics [16]. *Lactococcus lactis* has also been successfully employed [15], leading to improved clinical outcomes [17]. However, the need for further studies on the use of probiotics as adjuvant therapy for cancer patients to reduce the side effects of chemotherapy is imperative in order to define exact personalized doses [18]. While chemotherapeutics, including 5-FU, inhibit bacterial growth [19], bacteria have been reported to accumulate or even biotransform drugs and pollutants [20], and the study of such interactions is crucial [21]. A drug's oral bioavailability, and hence its efficacy, are affected by the biotransformation and elimination of drugs by the microbiome and should be assessed [22]. The bacterial metabolism of 5-FU modified its efficacy in the nematode *C. elegans* [23]. Moreover, strains of *L. lactis* resistant to 5-FU were isolated [24], and an enzyme isolated from *L. lactis* [25] presented great resemblance to DPD, which catalyzed the formation of the 5-FU inactive metabolite [26]. These findings indicate possible effects of the microbiota and its manipulation of the efficacy of chemotherapeutics. The interactions of probiotics with 5-FU have not yet been reported, and require the determination of the drug and its active metabolite FdUMP in cultures and in bacterial cells.

Several methods have been developed for the extraction and determination of 5-FU and its metabolites in biological samples. Both 5-FU and its active metabolite FdUMP present a high aqueous solubility and a low solubility to organic solvents, thus resulting in extraction difficulties [27]. Extraction usually employs the acidification of human serum samples as well as protein precipitation [27,28] followed by liquid-liquid extraction, employing different solvents such as propanol: diethyl ether [29] or ethyl acetate [30,31]. Solid phase extraction has also been employed to ensure improved 5-FU recovery using the C18 column matrix [32,33], ion exchange SPE [34–36], or the polymeric matrix SPE [37]. The methods employed for the analysis of 5-FU in human plasma, serum, and urine have been thoroughly reviewed [27,38,39] and employ HPLC-UV analysis [29,40] or GC analysis [41,42], and more recently, LC-MS/MS [43–45].

However, extraction from tissue samples is hindered by coeluting matrix components [40], hence fewer methods are specific for tissue in either human cancer [46–49] or human skin [50], and more elaborate extraction protocols, or even derivatization and column switching, have been proposed [51]. The need for tissue specific extraction methods has been emphasized [40].

Although the methods for the determination of these compounds in human samples are abundant, there is no report on a method specific for bacterial cultures that would allow the study of the possible biotransformation of the drug by the probiotic bacteria which are coadministered with chemotherapy. To this end, in the present study a simple method for the extraction and simultaneous determination of 5-FU, its active metabolite 5-fluorodeoxyuridin monophosphate, and uracil in the cultures of the probiotic *L. lactis* was developed and validated.

2. Materials and Methods

2.1. Chemicals and Reagents

Acetonitrile, methanol, formic acid, and NaCl were acquired from VWR chemicals (VWR International GmbH, Graumanngasse 7, A-1150 Wien, Austria); n-Hexane was acquired from Labscan (RCI Labscan Limited, 24 Rama 1 Rd., Rongmuang, Pathumwan, Bangkok, Thailand). $(NH_4)_2SO_4$ was acquired from Lach-ner (Tovární 157, 277 11 Neratovice, Czech Republic); KH_2PO_4, $CaCl_2$, $MgSO_4$, NH_4Cl, and $FeCl_3$ were acquired from Panreac (Panreac Química SLU, Castellar del Vallès, Spain). Brain Heart Infusion broth

(BHI broth), liquid culture medium, and yeast extract were acquired from Applichem (AppliChem GmbH, Darmstadt, Germany). Uracil and FdUMP were acquired from Merck (MERCK KGAA, Darmstadt, Germany). 5-fluorouracil (5-FU) and the internal standard 5-bromouracil (5-BrU) were purchased from Alfa Aesar (Ward Hill, Haverhill, MA, USA). Solid phase extraction columns, frits, C_{18} sorbent material, and 35–75 U were acquired from Agilent (Santa Clara, CA, USA). The composition of Basal Salts Medium (BSM) was KH_2PO_4 1 g/L, NH_4Cl 1 g/L, $MgSO_4$ 0.1 g/L, NaCl, and $CaCl_2$, all at 0.05 g/L each, as well as $FeCl_3$ 0.01 g/L and yeast extract 1 g/L diluted in double-distilled water (ddH_2O).

2.2. Standard Preparation

Stock solutions of the analytes 5-FU (1 mg/mL), uracil (1 mg/mL), FdUMP (100 µg/mL), and the internal standard 5-BrU (500 µg/mL) were prepared in 1:1 (v/v) methanol: ddH_2O. Calibration curves of 5-FU, FdUMP, and uracil were constructed in BSM for the supernatant. For the 5-FU calibration curve preparation in BSM, working standard solutions were prepared at concentrations of 5, 25, 50, 75, and 100 µg/mL. For uracil and for the FdUMP calibration curves, working standard solutions were prepared at concentrations of 5,10 15, and 20 µg/mL. Calibration curves in the cell precipitates were prepared for 5-FU, FdUMP, and uracil using cells suspended in 0.5 mL of ddH_2O containing 0.05% v/v formic acid. Moreover, 5-FU was added at concentrations of 5, 25, 50, 75, and 100 µg/mL, while FdUMP and uracil were added at concentrations of 5, 10, 15, and 20 µg/mL. The chemical structures of the analytes 5-FU, U, FdUMP, and the internal standard 5-BU are shown in Figure 1.

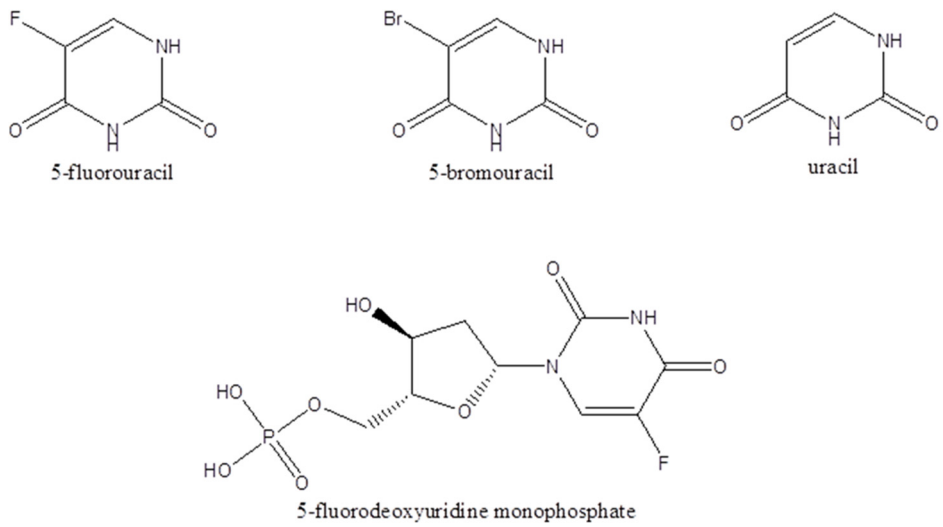

Figure 1. The chemical structures of 5-fluorouracil, 5-bromouracil, uracil, and 5-fluorodeoxyuridine monophosphate.

2.3. Sample Preparation

To prepare the bacterial pellet for analysis, 0.1 g of wet cell weight *L. lactis* was suspended in 0.5 mL of ddH_2O containing 0.05% v/v formic acid, and the desired amount of 5-FU was added to the sample. The sample was then homogenized while the tube was submerged in ice. The homogenized mixture was then transferred to an Eppendorf tube and centrifuged at 8000 rpm for 10 min. The supernatant was collected and 0.5 g of $(NH_4)_2SO_4$ was added. After thoroughly stirring the sample for 1 min, it was centrifuged for 20 min at 10,000 rpm, then the supernatant was collected. The sample was then loaded on a solid phase extraction (SPE) cartridge (0.5 g C_{18} sorbent) following conditioning with 5 mL methanol, 5 mL dH_2O, and 5 mL dH_2O containing 0.1% v/v formic acid (pH = 3.5–4). A manual flow of 1 mL/min was employed. The cartridge was washed with 2 mL n-Hexane, and 5-FU was eluted using 0.5 mL of dH_2O containing 0.05% formic acid and

acetonitrile at a ratio of 95:5 v/v. The analytical standard 5-BrU was added in the SPE eluate at a concentration of 50 μg/mL.

Bacterial supernatant samples (0.5 mL) were collected at 0 h and at 96 h and placed in an Eppendorf tube, 0.5 g $(NH_4)_2SO_4$ was added, and the procedure described for the cell pellet, including centrifuging and SPE, was followed.

All of the above procedures were performed in tubes covered in triplicate and all tubes were kept in the dark to avoid the photodegradation of the 5-FU. All of the samples were either analyzed directly or stored at −20 °C until analysis.

2.4. Sample Analysis Using HPLC-DAD

An $LC20_{AD}$ pump and an SPD-20A photodiode array detector (DAD) (Shimadzu, Kyoto, Japan) was used. The injection volume was 80 μL, and the separation was done at room temperature using a Nucleosil 100 C_{18} column (250 × 4.6 mm, 5 μm) purchased from Macherey-Nagel GmbH & Co. (Duren, Germany) with a binary mobile phase system. Mobile phase A consisted of ddH_2O containing 0.05% formic acid and acetonitrile at a ratio of 95:5 v/v, while mobile phase B consisted of ddH_2O containing 0.05% formic acid and acetonitrile at a ratio of 5:95 v/v. A gradient elution was used to separate the analytes, and the flow rate was constant at 0.5 mL/min. The gradient started at 100% during mobile phase A, which was brought down by 90% over 2 min, 80% over 5 min, 70% at 8 min, and lastly at 50% over 20 min, where it was kept until the end of the analyses. All analytes were detected at 260 nm with a bandwidth of 2.0 nm. Each of them was identified by matching the peak retention time (Rt) and peak purity via spectrum overlay with those of the pure standards. The concentration of the analyte was plotted against the peak area ratio of the analyte to the peak area of the internal standard and was used for internal standard calibration.

2.5. Peak Identification

Peaks originating from HPLC analysis for 5-FU and uracil were identified by retention time and spectrum analysis. Unidentified peaks were subjected to ESI-MS analysis on an LC-20AD Shimadzu connected to Shimadzu LCMS-2010EV equipped with a C_{18} analytical column (Reprospher 100 C_{18}-DE, 5 μm 250 × 4.6 mm, Dr Maisch GmbH, Ammerbuch, Germany) using 1% formic acid in 50:50 v/v acetonitrile: water. The obtained mass spectra of these compounds were compared to the standard spectra of the NIST Mass Spectral Library (NIST Chemistry WebBook, SRD 69, National Institute of Standards and Technology NIST 100 Bureau Drive, Gaithersburg, MD, USA) for 5-FU and uracil, while for 5FdUMP, the mass spectra were compared to the spectra of the pure standard and to the standard spectra of the Human Metabolome Database (HMDB).

2.6. Photodegradation Assay

To investigate the photodegradation of 5-FU in the bacterial cultures, a quantity of 5-FU (1 mg/mL) was added in a falcon containing 50 mL of BSM so that the final concentration would be 50 μg/mL. An identical tube was prepared and kept in the dark, wrapped in aluminum foil. The two falcon tubes were incubated at 28 °C under constant stirring. The 5-FU was quantified using the calibration curves and the methods described above in the beginning (0 h) and at the end of the 96 h period.

2.7. FU Administration

The bacterial strain *L. lactis* (ATCC 11454) was inoculated in 50 mL of Brain Heart Infusion broth (BHI broth). After a 24 h incubation period at 28 °C under constant stirring, the culture was centrifuged at 3000 rpm for 15 min at 4 °C and the precipitate was resuspended in 50 mL of Basal Salts Medium (BSM). Then, a quantity of 5-FU (1 mg/mL) was added so that the final concentration would be 50 μg/mL, with 5-FU serving as the main carbon source. The culture was left under constant stirring at 28 °C for 96 h. At 96 h the culture was centrifuged at 3000 rpm for 15 min at 4 °C, and a sample (0.5 mL) of the supernatant

was collected and analyzed using the method and calibration curves that were described above, while the precipitated cells were harvested and stored at −20 °C until analysis.

3. Results and Discussion

3.1. Optimization of Sample Preparation

Extraction of the analytes from BHI medium, recommended for growth of *L. lactis*, resulted in matrix peaks that might interfere with the analytes peaks. Hence, the analysis was performed in BSM medium and not in the medium which is recommended for *L. lactis* growth. The use of BSM also contributed to the biotransformation of 5-FU by increasing its uptake by the cells, since it was their main carbon source. The 5-bromouracil was selected as the internal standard due its structural similarity with 5-FU and because it is not expected to be present in microorganism liquid cultures. The same internal standard has been used previously during the analysis of 5-FU in plasma and tissue samples [48] or human plasma [52], pointing out the superiority of 5-bromouracil and 5-chlorouracil against 5-fluorocytosine. Extraction of 5-FU, uracil, and FdUMP from cells following homogenization was performed in acidic conditions employing formic acid, since 5-FU and uracil analytes are weak organic acids with corresponding pKa values of 8.02 and 9.45, respectively (PubChem Compound Summary for CID 3385, CID 1174), while 5-fluorodeoxyuridine monophosphate is acidic, with a pKa of 1.2 (Drugbank). Protein precipitation followed since proteins expected to be present in analytical samples such as bacterial cells comprise interfering matrix components [27,28]. Further purification of the samples and removal of salts from the preceding ammonium sulfate step employed solid phase extraction. In our preliminary experiments, following sample addition the column was washed with 5 mL of ddH_2O solution containing 0.05% NH_3, aiming at the ionization of 5-FU so that it would remain attached to the column during the washing step, to be eluted later. However, the analytes were washed out due to high water solubility, resulting in poor recoveries, and hence, washing with hexane, a nonpolar solvent, was employed. The recovery of our method ranged between 99.25–107.02% for the culture supernatant and between 98.93–105.42% for the cell precipitate (Table 1). Representative chromatograms of the analytes are presented in Figure 2 for the cells and in Figure 3 for the culture supernatant.

The peak appearing at 4.9 min does not correspond to any of the studied analytes and could be attributed to a BSM compound since it is present in all chromatograms originating from all samples of *L. lactis* cultures (Figures 2 and 3). The peak appearing in blank cells at 7.74 min (Figure 2A) might correspond to endogenous free uracil. This peak, however, corresponded to about 0.53 μg/mL, which is below our LOQ and was not taken into account in our calculations. The retention times of 5-FU, FdUMP, uracil, and of the internal standard 5-BU in cells were 8.37 ± 0.17, 5.81 ± 0.11, 7.76 ± 0.11, and 13.29 ± 0.12 min, respectively (Table 2). Similar retention times were acquired for supernatant samples, except for uracil, which was eluted at 7.252 ± 0.11 min.

3.2. Method Validation

The standard curves of all analytes, both for the culture supernatant and for the *L. lactis* precipitate, presented adequate coefficients of determination and acceptable F-test results (Table 1). Relative bias, estimated as the percentage of the difference of the mean calculated concentration to the nominal concentration divided by the nominal concentration of the analyte, was below the accepted range of ±10% in all cases. A total of three injections of the nominal concentration were performed on the same day to calculate the intraday precision, while the interday precision was calculated from a total of six injections of the nominal concentration performed over a two-day period; both are expressed as % R.S.D.

The limit of detection ((3.3 × $SE_{Intercept}$)/slope of the standard curve), the limit of quantitation ((10 × $SE_{Intercept}$)/ slope of the standard curve), and the retention time of the analytes in the two samples, namely the cells and cell-free culture supernatant, are shown in Table 2. Although lower values of 3.2 ng/mL [8] or 12.5 ng/mL [48] have been reported in human plasma in 5-FU determinations, their calculations were based on a signal-to-noise

ratio. In the present study, calculations were based on the residual standard error of the intercept of the calibration line, which is recommended, in order to obtain more accurate estimates [53]. Moreover, these estimations largely depend on the analyte concentrations employed. In the present study, the calibration curve was constructed in a manner such that the concentration of 50 μg/mL 5-FU employed in the administration experiment falls within the range of the curve while being slightly below the 50% minimum inhibitory concentration (MIC_{50}). The estimated recovery rates, all above 94.6%, were comparable to the value of 96.2% reported for human serum [35], further supporting the efficacy of our method.

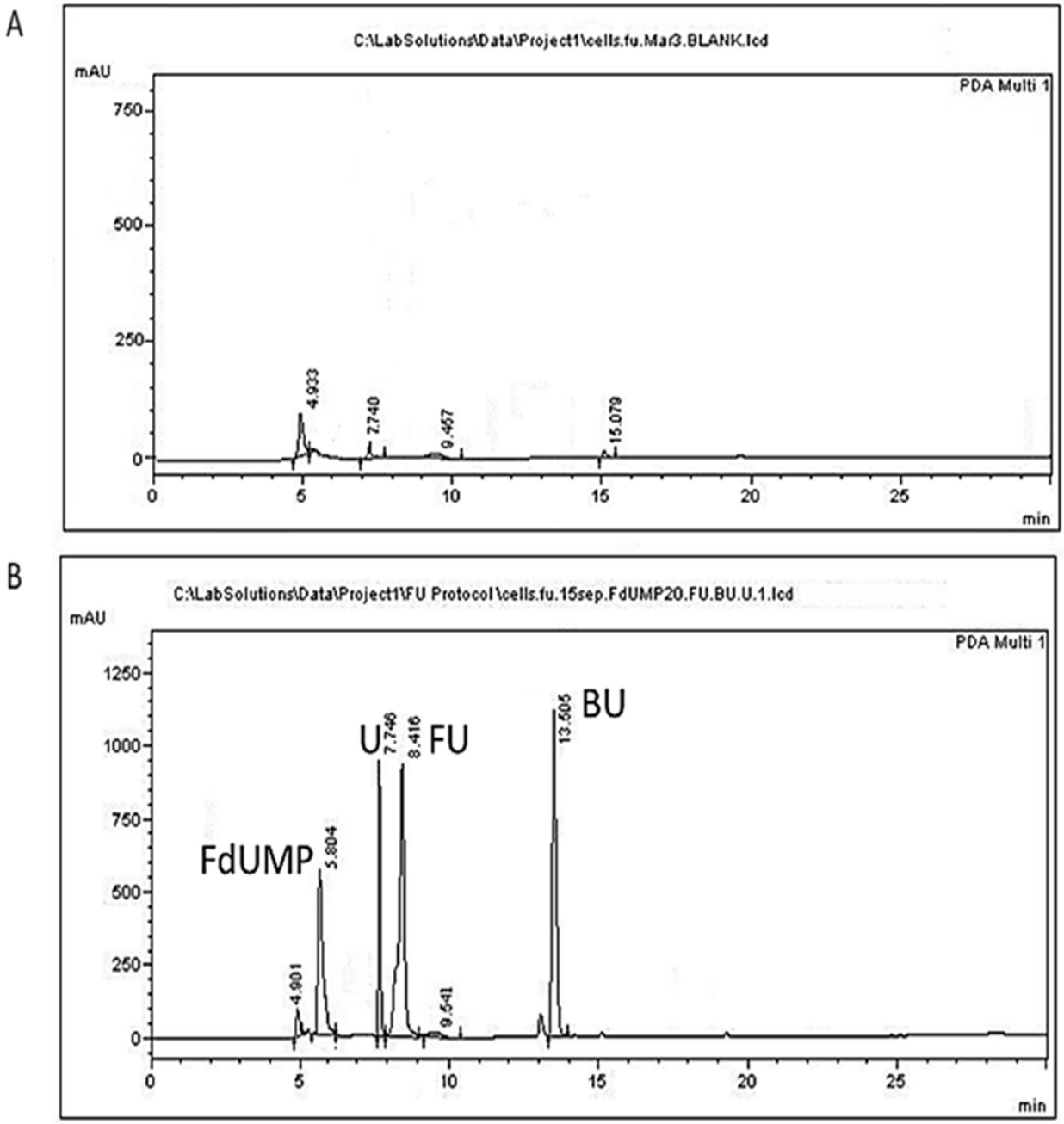

Figure 2. Representative chromatograms of *L. lactis* cells. (**A**) A blank sample; (**B**) cells spiked with 50 μg/mL of 5-FU (FU), 20 μg/mL uracil (U), 20 μg/mL FdUMP (FdUMP), and 50 μg/mL of the internal standard 5-BU (BU).

Table 1. Validation parameters for the determination of 5-FU, 5-FUMP, and uracil. Results for the Mean Calculated Concentrations and Recoveries are expressed as mean ± SD (n = 3). In calculations for Intraday Precision n = 3, and for Interday Precision n = 6 over three days.

Analyte in Medium/Std. Curve Equation/R^2 (F, $F_{critical, p}$)	Nominal Conc. (μg/mL)	Mean Calculated Concentration (μg/mL) (Mean ± SD)	Relative Bias (%)	Precision		Recovery (Mean ± SD)
				Intraday (RSD %)	Interday (RSD %)	
5-FU in BSM	5	5.3 ± 1.0	7.0	3.5	4.5	107.0 ± 19.4
y = 0.0054x + 0.104	25	24.8 ± 3.8	−0.7	2.7	3.3	99.2 ± 15.3
0.9999	50	50.1 ± 10.7	0.3	2.8	3.3	100.2 ± 21.4
(34,170.8, 6.4, 2.6×10^{-9})	75	74.6 ± 0.8	−0.5	0.9	1.0	99.5 ±1.1
	100	100.6 ± 7.9	0.6	6.4	7.8	100.6 ± 7.9
5-FU in *L. lactis* cells	5	4.7 ± 0.8	−5.3	0.1	0.2	94.6 ± 1.3
y = 0.0051x + 0.079	25	25.6 ± 2.7	2.7	2.2	3.1	102.6 ± 9.5
0.9999	50	50.4 ± 3.3	0.8	3.8	5.4	102.5 ± 3.9
(37,773, 6.4, 2.1×10^{-9})	75	75.8 ± 6.2	1.2	3.1	4.3	101.1 ± 3.4
	100	98.5 ± 5.6	−1.4	3.9	4.8	100.6 ± 5.6
FdUMP in BSM	5	5.1 ± 0.4	2.6	2.8	7.1	102.6 ± 7.2
y = 0.003 + 0.010	10	9.9 ± 0.4	−1.3	3.1	4.0	98.7 ± 4.0
0.9999	15	14.7 ± 1.2	−2.0	6.8	8.3	97.9 ± 8.2
(128,406.1, 9.3, 3.7×10^{-8})	20	20.2 ± 1.3	1.3	5.3	6.6	101.3 ± 12.2
FdUMP in *L. lactis* cells	5	4.8 ± 0.2	−3.5	0.9	3.9	96.4 ± 3.8
y = 0.004x + 0.012	10	10.1 ± 0.4	0.7	2.3	4.5	100.7 ± 4.5
0.9998	15	14.8 ± 1.2	−1.4	5.7	6.8	98.5 ± 7.9
(70,690.9, 6.4, 6×10^{-10})	20	19.7 ± 1.0	−1.4	4.6	5.2	98.6 ± 5.2
Uracil in BSM	5	5.3 ± 0.6	6.8	0.3	10.6	106.8 ± 11.1
y = 0.014x + 0.041	10	10.1 ± 1.1	0.8	7.3	10.9	101.1 ± 11.5
0.9993	15	14.8 ± 1.1	−1.5	1.0	7.1	99.8 ± 7.2
(5197, 9, 4.5×10^{-6})	20	20.0 ± 0.7	0.2	1.8	3.6	102.2 ±3.7
Uracil in *L. lactis* cells	5	4.8 ± 0.3	−3.03	2.9	3.5	96.9 ± 5.8
y = 0.13x + 0.046	10	10.9 ± 0.1	9.52	0.8	1.0	109.5 ± 1.5
0.9994	15	14.9 ± 0.8	−0.01	3.7	4.5	99.9 ± 5.5
(5649.3, 9.3, 4×10^{-6})	20	19.9 ± 0.4	−0.16	1.4	1.8	99.8 ± 2.1

Table 2. LOD, LOQ, and RT values for 5-FU, uracil, and 5FdUMP. Values are provided for both the culture supernatant (BSM) and the bacterial precipitate (*L. lactis* cells). Retention time values are means ± SD (n = 6).

Std. Curve	Retention Time (min) Mean ± SD (RSD %)	LOD (μg/mL)	LOQ (μg/mL)
5-FU in BSM	8.12 ± 0.11	1.2	3.7
5-FU in *L. lactis* cells	8. 37 ± 0.17	0.9	3.0
FdUMP in BSM	5.89 ± 0.09	0.4	1.2
FdUMP in *L. lactis* cells	5.81 ± 0.11	0.4	1.2
Uracil in BSM	7.25 ± 0.11	0.7	2.1
Uracil in *L. lactis* cells	7.76 ± 0.11	0.7	2.2

3.3. Photodegradation of 5-FU

The reduction in 5-FU levels observed when incubations were performed in the dark amounted to the negligible amount of 0.2% (Figure 4A,B), indicating that abiotic factors other than light do not result in the degradation of 5-FU. However, a reduction of 36.2% was observed following incubation under normal light for 96 h in combination with the occurrence of a peak with a retention time of 6.9 min. Similar reduction and two extra peaks were observed when 5-FU was incubated under UVB light [54], and the peaks were identified as photoproducts formed following the addition of water to one double bond of the molecule. Since the parent compound was reported to present improved toxicity against cancer cells compared to the products of transformation by light, care was taken to avoid photodecomposition of the drug in the present study, and all experiments were conducted in the dark.

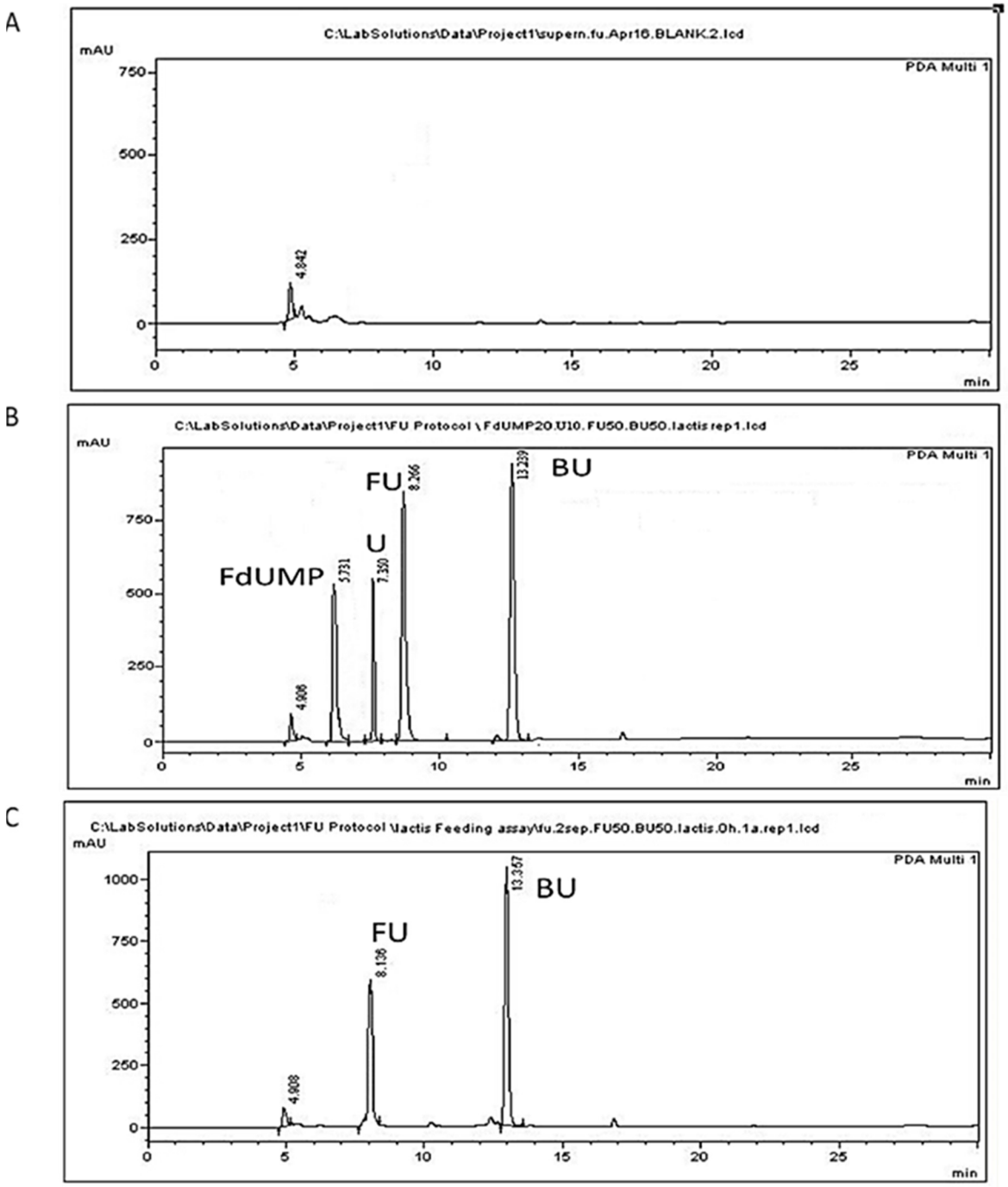

Figure 3. Representative chromatograms of L. lactis cell-free culture supernatants. (**A**) A blank sample; (**B**) a BSM sample spiked with 50 μg/mL of 5-FU (FU), 10 μg/mL uracil (U), 30 μg/mL FdUMP (FdUMP), and 50 μg/mL of the internal standard 5-BU (BU); (**C**) culture supernatant collected at 0 h following administration of 50 μg/mL 5-FU at 0 h.

3.4. 5-FU Administration

In preliminary experiments on the toxicity of 5-FU against *L. lactis* (data not shown), the MIC_{50} was determined at about 65 μg/mL. The 5-FU concentration selected to be used in the administration experiments was 50 μg/mL, which is below that of MIC_{50}. Representative chromatograms of the 5-FU administration assay are presented for *L. lactis* cell precipitate and culture supernatant in Figures 4B and 5A, respectively. The analyte 5-FU was detected in cell samples even after 96 h of incubation (Figure 5A), amounting to

10.5 μg/mL originating from a 0.1 g sample or to 26.2 μg in total cell weight (0.25 g) of the culture (Table 3).

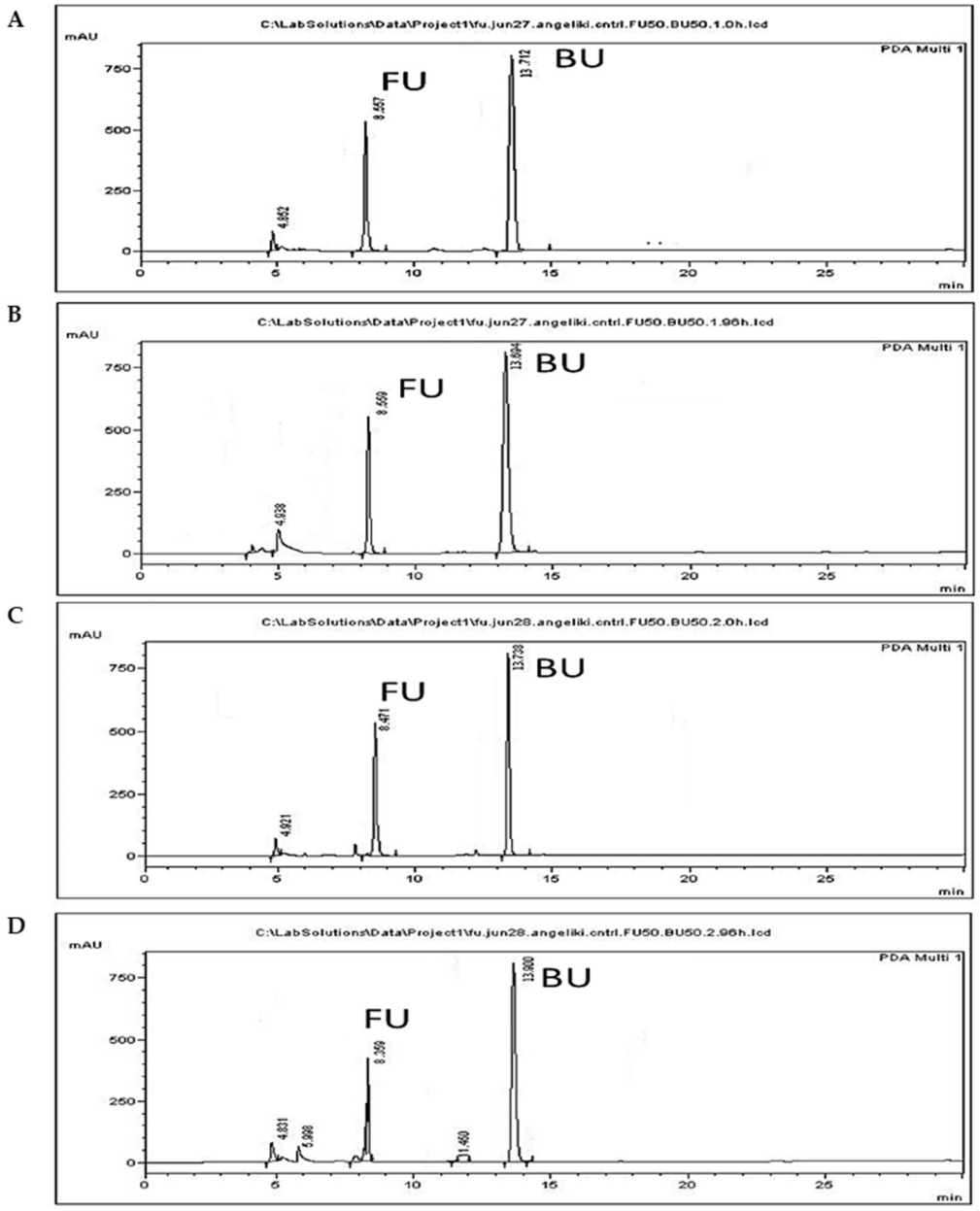

Figure 4. Photodegradation of 5-FU. Representative chromatograms from 5-FU (FU) (50 μg/mL) incubation in the dark at 0 h (**A**) and 96 h (**B**), and under normal light at 0 h (**C**) and 96 h (**D**).

The levels of 5-FU in the medium (Figure 3C) were determined to be 49.5 μg/mL at the onset of the experiment and 5.2 μg/mL at the end (Figure 5B); that is a reduction of 89.5%. These values correspond to 156 μg in total culture volume (30 mL), and added to the 5-FU levels in the cells (Table 2), they produce a sum of 182.2 μg 5-FU, a value which is quite lower than the originally added nominal amount of 50μg/mL of 5-FU or the determined amount of 1485 μg in total culture volume.

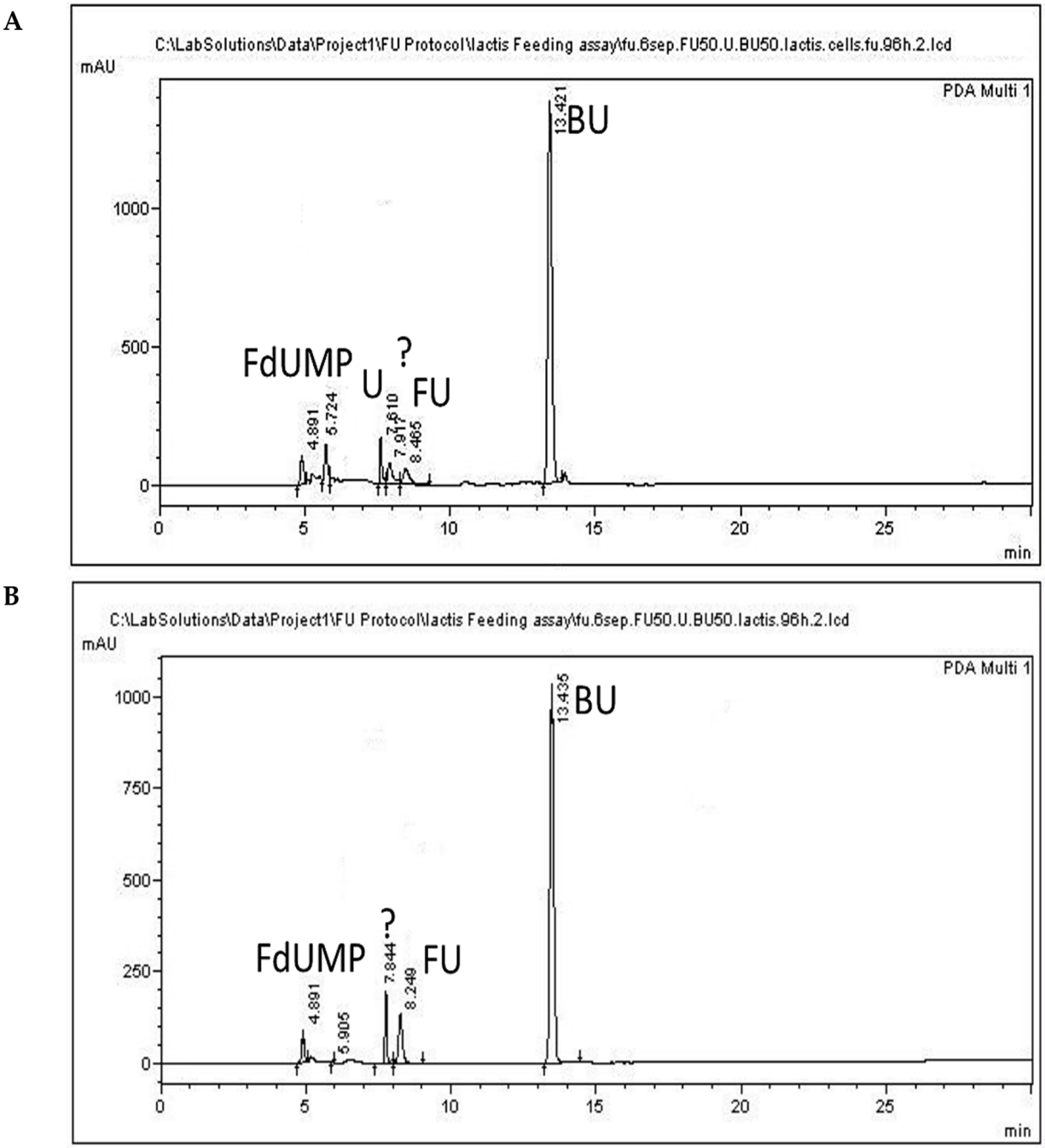

Figure 5. Representative chromatograms of cell precipitate (**A**) and cell-free culture supernatant (**B**) collected at 96 h, following administration of 5-FU (FU) (50 μg/mL) at 0 h. Uracil and FdUMP are indicated as (U) and (FdUMP), respectively. An unidentified compound eluting at 7.9 min is indicated with a question mark.

Uracil was not detected in the culture medium, and although it was detected in cells, its levels amounted to 1.15 μg/mL, a value below the LOQ (Table 2). The levels of the active metabolite of 5-FU, namely FdUMP, in the cells amounted to 21.25 μg/mL (Table 3), which corresponds to only 26% of the determined 5-FU in the 96 h sample and to about only 3% of the originally added 5-FU (1500 μg/mL). FdUMP was also detected in the supernatant; however, its levels amounted to 0.9 μg/mL and were below the LOQ. The determined 5-FU levels and the detection of the active metabolite in the cells indicate an uptake of 5-FU by *L. lactis* cells.

Table 3. Levels of 5-FU, FdUMP, and uracil in cells and in the culture medium following administration of 50 μg/mL 5-FU for 96 h. The estimated values are presented as mean ± SD_p (n = 3) in the first column, and the values in parentheses indicate the confidence interval (CI) at a = 0.025, df = 2, and t value = 4.303. Values in the second column are estimated by multiplying the average appearing in the first column with either the total weight of the cells or the total volume of the culture, as appropriate.

Analyte/Sample	Amount (μg/mL) Mean ± SD_p (CI, n = 3, df = 2)	Amount in Total Culture (0.25 g Cells/30 mL) (μg)
5-FU/medium	5.2 ± 0.8 (2.0)	156
5-FU/cells	10.5 ± 0.63 (1.6)	26.2
F-dUMP/medium	nq	-
F-dUMP/cells	21.2 ± 1.2 (2.55)	47.2
Uracil/medium	nd	-
Uracil cells	nq	-

nq: the compound was detected but not quantified as lower than LOQ; nd: the compound was not detected.

Moreover, our results presented in Table 3 suggest that only a small portion of 5-FU is transformed to its active metabolite, while the other part may be excreted or may lead to the formation of other products. The resistance of certain bacteria to 5-FU has been attributed to mutations in the uracil phosphotransferase (upp) or to the protein/uracil phosphoribosyl transferase (pyrR) genes, either by inhibiting the formation of FdUMP or by de novo production of UMP [19]. Although strain-specific differences occur, the activity of upp has been documented in *L. lactis* MG1363 [55]. However, both upp and pyrR mutations lead to decreased uracil metabolism [56]. Uracil concentrations in cells at 96 h following 5-FU administration amounted to 0.94 μg/mL and presented a distinct increase compared to control cells in which free uracil levels were below LOQ, indicating a decreased metabolism of uracil. These increased uracil levels agree with accumulating uracil due to its decreased metabolism. All peaks showed increased similarities in absorbance data comparisons (Figure S1A–C). The extra peak appearing at 7.8 min in culture supernatant, and at 7.9 min in the cells and the culture supernatant indicated by a question mark in Figure 5, showed a similarity of only 75% in the UV spectrum to commercial uracil or to commercial 5-FU (Figure S1D,E). This led us to further examinations of this peak using ESI-MS analysis, which revealed that it does not correspond either to uracil or to 5-FU, but that it presents close resemblance to the inactive metabolite 5,6-dihydro-5-fluorouracil (FUH_2) (Figure S1F). Although this metabolite was not quantified in the present study, its presence indicates that a considerable part of 5-FU is transformed into this inactive metabolite. This finding agrees with previous reports of inactivation of the majority of the administered 5-FU as FUH_2 [57]. A low 5-FU degradation rate in patients with metastatic colon cancer was correlated with an increased efficacy of the drug and improved chances of survival [58]. Further research on other bacterial strains and their combinations is needed to determine the levels of 5-FU that are inactivated through metabolism by the probiotic bacteria coadministered with the drug, possibly leading to a decreased efficacy of treatment.

4. Conclusions

The RP-HPLC-DAD method for the determination of uracil, 5-FU, and its possible metabolite 5FdUMP in bacterial culture media and in bacterial cells was developed and validated. The estimated validation parameters indicate that it is accurate, precise, specific, and sensitive, with similar limits of detection for all analytes and both biological samples. This renders the method suitable for the analysis of these compounds in bacterial cultures. The method was applied to the bacterial culture of the probiotic *L. lactis*, and the analytes were determined in the cells as well as in the culture medium, following administration of 5-FU to the bacterium for 96 h. All analytes were identified by retention time and peak purity analysis. The formation and presence of the inactive metabolite of 5-FU in both culture media and bacterial cells were verified by MS analysis. To the best of our knowledge, this

is the first quantitative determination of free uracil, 5-FU, and 5-FU metabolites originating from bacterial metabolism, facilitating studies on 5-FU biotransformation. Further research towards the quantification of 5-FU, 5,6-dihydro-5-fluorouracil, uracil, and FdUMP at time points of culture time will provide evidence on the mechanism of 5-FU biotransformation by probiotic bacteria, contributing to a personalized use of probiotics in chemotherapy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9110376/s1, Figure S1: Absorbance spectrum analysis of peak similarity for (A) 5-FU, (B) uracil, (C) 5FdUMP, (D) mismatch of peak with a retention time of 7.8 to 5-FU, and (E) mismatch of peak with a retention time of 7.8 to Uracil and (F) MS analysis.

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