UHPLC-ESI-QqTOF Analysis and In Vitro Rumen Fermentation for Exploiting Fagus sylvatica Leaf in Ruminant Diet

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Abstract: In recent years, animal husbandry has aimed at improving the conditions of livestock animals useful for humans to solve environmental and health problems. The formulation of animal feeds or supplements based on antioxidant plant compounds is considered a valuable approach and an alternative for livestock productivity. Forest biomass materials are an underestimated source of polyphenolic compounds whose sustainable recovery could provide direct benefits to animals and, indirectly, human nutrition. In this context, an alcohol extract from leaves of Fagus sylvatica L. was first investigated through an untargeted ultra-high-performance liquid chromatography–high-resolution tandem mass spectrometry (UHPLC-HRMS/MS) approach. Then, it was fractionated into a fatty acid-rich and a polyphenolic fraction, as evidenced by total lipid, phenol, and flavonoid content assays, with antiradical and reducing activity positively correlated to the latter. When tested in vitro with rumen liquor to evaluate changes in the fermentative parameters, a significant detrimental effect was exerted by the lipid-rich fraction, whereas the flavonoid-rich one positively modulated the production of volatile fatty acids (i.e., acetate, butyrate, propionate, etc.).

Keywords: Fagus sylvatica L.; UHPLC-ESI-QqTOF analysis; polyphenols; flavonoids; in vitro rumen fermentation; volatile fatty acids

1. Introduction

The growing prevalence of chronic diseases and the adoption of animal husbandry for greater revenue and productivity, together with the new emphasis on livestock health, are driving the development of innovative ruminant feeds able to improve animal productivity and to feasibly reduce the footprint of the entire food/feed system on the environment and climate [1–3]. The European project “Rumen-Up”, through the creation of sustainable plant-based solutions, highlighted, almost in a pioneering way, that plants or their parts could be used for livestock feed and to manipulate rumen fermentation, providing benefits to humans, as the modulation of rumen microbial fermentation markedly affects the lipid composition of milk and meat [4]. Thus, the recovery of new feed ingredients from agro-industrial byproducts and residual forest tree materials [5,6] is currently being explored as a valuable strategy to achieve sustainable animal production through the efficient use of resources and waste reduction [7,8], which is also requested by Agenda 2030 (goal 12; that is, “Responsible Consumption and Production: Reversing current consumption trends and promoting a more sustainable future”).
As recently reported [9], grape pomace, olive byproducts and citrus pulp are just a few examples of sources of high added value ingredients for sustainable livestock diets [10–12]. In the research and development of products that can contribute to reducing the environmental impact and safeguarding the environment, the valorization of chestnut and quebracho biomasses is actually applied for livestock, and several plant extracts diversely composed in specialized metabolites, such as tannins, saponins, and flavonoids, as well as in essential oils [13,14], are of interest. Hydrolysable and condensed tannins are especially considered as antimicrobial feed additives, due to their antibacterial and antiparasitic activity [15], whereas saponins are reported to affect rumen defaunation and to impair protein digestion [16]. In general, these non-nutritive compounds are able to modify ruminal fermentation characteristics, to inhibit ruminal methanogenesis, and to enhance livestock performance, overall representing an alternative to antibiotic feed additives [17–19]. In addition, plant material to be disposed that is rich in flavonoids might be an interesting source for novel food additives. Flavonoids are broadly known as antioxidant and anti-inflammatory compounds. Therefore, and due to their related precious benefits under a variety of stressful conditions, these compounds have received a lot of attention for productive performance and health [20]. Moreover, flavonoids might beneficially interact with rumen microbiota and impact carbohydrate fermentation, protein degradation, and lipid metabolism.

Different effects on the microbiome of ruminant digestive systems, whose microorganisms are responsible for feed transformation into products, are reported. Complex carbohydrates are broken down into simple sugars by means of microbial fermentation, and sugar monomers could be used as an energy source and for biosynthesising products, such as volatile fatty acids (VFAs), methane, and carbon dioxide [21]. In fact, due to their antioxidant efficacy, polyphenols could prevent lipoperoxidation and/or protein degradation [22,23]. Furthermore, research is pushing forward to manipulate farm animal feed to influence the sensory, nutritional, and technological characteristics, increasing the level of bioactive metabolites [24]. Additionally, plant extracts open up a fascinating scenario for enhancing both livestock productivity and dairy products (e.g., milk, meat, and cheese) quality. Indeed, different factors, mainly including the plant source and its chemical composition, which can vary based on harvesting time and/or extraction method applied, could affect the efficacy of plant extracts, impoverishing the in vivo applicability. Thus, in order to fully exploit plant compound diversity, a deepening insight into the chemical compositions of plant extracts and the optimization of fractionation procedures could be pursued to differently concentrate phytochemicals on the basis of their features (i.e., polarity, solubility, etc.). This is in line with a performing use of the different coexisting specialized metabolites for feed/food purposes.

In this context, based on a previous phytochemical investigation on *F. sylvatica* leaves [25] as a renewable source for feed and food, herein, a two-steps fractionation process has been proposed, achieving two main fractions. The latter, together with their parental extract, were preliminarily screened for their total phenolic (TPC), flavonoidic (TFC), and lipidic contents (TLC), as well as for their antiradical and reducing activity. The further chemical investigation by means of UV–Vis spectroscopy and ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight tandem mass spectrometry (UHPLC-QqTOF-MS/MS) analyses opened up the evaluation of their effect on in vitro ruminal fermentation (cumulative gas production; organic matter degradability; fermentation kinetics; and end products, i.e., ammonia-N, volatile fatty acids, branched-chain fatty acid proportion, and acetate/propionate ratio). To the best of our knowledge, such a rigorous approach, in which the bioactivity assessment is closely coupled to a detailed chemical investigation, has never been reported in the literature before.

2. Results and Discussion

The alcoholic extract of *F. sylvatica* leaf (Fs/1/1), whose chemical profiling was previously reported [25], consisted of an abundant polyphenol part and fatty acids. Polyphenols
were mainly flavonols, beyond hydroxycinnamoyl compounds, while mono- or polyhydroxylated fatty acids were identified, also according to the literature [26], at the highest retention time in the total ion chromatogram [25]. The Fs/1/1 extract exhibited a good antioxidant efficacy, which was supposed to be enhanced following fractionation and with the obtainment of fractions differently composed in terms of polarity and identity of their specialized metabolites. In particular, with the aim to achieve a fraction depauperated in fatty acids, a biphasic extraction was firstly employed on Fs/1/1, and the nonpolar fraction Fs/2/1 was partitioned from an hydroalcoholic fraction comprising hydrophilic metabolites. This latter was further chromatographed using XAD-4 polystyrene resin, suitable for the recovery of polyphenols (Figure 1). Thus, two organic fractions were collected (Fs/2/1 and Fs/3/2), and with the aim to explore the impact on microbial fermentation processes of the parental leaf extract and its most bioactive fractions, a spectrophotometric screening was first carried out.

The preliminary assessment of the total phenolic (TPC), total flavonoidic (TFC), and total lipidic contents (TLC) markedly differentiated the fractions Fs/2/1 and Fs/3/2. This latter appeared mainly constituted by phenol compounds and flavonoids, with the highest TPC and TFC values equal to 161.3 ± 15.0 gallic acid equivalents (GAEs) and 128.4 ± 1.9 mg quercetin equivalents (QUEs) per g of extract, respectively (Figure 2A(a,b)). The lipidic content of Fs/3/2 was not negligible, so much so that it was estimated to be equal to 212.0 ± 27.5 mg oleanolic acid equivalents (OAEs) per g of extract. It could be due to the saponins previously identified. In fact, the assay employed originally utilized to colorimetrically evaluate saponins was enlarged in its aims, as the presence of double bonds or free hydroxyl groups within lipid analytes is a feature that allows the positive response to be detected [27,28].

The organic fraction Fs/2/1 likely lacked phenolic compounds (62.2 ± 8.6 mg QUEs per g of extract) and, specifically, flavonoid constituents (19.7 ± 3.2 mg QUEs per g of extract), being richer in lipid compounds (Figure 2A). To further corroborate the diverse chemical constitution and to preliminarily discriminate the fractions based on their antiradical and reducing activities, DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azinobis(3-ethylbenzothiazolin-6-sulfonic acid) assays, as well as the Fe(III) reducing test, were performed. In this context, the data acquired highlighted that fraction Fs/3/2 was effective in scavenging both ABTS•+ and DPPH• with ID_{50} values equal to 0.74 ± 0.08 µg/mL and 40.8 ± 1.1 µg/mL, respectively. Furthermore, it was able to reduce ferric ions also at the lowest tested dose, exhibiting a Trolox Equivalent Antioxidant Capacity (TEAC) value equal to 28.3. All data underwent a Principal Component Analysis, and the main

![Figure 1. Extraction and fractionation of F. sylvatica L. leaf. UAM: ultrasound assisted maceration; LLE: liquid–liquid extraction; Haf: hydroalcoholic fraction; XAD-4 GPC: Gel Permeation Chromatography on Amberlite XAD-4 absorbent resin.](image-url)
components obtained (PC1 and PC2) allowed us to further interpret the original dataset. In particular, it was observed that phenol and flavonoid contents, as well as antiradical and reducing activity, were positively correlated to the fraction Fs/3/2, whereas the lipid content represented the main feature of the fraction Fs/2/1.

Figure 2. (A) (a) Total phenolic content (TPC), expressed as mg of gallic acid equivalents (GAE) per g of extract; (b) total flavonoidic content (TFC), expressed as mg of quercetin equivalents (QUE) per g of extract; (c) total lipidic content (TLC), expressed as mg of oleanolic acid equivalents per g of extract. Values reported are the mean ± SD of three independent measurements. (B) (a) Scavenging capability (SC%) vs. 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical cation, (b) scavenging capability (SC%) vs. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, and (c) Fe (III) reducing power (RP) of *F. sylvatica* extract and organic fractions therefrom. Values reported are the mean ± SD of three independent measurements. (C) PCA analysis based on colorimetric compositive assays and data from antiradical and reducing power tests.

2.1. Chemical Investigation on Fs/2/1 and Fs/3/2 Fractions

The fractions Fs/2/1 and Fs/3/2 were chemically investigated by means of UV spectroscopy and UHPLC-ESI-QqTOF tandem mass spectrometry.

UV data, with absorption bands at 235 and 205 nm, highlighted that the main constituents of the Fs/2/1 fraction could be fatty acids and their oxidized derivatives. Indeed, the fraction also showed very weak UV absorption at 328, 415, and 667 nm, in line with the trace presence of phenol, carotenoid, and chlorophyll compounds. The UV spectrum of the Fs/3/2 fraction, with bands at 330, 295, 270, and 203 nm, was in line with the occurrence of hydroxycinnamoyl compounds, flavonoids, and saponins (Figure S1).

UHPLC-QqTOF-MS/MS analysis, and the relative quantifications relying on the change of the levels of each identified compound (Table 1) in the parental Fs/1/1 extract and its fractions Fs/2/1 and Fs/3/2 (Figure 3) were carried out. It appeared that, according to fractionation-induced reduction of the plant extract complexity, Fs/2/1 mainly constituted octanoids and other fatty acids derivatives, where Fs/3/2 massively contained the phenol and polyphenol components of the parental extract, with a few differences. Thus, the polar constituents of beech methanolic extract were part of Fs/3/2, in which minor constituents could be also masked, such as a galloyl hexoside with the [M-H]− at *m/z* 331.0667 and a hydroxybenzoyl hexoside whose deprotonated molecular ion was at *m/z* 299.0770.
| Peak | Rt (min) | Tentative Assignment | Formula | [M-H]– Found (m/z) | [M-H]– Calc. (m/z) | Error (ppm) | RDB | MS/MS Fragment Ions (m/z) and Relative Intensity |
|------|----------|----------------------|---------|-------------------|-------------------|-------------|-----|-----------------------------------------------|
| 1    | 0.397    | Quinic acid          | C7H12O5 | 191.0357          | 191.0566          | 2.2         | 2   | 191.0557; 129.0192; 111.0096; 87.0098; 85.0307 |
| 2    | 0.454    | Citric acid          | C6H7O7  | 191.0203          | 191.0197          | 3           | 3   | 111.0089; 87.0089                            |
| 3    | 0.769    | Galloyl hexose       | C13H16O10 | 331.0667  | 331.0671          | 1.1         | 6   | 331.0662; 211.0236; 169.0139; 151.0033       |
| 4    | 1.311    | Dihydroxybenzoic acid hexose | C13H16O9 | 315.0720          | 315.719           | 0.1         | 6   | 315.0722; 271.0396; 227.0528; 195.0287; 153.0199; 152.0114 |
| 5    | 1.574    | Hydroxybenzoyl hexose| C13H16O8 | 299.0770          | 299.0772          | 0.8         | 6   | 299.0770; 239.0557; 179.0345; 151.0394; 137.0237 |
| 6    | 1.890    | Hydroxyphenylactic acid hexose | C14H18O8 | 313.0939          | 313.0929          | 3.2         | 6   | 313.0910; 151.0390                           |
| 7    | 2.038    | 3-O-Caffeoyl quinic acid (cis) | C16H18O8 | 353.0885          | 353.0878          | 2           | 8   | 353.0888; 191.0565; 179.0353; 135.0451     |
| 8    | 2.235    | 3-O-Caffeoyl quinic acid (trans) | C16H18O8 | 353.0888          | 353.0878          | 2.2         | 8   | 353.0888; 191.0565; 179.0353; 135.0451     |
| 9    | 2.444    | Caffeoyl threonic acid (1) | C13H14O8 | 297.0614          | 297.0622          | 2           | 7   | 179.0340; 161.0245; 135.0310; 117.0192; 89.0248 |
| 10   | 2.603    | Caffeoyl acid hexose  | C13H14O8 | 341.0874          | 341.0878          | 7           | 1.2 | 179.0344; 161.0244; 135.0444; 134.0296     |
| 11   | 2.822    | Caffeoyl threonic acid (2) | C13H14O8 | 297.0618          | 297.0616          | 0.7         | 7   | 179.0344; 135.0301                           |
| 12   | 2.839    | 3-O-p-Coumaroyl quinic acid | C16H18O8 | 337.0926          | 337.0929          | 0.9         | 8   | 191.0560; 163.0401; 119.0499                  |
| 13   | 3.210    | 3-O-p-Coumaroyl quinic acid | C16H18O8 | 337.0926          | 337.0929          | 0.9         | 8   | 191.0556; 163.0340; 119.0502                 |
| 14   | 3.444    | Procyandin          | C30H26O12 | 577.1353          | 577.1352          | 0.3         | 18  | 577.1393; 451.1046; 425.0883; 407.0781; 289.0711; 245.0463; 125.0236 |
| 15   | 3.523    | p-Coumaroyl acid hexose | C15H18O8 | 325.0926          | 325.0929          | 0.9         | 7   | 163.0393; 119.0500                           |
| 16   | 3.676    | p-Coumaroyl threonic acid (1) | C13H14O7 | 281.0662          | 281.0666          | 1.7         | 7   | 163.0396; 135.0298; 119.0502                 |
| 17   | 3.950    | Caffeic acid         | C6H4O5  | 179.0357          | 179.0350          | 4.0         | 6   | 135.0454; 134.0377; 117.0348; 107.0508; 89.0403 |
| 18   | 4.259    | p-Coumaroyl threonic acid (2) | C13H14O7 | 281.0666          | 281.0666          | 0           | 7   | 177.0570; 163.0399; 145.0293; 135.0298; 119.0503; 117.0196; 87.0089 |
| 19   | 4.624    | 5-O-Caffeoyl quinic acid (trans) | C16H18O8 | 353.0879          | 353.0878          | 0.3         | 8   | 191.0571; 85.0306                             |
| 20   | 5.665    | Caffeoyl threonic acid (3) | C13H14O8 | 297.0616          | 297.0612          | 1.3         | 7   | 179.0343; 161.0242; 135.0242                 |
| 21   | 5.693    | Caffeoyl propionic acid | C12H12O5 | 253.0718          | 253.0718          | 0           | 2   | 253.0705; 179.0344; 161.0243; 135.0450; 134.0375; 133.0375 |
| 22   | 5.759    | 5-O-Caffeoyl quinic acid (cis) | C16H18O9 | 353.0874          | 353.0878          | 1.1         | 8   | 191.0557; 161.0240; 85.0293                 |
| 23   | 6.090    | Eriodictyol 7-O-hexose | C21H22O11 | 449.1092          | 449.1089          | 0.3         | 11  | 449.1093; 421.1144; 313.0705; 287.0553; 283.0602; 259.0607; 243.0665; 215.0702; 178.9980; 125.0242 |
| 24   | 6.645    | Tuberic acid         | C18H18O5 | 387.1662          | 387.1661          | 0.4         | 5   | 387.1655; 207.1024; 163.1130; 89.0243       |
| 25   | 6.981    | 5-O-p-Coumaroyl quinic acid | C16H18O8 | 337.0930          | 337.0929          | 0.3         | 8   | 191.0559                                   |
Table 1. Cont.

| Peak | Rt (min) | Tentative Assignment                  | Formula     | [M-H] – Found (m/z) | [M-H] – Calc. (m/z) | Error (ppm) | RDB | MS/MS Fragment Ions (m/z) and Relative Intensity |
|------|----------|---------------------------------------|-------------|---------------------|---------------------|-------------|------|-------------------------------------------------|
| 27   | 7.871    | Caffeoylshikimic Acid                 | C_{18}H_{16}O_{8} | 335.0766            | 335.0772            | –1.9        | 9    | 335.0766; 179.0355; **161.0246**; 135.0450       |
| 26   | 7.312    | p-O-Coumaroylmalic acid (I)           | C_{13}H_{12}O_{7} | 337.0925            | 337.0929            | –1.2        | 8    | **191.0560**; 173.0461; 93.0347; 85.0296         |
| 28   | 8.101    | 5-O-p-Coumaroyl quinic acid           | C_{16}H_{18}O_{8} | 335.0770            | 335.0772            | –0.7        | 9    | **179.0350**; 161.0243; 135.0453                 |
| 29   | 8.314    | Caffeoylshikimic Acid                 | C_{18}H_{16}O_{8} | 337.0925            | 337.0929            | –1.2        | 8    | **191.0560**; 173.0461; 93.0347; 85.0296         |
| 30   | 9.288    | p-O-Coumaroylmalic acid (II)          | C_{13}H_{12}O_{7} | 279.0508            | 279.0510            | –0.1        | 8    | 179.0348; **135.0451**                          |
| 31   | 10.091   | Naringenin-C-hexoside (1)             | C_{21}H_{22}O_{10} | 433.1145            | 433.1140            | 1.1         | 11   | 433.1153; 415.1027; 343.0826; 325.0715; **313.0715** |
| 32   | 10.712   | Naringenin-C-hexoside (2)             | C_{21}H_{22}O_{10} | 433.1140            | 433.1140            | 1.6         | 11   | 433.1151; 415.1043; 343.0826; 325.0710; **313.0718** |
| 33   | 11.960   | Myricetin-3-O-hexoside                | C_{21}H_{20}O_{13} | 479.0839            | 479.0831            | 1.6         | 12   | 479.0841; 317.0286; **316.0221**; 287.0193; 271.0239 |
| 34   | 14.293   | Quercetin-3-O-hexoside (1)            | C_{21}H_{20}O_{12} | 463.0893            | 463.0882            | 2.4         | 12   | 463.0898; 301.0355; **300.0270**; 271.0246; 255.0298; 178.9989; 151.0035 |
| 35   | 14.445   | Quercetin-3-O-hexuronide              | C_{21}H_{18}O_{13} | 477.0679            | 477.0675            | 0.9         | 13   | 477.0688; **301.0347**; 178.9979; 151.0033       |
| 36   | 14.728   | Quercetin-3-O-hexoside (2)            | C_{21}H_{20}O_{12} | 463.0891            | 463.0882            | 1.9         | 12   | 463.0899; 301.0355; **300.0271**; 271.0247; 255.0295; 243.0296; 178.9985; 151.0035 |
| 37   | 14.739   | Kaempferol-3-O-hexuronide (1)         | C_{21}H_{18}O_{12} | 461.0733            | 461.0725            | 1.6         | 13   | **285.0398**                                   |
| 38   | 14.940   | Naringenin-C-hexoside (3)             | C_{21}H_{22}O_{10} | 433.1145            | 433.1140            | 1.1         | 11   | 433.1146; 415.1026; 343.0827; 325.0731; **313.0713** |
| 39   | 15.083   | Neolignan (I)                         | C_{25}H_{34}O_{11} | 509.2033            | 509.2028            | 0.9         | 9    | 509.2033; 491.1937; 461.1823; 367.1396; 313.1288; **179.0711** |
| 40   | 15.218   | Neolignan (II)                        | C_{25}H_{34}O_{11} | 509.2043            | 509.2028            | 2.9         | 9    | 509.2052; 491.1949; 473.1834; 461.1832; 367.1406; 313.1301; **179.0717** |
| 41   | 15.338   | Quercetin-3-O-pentoside               | C_{26}H_{18}O_{11} | 433.0783            | 433.0776            | 1.5         | 12   | 433.0798; 301.0359; **300.0277**; 271.0250; 255.0299; 243.0294; 178.9986 |
| 42   | 15.423   | Kaempferol-3-O-hexoside (1)           | C_{21}H_{20}O_{11} | 447.0944            | 447.0933            | 2.5         | 12   | 447.0945; **285.0398**; **284.0318**; 255.0292; 227.0340; 151.0031 |
| 43   | 15.517   | Quercetin-3-O-(acetyl)hexoside        | C_{23}H_{22}O_{13} | 505.1003            | 505.0988            | 3.0         | 13   | 505.1018; 463.0897; 447.0945; 301.0357; **300.0277** |
| 44   | 15.743   | Kaempferol-3-O-hexuronide (2)         | C_{21}H_{18}O_{12} | 461.0741            | 461.0725            | 3.4         | 13   | 461.0739; **285.0406**; 257.0460; 229.0511; 113.0248 |
| 45   | 15.903   | Kaempferol-3-O-hexoside (2)           | C_{21}H_{20}O_{11} | 447.0951            | 447.0933            | 4.1         | 12   | 447.0944; 327.0504; 285.0397; **284.0317**; 255.0295; 227.0345; 151.0040 |
| Peak | Rt (min) | Tentative Assignment | Formula | [M-H] – Found (m/z) | [M-H] – Calc. (m/z) | Error (ppm) | RDB | MS/MS Fragment Ions (m/z) and Relative Intensity |
|------|----------|----------------------|---------|---------------------|---------------------|------------|------|-----------------------------------------------|
| 46   | 16.237   | Kaempferol-3-O-pentoside (1) | C_{20}H_{18}O_{10} | 417.0843            | 417.0827            | 3.3        | 12   | 417.0836; 285.0399; 284.0323; 255.0295; 227.0345; 151.0036 |
| 47   | 16.456   | Kaempferol-3-O-pentoside (2) | C_{20}H_{18}O_{10} | 417.0833            | 417.0827            | 1.4        | 12   | 417.0841; 285.0406; 284.0330; 255.0300; 227.0349 |
| 48   | 16.748   | Kaempferol-3-(acetyl)hexoside | C_{23}H_{22}O_{12} | 489.1049            | 489.1039            | 2.1        | 13   | 489.1069; 429.0800; 369.0996; 285.0412; 284.0333; 255.0301; 227.0350; 151.0034 |
| 49   | 17.073   | Kaempferol-3-O-deoxyhexoside | C_{21}H_{20}O_{10} | 431.0991            | 431.0984            | 1.4        | 12   | 431.1002; 285.0408; 284.0332; 255.0303; 227.0352; 229.0506 |
| 50   | 18.232   | Neolignan (III) | C_{27}H_{38}O_{12} | 553.2313            | 553.2291            | 4.1        | 9    | 553.2330; 343.1403; 328.1162; 183.0655 |
| 51   | 19.380   | Kaempferol p-coumaroylhexoside | C_{30}H_{26}O_{13} | 593.1325            | 593.1320            | 3.3        | 18   | 593.1322; 447.0935; 307.0813; 285.0387; 284.0323; 255.0294 |
| 52   | 19.623   | Kaempferol | C_{15}H_{10}O_{6} | 285.0404            | 285.0405            | −0.2       | 11   | 285.0400; 257.0445; 255.0293; 229.0495; 211.0393; 187.0393; 151.0034 |
| 53   | 18.438   | Dodecenedioic acid | C_{12}H_{20}O_{4} | 227.1289            | 227.1300            | 4.0        | 3    | 183.1394; 165.1278 |
| 54   | 18.867   | 9,12,13-trihydroxy-10,15-octadecadienoic acid | C_{18}H_{32}O_{5} | 327.2191            | 327.2177            | 0.9        | 3    | 309.2076; 291.1970; 229.1288; 229.1446; 221.1179; 211.1338; 191.1236; 183.1391; 171.1022; 137.0966 |
| 55   | 21.080   | Neolignan (IV) | C_{38}H_{52}O_{16} | 763.3187            | 763.3183            | 0.6        | 13   | 763.3225; 343.1403; 328.1154; 183.0654 |
| 56   | 19.695   | 9,12,13-trihydroxy-10-octadecenoic acid | C_{18}H_{34}O_{5} | 329.2337            | 329.2340            | 2.0        | 2    | 329.2343; 229.1448; 211.1344; 183.1393; 171.1030 |
| 57   | 20.297   | Dihydroxyhexadecanoic acid | C_{16}H_{32}O_{4} | 287.2228            | 287.2240            | 4.2        | 1    | 287.2235; 259.2127 |
| 58   | 20.558   | 9,16-diidrossi-octadeca-10,12,14-trienoic acid (1) | C_{18}H_{30}O_{4} | 309.2072            | 309.2071            | 0.2        | 4    | 309.2078; 291.1972; 273.1841; 251.1654; 239.1650; 221.1544; 197.1162; 183.1026; 171.1032; 107.0865 |
| 59   | 20.846   | 16-idrossi-9-ossooctadeca-12,14-dienoic acid | C_{18}H_{30}O_{4} | 309.2071            | 309.2071            | −0.1       | 4    | 309.2059; 291.1962; 251.1655; 171.1028; 125.0971 |
| 60   | 21.053   | 9,16-diidrossi-octadeca-10,12,14-trienoic acid (2) | C_{18}H_{30}O_{4} | 309.2071            | 309.2071            | 0.2        | 4    | 309.2079; 291.1967; 251.1649; 185.1180; 171.1021; 137.0971 |
| 61   | 21.350   | 16-idrossi-9-ossooctadeca-12,14-trienoic acid | C_{18}H_{28}O_{4} | 307.1915            | 307.1925            | 3.3        | 5    | 289.1793; 249.1492; 235.1342; 211.3430; 185.1187; 125.0976; 121.0659 |
| Peak | Rt (min) | Tentative Assignment | Formula | \([\text{M-H} - \text{Found} (m/z)]\) | \([\text{M-H} - \text{Calc.} (m/z)]\) | Error (ppm) | RDB | MS/MS Fragment Ions (m/z) and Relative Intensity |
|------|---------|----------------------|---------|---------------------------------|---------------------------------|-------------|------|--------------------------------------------------|
| 62   | 21.350  | Dihydroxyoctadecenoic quinic acid | C_{25}H_{42}O_{9} | 485.2776 | 485.2756 | 4.1 | 5 | 485.2791; 311.2238; 223.1706; 191.0568 |
| 63   | 22.220  | 16-idrossi-9-ossooctadeca-6,12,14,16-tetraenoic acid | C_{18}H_{26}O_{4} | 305.1758 | 305.1760 | 0.5 | 6 | 305.1744; 249.1498; 205.1587; **135.0817** |
| 64   | 22.435  | Kaempferol \(p\)-coumaroyldeoxyhexoside (1) | C_{30}H_{26}O_{12} | 577.1368 | 577.1352 | 2.9 | 18 | 577.1389; 431.0988; *285.0404*; 284.0316; 257.0452; 299.0495 |
| 65   | 22.553  | Kaempferol \(p\)-coumaroyldeoxyhexoside (2) | C_{30}H_{26}O_{12} | 577.1357 | 577.1352 | 1.0 | 18 | 577.1382; *285.0399*; 284.0328 |
| 66   | 22.842  | Kaempferol di-\(p\)-coumaroypentoside | C_{38}H_{30}O_{14} | 709.1463 | 709.1590 | 3.8 | 24 | 709.1641; 563.1244; 423.1116; *285.0416*; 284.0332; 145.0290 |
| 67   | 23.154  | 15,16-dihydroxy-9Z,12Z-octadecadienoic acid | C_{18}H_{32}O_{4} | 311.2242 | 311.2228 | 1 | 3 | 311.2238; 293.2112; 275.2011; 235.1708; *223.1705*; 201.1135; 87.0454 |
| 68   | 23.310  | 3-O-(dihexosyl)hexuronidyl oleanonic acid | C_{48}H_{76}O_{19} | 955.4928 | 955.4908 | 2.1 | 11 | *955.4984*; 793.4427; 731.4414; 613.3786; 569.3871; 523.3830 |
| 69   | 23.582  | 3-O-(dihexosyl)hexuronidyl oleanonic acid | C_{48}H_{76}O_{19} | 955.4923 | 955.4908 | 1.6 | 11 | *955.4972*; 793.4423; 569.3868 |
| 70   | 24.029  | Dihydroxyoctadecenoic acid | C_{18}H_{34}O_{4} | 313.2382 | 313.2384 | −0.7 | 2 | 313.2377; 255.2270; 277.2163; *183.1384*; 129.0916 |
| 71   | 24.050  | 3-O-(hexosylpentosyl)hexuronidyl oleanonic acid | C_{47}H_{74}O_{18} | 925.4801 | 925.4802 | −0.2 | 11 | *925.4840*; 763.4277 |
| 72   | 24.128  | 3-O-(hexosyl)hexuronidyl oleanonic acid | C_{42}H_{66}O_{14} | 793.4395 | 793.4380 | 0.9 | 10 | *793.4407*; 631.3873; 569.3854 |
| 73   | 24.457  | Kaempferol di-\(p\)-coumaroylexyhexoside | C_{30}H_{32}O_{14} | 723.1720 | 723.1719 | 0.1 | 24 | *723.1784*; 577.1396; 559.1250; 437.1265; 285.0409; 284.0332; 187.0391; 163.0401; 145.0294 |
| 74   | 24.483  | Kaempferol \(p\)-coumarol feruloyl pentoside | C_{48}H_{34}O_{15} | 753.1825 | 753.1823 | −0.3 | 24 | 753.1884; 607.1502; 589.1358; 467.1378; *285.0407*; 284.0320; 193.0495 |
| 75   | 25.302  | 9-oxooctadeca-10,12-dienoic acid | C_{18}H_{30}O_{3} | 293.2122 | 293.2130 | 2.7 | 4 | 293.2126; *275.2019*; 235.1708; 211.1340; 183.1392; 171.1025; 121.1026 |
| 76   | 25.302  | 9-oxooctadeca-10,12,15-trienoic acid | C_{18}H_{28}O_{3} | 291.1971 | 291.1966 | 1.8 | 5 | *291.1969*; 273.1851; 247.2069; 223.1701; 195.1382 |
| Peak | Rt (min) | Tentative Assignment | Formula | [M-H] – Found (m/z) | [M-H] – Calc. (m/z) | Error (ppm) | RDB | MS/MS Fragment Ions (m/z) and Relative Intensity |
|------|---------|----------------------|---------|---------------------|---------------------|-------------|------|-------------------------------------------------|
| 77   | 25.611  | 13-hydroxyoctadeca-9,15-dienoic acid | C_{18}H_{32}O_3 | 295.2283 | 295.2279 | 1.5 | 3 | 295.2284; 277.2176; 183.1389 |
| 78   | 25.783  | 13-hydroxyoctadeca-9,11-dienoic acid | C_{18}H_{32}O_3 | 295.2286 | 295.2279 | 1.8 | 3 | 295.2272; 277.2166; 195.1387; 183.1023; 155.1065; 113.0971 |
| 79   | 25.937  | 13-oxo-9Z,11E-octadecadienoic acid | C_{18}H_{30}O_3 | 293.2121 | 293.2122 | 2.0 | 4 | 293.2119; 275.2022; 249.2220; 195.1390; 185.1179; 153.1287; 113.0974 |
| 80   | 26.267  | 9-hydroxyoctadec-12-enoic acid | C_{18}H_{34}O_3 | 297.2444 | 297.2435 | 3.0 | 2 | 297.2439; 279.2331; 171.1027; 155.1075 |
| 81   | 27.520  | α-Linolenic acid | C_{18}H_{36}O_2 | 277.2180 | 277.2173 | 2.5 | 4 | 277.2181 |
| 82   | 28.089  | Linoleic acid | C_{18}H_{32}O_3 | 279.2338 | 279.2330 | 3.0 | 3 | 279.2347; 259.2098 |
| 83   | 28.552  | Palmitic acid | C_{16}H_{32}O_2 | 255.2336 | 255.2330 | 2.5 | 1 | 255.2334; 237.2220; 201.8341 |
| 84   | 28.638  | Oleic acid | C_{18}H_{34}O_2 | 281.2491 | 281.2486 | 1.8 | 2 | 281.2493 |
| 85   | 29.311  | Stearic acid | C_{18}H_{36}O_2 | 283.2656 | 283.2643 | 4.8 | 1 | 283.2652; 265.2517 |
Figure 3. Total ion current (TIC) chromatograms of Fs/1/1 extract (A), Fs/2/1 fraction (B), and Fs/3/2 fraction (C). The relative content of each extract/fraction in a derivative of benzoic acid (BA) and hydroxycinnamic acid (HCA), as well as of flavonoids (Fl), lignans (Lg), fatty acids (FA), and saponins (Sap), is shown next to each chromatogram.

Dihydroxybenzoic acid hexoside ([M-H]− at m/z 315.722) and a hydroxyphenylacetic acid hexoside ([M-H]− at m/z 313.0939) was also recognized. However, hydroxycinnamoyl derivatives and flavonoids were the main compounds of the Fs/3/2 fraction. Among hydroxycinnamoyl-based compounds, 3-O- and 5-O-caffeoyl quinic acids (7, 8, 19, and 22) were identified, together with 3-O- and 5-O-p-coumaroyl quinic acids (12, 13, 25, and 28). Two isomers of caffeoyl shikimic acids were first-time distinguishable as 4-O-CSA (27), based on the base peak at m/z 161.024, and 5-O-CSA (29), whose TOF-MS/MS base peak was at m/z 179.0350, respectively [29], and different caffeoyl and p-coumaroyl esters of threonic acid (9, 11, 16, 18, and 20). Threonic acid, which is a product of ascorbate catabolism [30], was identifiable thanks to the neutral loss of 118.02 and the fragment ion at m/z 117.02. The hydroxycinnamoyl moiety of the threonic acid depsides was identified based on the characteristic deprotonated ion, which was detected at m/z 179.03 for caffeoate and at m/z 163.04 for p-coumarate, and also based on their relative decarboxylated ions, which were, respectively, at m/z 135.03 [caffeic acid−CO2−H]+ and 119.05 [p-coumaric acid−CO2−H]+.

Caffeoyl esters of threonic acid, in which the hydroxycinnamoyl moiety was at carbons C2 or C4 of the threonic acid, were found in Chelidonium majus L., an herbal drug tradi-
tionally used against diseases of the liver, the gallbladder, and various skin disorders [31], and 4-E-caffeoyl-L-threonic acid was observed as an oviposition stimulant for Papilio bianor from Orixa japonica leaves [32]. Coumaroyl malic acids (26 and 30) were also herein tentatively identified, with the malate ion appearing at \( m/z \) 133.0301 on the TOF-MS/MS spectrum. Other hydroxycinnamoyl compounds were the hexoside of caffeic acid (10, with the deprotonated molecular ion at \( m/z \) 341.0874), and coumaric acid (15, \([M-H]\) at \( m/z \) 325.0926) and, again, caffeoyl propionic acid (21) and caffeic acid (17).

Flavonoids accounted for a large part of Fs/3/2. Among them, a procyanidin B-type dimer (14), whose deprotonated molecular ion at \( m/z \) 577.1353 provided fragment ions at \( m/z \) 451.1046, 425.0883, 407.0781, and 289.0711; a flavanone O-glycoside (23); and three flavanone C-glycosides (31, 32, and 38) and flavonol glycosides were the main constituents. Myricetin (in glycoside 33), quercetin, and kaempferol were found to be the core aglycones, with kaempferol glycosides as the most abundant compounds.

Quercetin 3-O-galactopyranoside (34), quercetin 3-O-glucopyranoside (36), quercetin glucuronide (35), a quercetin 3-O-pentoside (41), and quercetin 3-O-acetylhexose (43) were constituents of the fraction. Neutral losses of 162.05 Da (dehydrated hexose) and 132.04 Da (dehydrated pentose), 146.06 (dehydrated deoxyhexose), 204.07 Da (dehydrated acetyhexose), and 176.03 (dehydrated hexuronic acid) suggested the glyconic moiety identity.

Kaempferol 3-O-glucopyranoside (42), 3-O-galactopyranoside (45), and two kaempferol 3-O-pentosides (46, 47) were identifiable based on a relative neutral loss of 162.05 Da (hexose) and 132.04 Da (pentose) from the deprotonated molecular ion and the formation of the abundant \([\text{aglycone-H}]^-\) ion at \( m/z \) 284.03 (base peak), whose intensity was diagnostic for the localizing glyconic moiety.

A kaempferol deoxyhexoside (49) was also tentatively identified, whereas the detected \([M-H]^-\) ion at \( m/z \) 489.1049 for compound 48 and its relative TOF-MS/MS spectrum were in accordance with the acetyl derivative of kaempferol hexoside. Coumaroyl kaempferol glycosides (51, 64, 65, 66, 73, and 74) were also poorly detected, together with the kaempferol hexuronides (37 and 44) and the kaempferol aglycone (52). Compounds belonging to the neolignan class (39, 40, 50, and 55) were detected, although the TOF-MS/MS low fragmentiation did not allow to unambiguously identify them. Oleanolic acid-based saponins 68, 69, 71, and 72 appeared to be fully transferred into this fraction.

Kaempferol glycosides and acylated derivatives were found also in the apolar Fs/2/1 fraction, which mainly contained octadecanoids and other fatty acid derivatives. In particular, traces of kaempferol hexoside, pentoside, and deoxyhexoside were tentatively identified, whereas kaempferol p-coumaroyldeoxyhexosides (at \( m/z \) 577.1368 and 577.1357; 64 and 65, respectively), di-p-coumaroyldeoxyhexoside (at \( m/z \) 723.1720; 73), and p-coumaroyl feruloyl deoxyhexoside (at \( m/z \) 753.1825; 74), as well as kaempferol di-p-coumaroylpentoside (at \( m/z \) 709.1463, 66), eluting at higher retention times, were the most abundant. In Figure 4, TOF-MS/MS spectra of compounds 66, 73 and 74 are reported. Neutral losses of 146.04 Da (dehydrated p-coumaric acid), 292.09 Da (p-coumaroyldeoxyhexose-H2O), and 438.13 Da (di-p-coumaroyldeoxyhexoside-H2O) suggested the tentative identification of kaempferol p-coumaroyl- and di-p-coumaroyl deoxyhexoside.

The interest in these compounds is mainly related to their antimicrobial activity, so much so that antibacterial kaempferol coumaroyl rhamnosides, commonly called platano-rhamnosides, were previously isolated from the leaves of Platanus occidentalis [33]. Kaempferol-3-O-(bis-p-coumaroyl) rhamnoside was also from Persea lingue and was found to exert efflux inhibitory activity towards the NorA transporter of Staphylococcus aureus [34], whereas kaempferol 7-O-(2,3-di-E-p-coumaroyl-α-L-rhamnoside) was isolated from the flowers and fruit of Tetrapanax papyriferus [35]. The deprotonated molecular ion at \( m/z \) 753.1823 for compound 73 likely consisted of a derivative of the previous compound in which a feruloyl moiety was in the place of a p-coumaroyl residue. Finally, kaempferol di-p-coumaroylpentoside (66) showed the deprotonated molecular ion at \( m/z \) 709.1641 and TOF-MS/MS fragment ions at \( m/z \) 563.1244 (due to dehydrated p-coumaric acid), 423.1116 (coumaroylpentose-H2O), and 285.0416 (deprotonated kaempferol aglycone). Kaempferol
3-O-[2”,5”-di-O-(E)-p-coumaroyl]-α-L-arabinofuranoside was also isolated from Pseudosuga menziesii [36]. Fatty acid derivatives, especially octadecanoids, accounted for the greater part of the tentatively identified compounds. In fact, beyond a dodecanedioic acid (53), likely traumatic acid, whose [M-H]$^-$ ion fragmented gave the ions at m/z 183.1394 and 165.1278 by decarboxylation and following dehydration, respectively, and a dihydroxyhexadecanoic acid (57) with deprotonated molecular ion at m/z 287.2228, all the other fatty acids derivatives shared a carbon skeleton of 18 carbons.

![Figure 4. TOF-MS/MS spectra of the acylated kaempferol glycosides (A) 66, (B) 73, and (C) 74 with [M-H]$^-$ ions at m/z 709.1463, 723.1720, and 753.1825, which were detected in the lipophilic Fs/2/1 fraction.](image)

Characteristic TOF-MS/MS fragment ions and peculiar neutral losses tentatively distinguished hydroxylation and unsaturation sites. The fragment ions at theoretical m/z 171.1027, 183.1027, and 183.1391, due to oxononanoate, oxodecenoate, and undecenoate, respectively, were mostly detectable. The octadecanoid 9,12,13-trihydroxy-10,15-octadecadienoic acid (54) was putatively identified. This compound, which showed the [M-H]$^-$ ion at m/z 327.2191, was recently identified in aerial parts of Trifolium pratense [37]. The neutral loss of 98.0732 (theoretical value), likely due to hex-3-enal, provided the ion at m/z 229.1446, which dehydrated, giving the ion at m/z 211.1338 or, through CO loss, furnished the ion at m/z 183.1391. Compound 56 with deprotonated molecular ion at m/z 329.2337, based on the neutral loss of 100.0888 at the methyl end to achieve the ion at m/z 229.1448, was tentatively recognized as 9,12,13-trihydroxy-10-octadecenoic acid. This compound, herein identified for the first time in F. sylvatica, was firstly isolated from
Allium cepa, and a prostaglandin E-like activity was observed [38]. Furthermore, its isolation was also from Panax quinquefoliual [39]. Three isomers with the [M-H]− ion at m/z 309.207, according to the C₁₈H₃₀O₄ molecular formula, were also detected (compounds 58, 59, and 60), and one of the hydroxyl functions was tentatively localized at C-16 carbon based on the neutral loss of 58.0419. This latter was identifiable also in compound 61 with the [M-H]− ion at m/z 307.1915, suggesting that a dihydro derivative of the previous compounds occurred. Furthermore, two compounds with [M-H]− ions at m/z 293.2122 (75) and 293.2121 (79), respectively, according to molecular formula C₁₈H₃₀O₄, largely differing in their TOF-MS/MS fragment ions, were detected. TOF-MS/MS spectra of these compounds, beyond the common neutral loss of water, showed, in the first case, the ions at m/z 211.1340, 183.1392, and 171.1025, whereas the second appeared to be most sensitive to CO₂ neutral loss, providing the ion at m/z 249.2220. In the TOF-MS/MS spectrum of the second compound, the ion at m/z 185.1179 was observed. A further unsaturated compound (76) was at m/z 291.1971 [40], which shared with the previous ones the loss of water and carbon dioxide to give the ions at m/z 273.1851 and 247.2069. The [M-H]− ions at m/z 295.2283 and 295.2286 for compounds 77 and 78, respectively, and at m/z 297.2444 for compound 80 were in accordance with the oxygenated derivatives of α-linolenic (81 and 82) and linoleic acid, respectively. Compound 79 was likely a dihydro derivative of compound 78. These C-18 n:3 (80) and n:2 (81) were also detected, together with monounsaturated oleic acid (at m/z 281.2491; 84) and the saturated fatty acids palmitic (at m/z 255.2336; 83) and stearic acid (at m/z 283.2656; 85).

2.2. Effects of Beech Leaf Alcoholic Extract and Its Fractions on Fermentative Parameters

The effect of the chemical composition of the beech Fs/1/1 extract and its Fs/2/1 and Fs/3/2 fractions was evaluated based on rumen fermentation parameters. These are attributable to the ecological microbiota community, which is, in turn, affected by different factors, such as animal age, breeding system, and diet and feeding technique. The effects of common beech are not available in the literature, even if plants such as Quercus robur L. [41,42] and Castanea sativa L. [43,44], both belonging to the Fagaceae family, were investigated. Odeyinka et al. [45] reported the effects of twelve Scottish plants, including the leaves of F. sylvatica L., on rumen fermentation. The authors observed a reduction in diet digestibility, suggesting the possible influence of the high phenolic content. The in vitro fermentation characteristics are listed in Table 2.

The in vitro gas production was investigated to preliminarily ascertain the fermentation features of common beech extract and its different chemically constituted fraction. For this purpose, the in vitro gas production technique (IVGPT) was applied. The IVGPT reproduces at the laboratory scale the feed degradation occurring in the rumen and studies the fermentation kinetics, also estimating its nutritional value [46]. The organic matter degradability (OMD) and gas produced after 120 h of incubation (OMCV and A) showed lower values for all the beech-based diet samples, except Fs/3/2 at the 200-mg dose level, than the control diet. The lipophilic fraction Fs/2/1 at the 200-mg dose level exhibited the lowest value: the fraction supplement was able to decrease by 1.7, 2.7, and 2.9-fold the OMD, OMCV, and A values, respectively. In vitro fermentation kinetics highlighted the peculiar behavior of the Fs/2/1 200-mg sample, which showed the lowest values for B, C, Tₘₐₓ, and Rₘₐₓ. The Fs/3/2 fraction, on the other hand, showed a dose-dependent increase in the B and Tₘₐₓ parameters, while a very weak decrease in Rₘₐₓ was dose-dependently observed. The differences between substrates in the fermentation process are clearer in Table 2B and Figure 5, where the gas production rate and in vitro fermentation rate over time are shown. The curve of the Fs/2/1 fraction at the 200-mg dose level reached rapidly the asymptote (Tₘₐₓ: 7.39 and Rₘₐₓ: 2.36; p < 0.01), showing a slowdown of the process after 40 h of incubation. Otherwise, the curve related to Fs/3/2 200 mg reached half of the asymptote later (B: 35.24 h; p < 0.01), and the fermentation process continued throughout the 120 h of incubation. The gas production kinetics obtained incubating the Fs/3/2 lowest dose appeared similar to that exhibited by the control diet. Data acquired in terms of the
pH and concentration of the fermentation end products registered after 120 h of incubation are listed in Table 3. The rumen pH, which depends mainly on diet characteristics and physiological condition ranges between 6.2 and 7.5 [47], was not modified by the extract addition, and no significant difference was observed among the different extract concentrations. Oskoueian et al. [48], evaluating the effect of several flavonoids on the pH and ammonia nitrogen production, highlighted that the flavonoid addition did not alter these parameters. Accordingly, beech polyphenol containing extracts, such as Fs/1/1 and its fraction Fs/3/2, displayed similar ruminal pH to that exhibited by the control diet. Otherwise, the ruminal NH₃-N production was significantly (p < 0.001) affected by the addition of beech extracts. NH₃-N is a crude predictor of the efficiency of dietary N conversion into the microbial N total and a primary source of microbial growth [49]. Fraction Fs/2/1 slightly decreased the NH₃-N concentration at both the tested concentrations, suggesting an interacting role of oxygenated unsaturated fatty acids with dietary proteins, which could reduce their utilization by the rumen microorganism. Moreover, the significant decrease of VFA production in both Fs/2/1 doses seems to confirm the detrimental effect of lipids of the rumen fermentative process [50].

Table 2. (A) Cumulative gas production, organic matter degradability, and fermentation kinetics parameters of different F. sylvatica extracts. OMD: organic matter degradability; OMCV: cumulative volume of gas related to incubated organic matter. (A) Asymptotic gas production, (B) time at which one-half of the asymptote is reached, and (C) switching characteristics of the curve. Rₓmax: maximum fermentation rate; Tₓmax: time at which Rₓmax occurs. * p < 0.01 and ** p < 0.001. (B) In vitro gas production over time of F. sylvatica Fs/1/1 extract and its fractions Fs/2/1 and Fs/3/2 at 50-mg (●) and 200-mg (●) dose levels and control diet (●).
Figure 5. In vitro fermentation rate over time of *F. sylvatica* *Fs/1/1* extract (A) and its fractions *Fs/2/1* (B) and *Fs/3/2* (C) at the 50-mg (●) and 200-mg (●) dose levels and control diet (●).

Table 3. Effects of *F. sylvatica* L. extracts at different doses (50 mg and 200 mg) on fermentation end products after 120 h of incubation. N-NH$_3$ : ammonia-N; Total VFAs: total volatile fatty acid (acetate + propionate + butyrate + iso-butyrate + valerate + iso-valerate); BCFA: branched-chain fatty acid proportion (iso-butyrate + iso-valerate)/tVFA; A/P: acetate/propionate ratio. * $p < 0.01$, ** $p < 0.001$, and ‡ $p < 0.05$.

| Control Diet  | 50 mg | 200 mg | 50 mg | 200 mg | 50 mg | 200 mg |
|---------------|-------|--------|-------|--------|-------|--------|
| pH            | 6.99 ± 0.02 | 6.92 ± 0.01 | 6.96 ± 0.02 | 7.05 ± 0.07 | 7.09 ± 0.04 | 7.01 ± 0.04 | 6.98 ± 0.03 |
| NH$_3$-N (mmol/g) | 5.32 ± 0.04 | 5.15 ± 0.05 * | 5.15 ± 0.05 * | 5.08 ± 0.04 ** | 5.06 ± 0.02 ** | 5.26 ± 0.07 | 5.15 ± 0.05 * |
| Total VFA (mmol/g) | 122.42 ± 2.40 | 92.15 ± 2.64 ** | 80.52 ± 3.85 ** | 92.86 ± 10.7 ** | 49.54 ± 0.46 ** | 137.53 ± 0.03 ‡ | 124.65 ± 2.99 |
| BCFA (%VFA) | 3.32 ± 0.13 | 3.56 ± 0.19 | 2.96 ± 0.18 | 3.70 ± 0.33 | 6.20 ± 0.26 ** | 3.47 ± 0.11 | 2.30 ± 0.17 ** |
| A/P           | 3.08 ± 0.10 | 2.88 ± 0.06 | 2.95 ± 0.29 | 3.42 ± 0.27 | 4.26 ± 0.25 ** | 2.44 ± 0.30 ‡ | 3.15 ± 0.17 |

The in vitro fermentation end products were reported as a proportion (%) of the single volatile fatty acids towards the total volatile fatty acids content (Table 4). Volatile fatty acids (VFAs), among which acetate, propionate, and butyrate are the major ones, provide approximatively 70% of the ruminant energy requirement. Acetate is used as primary energy source in the lipogenic process, whereas propionate is a gluconeogenesis precursor, and butyrate is mainly metabolized in D-3-hydroxybutyrate [51]. Other fatty acids of microbial origin are odd-chain and branched FAs (OBCFA), which could be found as milk constituents and utilized to predict volatile fatty acids production in the rumen [51,52]. The fraction *Fs/3/2* positively affects VFAs production. These compounds, with adenosine triphosphate (ATP), CO$_2$, H$_2$, and CH$_4$, and other minor compounds, are formed by the degradation of carbohydrates in the rumen.

The effect observed appeared to be dose-dependent. In fact, when the dose of 50 mg was considered, an increase equal to 12.3% was observed with respect to the control diet, whereas a VFAs content similar to the control diet was detected for the 200-mg dose. This finding is in line with the observation that dietary polyphenols could differently affect nutrient utilization efficiency based on the level of their inclusion in the diet and that high polyphenol doses alter the membrane permeability [53]. Analogously, the dose level massively impacted the total volatile fatty acids amount, according to the double-edge sword role of polyphenol compounds in redox status maintenance and their antimicrobial efficacy [54]. In fact, the *Fs/3/2* fraction at the 50-mg dose level was able to increase the three main VFAs absorbed from the rumen: acetic, propionic, and butyric acids by 5.0, 34.4, and 36.8%, respectively, while a lower increase of butyric acid concentration (13%) was observed in the diet enriched with the *Fs/3/2* fraction at the 200-mg dose level.
Table 4. Effects of *F. sylvatica* Fs/1/1 extract and its fractions Fs/2/1 and Fs/3/2 at 50-mg and 200-mg dose levels on fermentation end products after 120 h of incubation. AcA = acetic acid; PrA = propionic acid; ButA = Butyric acid; ValA = valeric acid; iso-ButA = iso-butyric acid; iso-ValA = iso-valeric acid. * p < 0.01, ** p < 0.001, and ‡ p < 0.05. In the lower panel, the percentage increase or decrease of each volatile fatty acid was plotted for the different tested dose levels (■ 50 mg and ■ 200 mg) vs. FA% in the control diet.

| % VFA | Control Diet | Fs/1/1 | Fs/2/1 | Fs/3/2 |
|-------|--------------|--------|--------|--------|
|       | 50 mg  | 200 mg | 50 mg  | 200 mg | 50 mg  | 200 mg |
| AcA   | 65.33 ± 0.53 | 65.89 ± 0.06 | 62.90 ± 2.47 ‡ | 67.63 ± 1.35 ‡ | 61.59 ± 1.23 | 68.62 ± 1.38 | 66.24 ± 0.47 |
| PrA   | 21.18 ± 0.60 | 23.38 ± 0.69 | 21.39 ± 1.30 | 19.86 ± 1.18 | 14.47 ± 1.24 ** | 28.47 ± 2.99 ** | 21.04 ± 1.00 |
| ButA  | 7.37 ± 0.15  | 8.36 ± 0.22  | 5.59 ± 0.45  | 7.84 ± 0.75  | 4.61 ± 2.58  | 10.08 ± 0.04  | 8.38 ± 0.41  |
| ValA  | 1.90 ± 0.05  | 1.82 ± 0.22  | 1.45 ± 0.06 ‡ | 1.20 ± 0.05 ‡ | 1.15 ± 0.05 ‡ | 1.76 ± 0.14 ‡ | 1.83 ± 0.09 ‡ |
| iso-ButA | 1.57 ± 0.07 | 1.21 ± 0.04 | 0.89 ± 0.11 * | 1.16 ± 0.02 ‡ | 1.22 ± 0.01 | 1.98 ± 0.42 ‡ | 0.93 ± 0.03 * |
| iso-ValA | 2.57 ± 0.02 | 2.07 ± 0.16 ‡ | 1.50 ± 0.15 ** | 2.09 ± 0.16 ‡ | 2.15 ± 0.32 | 2.62 ± 0.23 * | 2.02 ± 0.10 |

The influence of the Fs/3/2 fraction dose level on rumen microbiota [55] in terms of population or activity was suggested also by the calculated A:P ratios, which were found equal to 2.32 and 3.20 for the low-dose and high-dose treatments, respectively. Thus, the low dose of the polyphenol beech fraction, by contrast with its high dose, was able to reduce the ruminal acetate-to-propionate ratio with respect to the control diet, improving the diet utilization efficiency. The total VFA was positively correlated to the low dose of Fs/3/2 with the highest content of total phenolic (r = 0.983) and flavonoid amount (r = 0.986) (Figure S2), according to previous findings. In fact, Acacia nilotica leaf exhibited a higher substrate degradability, total volatile fatty acid, mainly propionic acid, production, and lower methanogenesis [56]. Analogously, mulberry leaf, or different phenolic compounds from propolis extract, showed an increase of total volatile fatty acids and acetate, butyrate, and propionate [57,58]. Several reports have illustrated that flavonoids may regulate ruminal microbiota and that different classes of flavonoids have distinct effects. Similarly, the ruminal biomass can be influenced by in vitro incubation with different plant-derived substrates [59,60]. The increase of iso-butyric and iso-valeric acids was also observed in the Fs/3/2 50-mg diet sample. This could be due to the possible increase of protein digestibility and/or to changes in the composition of the rumen bacteria population [61]. In fact, iso-butyric and iso-valeric acids are synthesized by rumen microorganisms via an oxidative deamination and an oxidative decarboxylation starting from the branched-chain amino acids, valine, and leucine, respectively [47]. Additionally, other studies observed that flavonoids, pure [48,62] or in a mixture [63], are able to improve VFAs production; the flavanone naringenin appeared to mainly increase acetate [48].

The impact of the Fs/2/1 diet samples had a different behavior as a marked decrease of total VFA was measured for both the dose-level treatments considered. In particular, butyric acid, which is a signaling molecule that indirectly stimulates epithelial proliferation in growing calves before weaning [64], was slowed down by 37% in the Fs/2/1 200-mg diet sample. Propionic acid appeared to be massively decreased following both the treatments, so much so that a percentage decrease by 6.2% was observed in the Fs/2/1 50-mg diet sample and by 31.7% in the Fs/2/1 200-mg diet sample. In this context, the A:P ratios appeared to weakly augment a peculiar increase in BCFA following the Fs/2/1 diet sample
3. Materials and Methods

3.1. Plant Collection and Fractionation

The leaves of *F. sylvatica* L. were collected in June 2017 in the Tannenberger Gehölz National Forest area (Kiel, Schleswig-Holstein, Germany, N 54°21′52.6″, E 10°06′35.9″, 25 m a. m. s. l, Google Earth). Voucher specimens were deposited in the herbarium of Kiel University (KIEL) and the private herbarium of Christian Zidorn (voucher code: FS_20160705A-1). The leaves were first air-dried and minced and then ultrasound-assisted macerated (Branson Ultrasonics™ Bransonic™ M3800-E, Danbury, CT, USA) using methanol (leaves/solvent ratio 1:8, g:mL) at room temperature (set at 25 °C). Three sonication cycles were carried out, each one of 30 min, for obtaining the Fs/1/1 extract. The extract yield (%) was equal to 14.8% (45.5 g). The alcoholic extract was then dissolved in a biphasic solution CHCl₃:MeOH:H₂O (13:7:6, v:v:v), and discontinuous liquid–liquid extraction (LLE) was performed. Thus, an organic fraction (Fs/2/1; yield 31.2% of Fs/1/1) and a hydroalcoholic one (Fs/2/2) were obtained. The fraction Fs/2/2 was chromatographed on XAD-4 resin using water first and then methanol. The alcoholic fraction Fs/3/2 was obtained with a yield equal to 12.4%.

3.2. UHPLC-HRMS and MS/MS Parameters and UV-Vis Analyses

Fs/1/1 extract and the fractions therefrom were first analyzed by UV–Vis spectrophotometry in the range 200–800 nm by a Cary 100 spectrophotometer. The three samples (10 mg/mL) were then profiled by a Shimadzu NEXERA UHPLC system (Shimadzu, Tokyo, Japan). A Luna® Omega C18 (1.6-µm particle size, 50 × 2.1 mm i.d.) was utilized, and 2.0 µL of each sample were injected. The separation was achieved using a binary solution: (A) H₂O (0.1% HCOOH) and (B) CH₃CN (0.1% HCOOH). A linear gradient was used in which the percentage of solvent B increased as follows: 0–5 min, 5%→12% B; 5–13 min, 12%→22% B; 13–23 min, 22%→45% B; 23–26 min, 45%→65% B; 26–30 min, 65%→95%; 30–33 min, 95% B; and 33.01–35 min, column re-equilibration. The flow rate was set at 400 µL/min. MS analysis was performed using a hybrid Q-TOF MS instrument, the AB Sciex Triple TOF® 4600 (AB Sciex, Concord, ON, Canada), equipped with a DuoSpray™ ion source, which was operated in the negative Electrospray (ESI) mode. The APCI probe was used for automated mass calibration using the Calibration Delivery System. A full-scan time-of-flight (TOF) survey (dwell time 250 ms, 100–1500 Da) and eight IDA MS/MS scans (dwell time 100 ms, 80–1300 Da) were acquired, using the following parameters: curtain gas (CUR) 35 psi, nebulizer (GS1) and heated (GS2) gases 60 psi, ion spray voltage (ISVF) 4500 V, ion source temperature (TEM) 600 °C, and declustering potential (DP) −70 V. The collision energy (CE) applied was −35 V, with a collision energy spread (CES) of 15 V. The instrument was controlled by Analyst® TF 1.7 software (AB Sciex, Concord, ON, Canada, 2016), while data processing was carried out using PeakView® software version 2.2 (AB Sciex, Concord, ON, Canada, 2016).
3.3. Radical Scavenging Capacity: DPPH and ABTS Tests

The leaf extract and fractions therefrom were tested at 200, 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL (final concentration levels) towards ABTS (2,2′-azinobis-(3-ethylbenzothiazolin-6-sulfonic acid)) radical cation and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Trolox (4, 8, 16, and 32 µM) was used as the standard, and all recorded activities were compared to a blank sample, arranged in parallel. ABTS•+ was generated by mixing (2,2′-azinobis-(3-ethylbenzothiazolin-6-sulfonic acid); 7 mM) and potassium persulfate (K₂S₂O₈; 2.45 mM), in the dark for 12 h. ABTS•+ was diluted with PBS (pH 7.4) until an absorbance equal to 0.7 at 734 nm was reached. Thus, the extract doses were dissolved in ABTS•+ solution, and after 6 min, the absorbance was measured using a Wallac Victor3 spectrophotometer in reference to a blank [65]. DPPH• methanol solution (9.4 × 10⁻⁵ M) and extract doses were utilized to assess the DPPH• scavenging capability. The mixtures were stirred for 15 min, and the absorption was read at 517 nm by a Wallac Victor3 spectrophotometer in reference to a blank. The results were expressed in terms of the percentage reduction of the initial radical adsorption by the tested samples [66]. Trolox (4, 8, 16, and 32 µM) was used as the positive standard. All data were expressed as the mean ± standard deviation (SD).

3.4. Fe (III) Reducing Power

The ability to reduce the Fe(III) of beach leaf Fs/1/1 extract and its fractions (at the 200, 100, 50, 25, 12.5, 6.25, and 3.125-µg/mL final concentration levels) was evaluated using the ferricyanide FRAP assay, according to PFRAP procedure [25]. The absorbance was measured at 700 nm. The increase in absorbance with reference to the blank was considered. Trolox (4, 8, 16, and 32 µM) was used as the positive standard. All data were expressed as the mean ± standard deviation (SD).

3.5. Determination of Total Phenolic Content

The total phenolic content was determined according to the Folin–Ciocalteu procedure [65]. Aliquots of the samples (0.25 mg and 0.125 mg) were mixed with 2.25 mL of Na₂CO₃ (7.5% w/v) and 0.25 mL of Folin–Ciocalteu reagent. The tubes were mixed and allowed to stand for 3 h at room temperature (T = 25 °C). The absorbance was read at 765 nm using a Synergy Biotek spectrophotometer. The total phenol content was expressed as milligrams of gallic acid equivalents (GAEs) per g of extract.

3.6. Determination of Total Flavonoidic Content

NaNO₂ (5%, w/v; 0.3 mL) was added to the samples (1 mg and 2 mg), firstly solubilized into 5 mL of distillate water. After 10 min, AlCl₃ (10%, w/v; 0.6 mL) was added. The reaction was carried out for 6 min. Then, NaOH (1.0 M, 2.0 mL) was added, and the mixture was diluted to 10 mL with distillate water. The absorbance was read at 510 nm against the blank (water) using a Synergy HT Biotek spectrophotometer. The flavonoid content was expressed as milligrams of quercetin equivalents (QUEs) per g of extract [67].

3.7. Determination of Total Lipidic Content

Beech samples (at 0.5 mg, 0.25 mg, and 0.125 mg) were mixed with 150 µL of isovanillin/glacial acetic acid (5% w/v) and 500 µL of oxidant perchloric acid [68]. The mixture was incubated at 60 °C for 45 min and cooled down in an ice bath. Ethyl acetate was added in order to obtain 2 mL as the total volume. Absorbance was read using a Synergy HT Biotek spectrophotometer at 550 nm. The total saponins were quantified by using a standard curve of calibration of oleanolic acid and expressed as milligrams of oleanolic acid equivalents (OAEs) per g of extract.

3.8. In Vitro Fermentation

Beech Fs/1/1 extract and its Fs/2/1 and Fs/3/2 fractions were incubated at 0 (control), 50, and 200-mg dose levels with one gram of a diet composed by mixed hay, corn silage, and concentrate (crude protein: 15.0%, NDF: 46.0%) in hermetically sealed serum flasks (120 mL...
each, three replications for each treatment) with buffered rumen fluid (10 mL) at 39 °C under anaerobic conditions [46]. The rumen liquor was collected at a slaughterhouse [69] from four buffalos fed a total mixed ratio. All procedures involving animals were approved by the Ethical Animal Care and Use Committee of the University of Naples Federico II (Prot. 2019/0013729 on 08/02/2019). The gas produced during the 120 h of incubation into the fermenting flasks was recorded using a manual pressure transducer (Cole and Palmer Instrument Co, Vernon Hills, IL, USA) and related to incubated OM (OMCV, mL/g). At the end of the incubation period, the pH of the fermentation liquor was measured by a pH meter (ThermoOrion 720 A+, Fort Collins, CO, USA). The organic matter degradability (OMD, %) was assessed by weight differences of the incubated OM, and the undegraded filtered (sintered glass crucibles; Schott Duran, Mainz, Germany, porosity # 2) residue burned at 550 °C for 3 h.

3.9. Fermentation End Products Assessment

To determine the volatile fatty acids (VFAs, mmol/g) production, the fermentation liquor was first cooled at 4 °C and centrifuged at 12,000 × g for 10 min (Universal 32R centrifuge, Hettich FurnTech Division DIY, Melle-Neuenkirchen, Germany). The supernatant (1 mL) was then mixed with oxalic acid (1 mL; 0.06 mol). The VFA was measured by gas chromatography (ThermoQuest 8000top Italia SpA, Rodano, Milan, Italy) equipped with a fused silica capillary column (30 m, 0.25 mm ID, 0.25-µm film thickness). Quantitation was based on an external standard solution of acetic, propionic, butyric, iso-butyric, valeric, and iso-valeric acids. Branched-chain fatty acids (BCFAs) percentages were calculated as follows: (iso-butyric acid +iso-valeric acid/tVFAs)/100. The ammonia nitrogen (N-NH₃, mmol/g) production was colorimetrically assessed [70].

3.10. Data Processing and Statistical Analysis

Colorimetric tests were carried out performing three replicate measurements for three samples (n = 3) of the extract (in total, 3 × 3 measurements). All data were expressed as the mean ± standard deviation (SD).

To estimate the fermentation kinetic parameters, the gas production profiles were fitted to the sigmoidal model [71]: G = A/(1 + B/t)C, where G is the total gas produced (mL/g of incubated OM) at time t (h), A is the asymptotic gas production (mL/g), B is the time at which one-half of A is reached (h), and C is the curve switch. The maximum fermentation rate (R_max, mL/h) and the time at which it occurs (T_max, h) were calculated utilizing the formula suggested by Bauer et al. [72]:

\[
R_{\text{max}} = \frac{A \times C^B} {1 + C^B} \times \left( \frac{B^{B-1}} {T_{\text{max}}^B} \right) \times \left( T_{\text{max}}^{B-1} \right) \times \left( \frac{B - 1} {B + 1} \right)^{1/B}
\]

\[
T_{\text{max}} = C \times \left( \frac{B - 1} {B + 1} \right)^{1/B}
\]

Statistical analyses were performed by ANOVA for one-way using software SigmaPlot 14.0 to evaluate the substrate effect. The in vitro parameters (OMCV, OMD, A, B, C, T_max, and R_max) and the end products data (pH, N-NH₃, VFA, and BCFA) were statistically analyzed, and the significant levels were verified using HSD Tukey’s test at p < 0.05, p < 0.01, and p < 0.001. The correlations between colorimetric assays values and fermentation parameters were also evaluated using Pearson’s correlation coefficient (JASP 14.0; Figures S2 and S3).

4. Conclusions

Plant materials with high nutrient contents and elevated digestibility can be used as a supplementary resource for the feeding of small and large ruminants. When used in animal nutrition, byproducts of biorefining of a forest biomass will provide bioactive
compounds that could modulate rumen microbes with beneficial effects for the environment or increasing the nutraceutical value of human food from animal sources (e.g., fatty acids, antioxidants, etc.). Herein, beech leaves appear as a promising material affecting ruminal fermentation, and the chemical composition of the beech prepared extract/fraction is the main actor in the recorded effects. In particular, it was observed that the addition of 50 mg of the polyphenols-rich fraction (named Fs/3/2) to a ruminant diet significantly ($p < 0.05$) increased the production of the total VFAs and the relative contents of acetic ($p < 0.05$), propionic ($p < 0.001$), and butyric acids, also reducing the gas production and fermentation rate. The data acquired are a starting point for further research, aimed at exploring the in vivo feasibility of *F. sylvatica* L. leaf extract/fraction as supplements in ruminant nutrition. The dose–response efficacy of the beech extract/fraction further emphasizes the need for future investigations.

**Supplementary Materials:** The following supporting information can be downloaded at [https://www.mdpi.com/article/10.3390/molecules27072217/s1](https://www.mdpi.com/article/10.3390/molecules27072217/s1), Figure S1: UV spectra of Fs/2/1 and Fs/3/2 beech leaf fractions, Figure S2: Heatmap of the correlation using Pearson’s coefficient correlation between antiradical (DPPH• and ABTS**+) activities, reducing the activity (PFRAP), total phenol content (TPC), total flavonoid content (TFC), and total saponin content (TSC) with the fermentation parameters at the dose level of 50 mg, Figure S3: Heatmap of the correlation using Pearson’s coefficient correlation between antiradical (DPPH• and ABTS**+) activities, reducing the activity (PFRAP), total phenol content (TPC), total flavonoid content (TFC), and total saponin content (TSC) with the fermentation parameters at the dose level of 200 mg.

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**Data Availability Statement:** Data are within the manuscript and related Supplementary Materials.

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**Sample Availability:** Samples of the compounds are available from the authors.

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