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Comparison of Aqueous and Lactobacterial-Fermented *Mercurialis perennis* L. (Dog's Mercury) Extracts with Respect to Their Immunostimulating Activity

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Abstract: Lactic acid (LA) fermentation of dog's mercury (*M. perennis* L.) herbal parts was investigated in samples inoculated with either Lactobacteria (*Lactobacillus plantarum* and *Pediococcus pentosaceus*, LBF) or whey (WF). Depending on fermentation time, LA concentrations were monitored in a range of 3.4–15.6 g/L with a concomitant pH decline from 6.5 to 3.9. A broad spectrum of cinnamic acids depsides containing glucaric, malic and 2-hydroxyglutaric acids along with quercetin and kaempferol glycosides were detected by LC-DAD-ESI-MS^n. Moreover, in this study novel constituents were also found both in unfermented and fermented extracts. Furthermore, amino acids and particular Lactobacteria metabolites such as biogenic amines (e.g., putrescine, 4-aminobutyric acid, cadaverine) and 5-oxoproline were assigned in WF extracts by GC-MS analysis after silylation. Enhanced NFκB and cytokine expression (IL-6, TNFα, IL-8 and IL-1β) was induced by all extracts, both non-fermented and fermented, in NFκB-THP-1 reporter cells, showing a concentration-dependent immunostimulatory effect. The WF extracts were tested for micronuclei formation in THP-1 cells and toxicity in luminescent bacteria (*V. fischeri*), whereby no mutagenic or toxic effects could be detected, which corroborates their safe use in pharmaceutical remedies.

Keywords: *M. perennis*; lactic acid fermentation; *Lactobacillus*; *Pediococcus*; depsides; flavonoids; cytokine expression; immunostimulation; genotoxicity

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1. Introduction

Wound healing is a complex process comprising three main phases: inflammation, formation of new tissue and remodeling. The inflammatory phase starts right after an injury with the activation, coagulation and attraction of immune cells. Neutrophils are the first to arrive, shortly followed by monocytes, which then differentiate to macrophages. The latter play a crucial role in further modulating the inflammatory response. Differentiation as well as modulation are triggered by an array of cytokines, which are regulated by the activation of the transcription factor NFκB [1–4]. In contrast to acute inflammatory events, which are necessary for wound healing and usually self-limiting, persistent inflammation processes may require specific therapeutic strategies. Depending on the condition of the wound, appropriate medicinal therapies should be chosen. Topical treatments with plant extracts

have traditionally been used to support wound healing by interaction with different phases of the healing process, with a particular emphasis on the inflammatory phase [4].

While anti-inflammatory plant constituents, such as phenylpropanoids [5–7] and terpenes [8,9] or other have been in the focus of research for years, immuno-stimulating natural products are a research topic of increasing relevance [10,11]. For instance, *beta*-glucans, arabino-galactans and other bioactive polysaccharides from plants may exhibit strong immuno-modulating/stimulating effects [12,13] to fulfil wound healing capacities.

In search of plant extracts with wound healing properties, our focus was drawn on the less known medicinal plant dog's mercury (*Mercurialis perennis* L.), which has been particularly used in remedies of the European folk medicine. *M. perennis* belongs to the Euphorbiaceae (spurge) family, growing in shady beech forests on calcareous soils. In the Middle Ages the plant was primarily applied as a strong laxative and for the treatment of menstrual cramps, but was also used against amenorrhea, anorexia, oedema, rheumatism, gout and bronchial catarrh, etc. [14]. For medical purposes, flowering herbal parts of *M. perennis* are harvested in early spring (March–April). However, the herb is considered weakly poisonous because of strong laxative effects when used internally [15,16]. Nowadays, fermented extracts of the herb are applied in complementary medicine for the topical treatment of hard-to-heal wounds, burns, inflammations, suppurations, hemorrhoids and also against conjunctivitis of the eye [14]. Therefore, lacto-fermented *M. perennis* extracts were prepared according to an official pharmaceutical procedure (German Homoeopathic Pharmacopoeia, GHP no. 34 c [17]). For that purpose, crushed plant material was blended with water and whey, resulting in spontaneous lactic acid (LA) fermentation by the action of the genuine microflora, while subsequently microbial enzymes disintegrated the plant cellular matrix and leached secondary metabolites into the extracts. We recently investigated the impact of LA fermentation on the conversion of certain *M. perennis* constituents such as alkaloids and cinnamic acid depsides [18,19].

The current study focused on investigations of both non-fermented and fermented herbal *M. perennis* extracts in monocytic immune cells as a model to study immune-triggered inflammatory processes. In addition, the spectrum of natural constituents of the extracts was thoroughly analyzed by LC-MS^n and GC-MS and safety issues were assessed.

2. Materials and Methods

2.1. General

BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetamide) for silylation, containing 1% trimethylchlorosilane, was obtained from Sigma-Aldrich (Merck KGa, St. Louis, MO, USA). MRS bouillon was from VWR International (Leuven, Belgium). Strains of *L. plantarum* and *P. pentosaceus*, gene bank accession numbers: MK841313.1 and MK841045.1, respectively, were formerly isolated from a fermentation batch of *M. perennis* and characterized by DNA genotypization (16*S* rRNA gene sequencing) [18].

2.2. Plant Material

Herbal parts of *M. perennis* were collected in the mountain forest above Bad Boll/Eckwaelden (Germany) in March 2021. The herb was cleaned from impurities, rinsed with cold tap water, wiped with paper towels and stored at −80 °C until investigation. A voucher specimen (voucher number: HOH-020290) was identified by *Dr. R. Duque-Thüs* and deposited at the herbarium of the Institute of Botany, Hohenheim University (Stuttgart, Germany).

2.3. Preparation of Aqueous Extracts (AE) and Model Fermentation after Inoculation with Lactobacteria Starter Cultures (LBF)

For extraction and fermentation, the frozen herb material of *M. perennis* (−80 °C) was pre-crushed with a hammer. Then, deionized water (500 mL, 0.055 µS/cm) was added to the ground plant material (100.0 g), the slurry was bubbled 15 min with nitrogen (N_2) to prevent air oxidation, then minced by Ultra-turrax® treatment (3 min at 15.000 rpm, IKA-

Werke GmbH and Co. KG, Staufen, Germany) and again treated with N_2. For preparation of the AE, the slurry was left over night in the refrigerator and then filtered over Celite by vacuum suction to yield the aqueous extract. For model fermentation (LBF), the slurry was directly inoculated with 1 d cultures of *L. plantarum* and *P. pentosaceus* in MRS bouillon, with a bacteria count of 1.7×10^8 cells/mL and 2.1×10^9 cells/mL, respectively. Incubation was performed for 7 d at 33 °C with shaking of the culture broth once a day (appox. 30 s). To monitor pH values and lactic acid concentrations, samples (approx. 12 mL) were taken daily, filtered over paper filter and frozen at −80 °C until investigation. After 7d the culture broth was filtered as explained above.

For cell culture experiments all samples were repeatedly (twice) frozen, thawed and filtered through a sterile 0.2 µm cellulose acetate membrane filter (VWR International GmbH, Darmstadt, Germany) to reduce endotoxin contents.

2.4. Whey-Inoculated Fermentation (WF) According to GHP Procedure No. 34c

Whey was prepared according to an in-house procedure. In brief, non-pasteurized cow milk (1 L) was inoculated with a mixture of pure cultures of *Lactococcus lactis* and *Lactobacillus helveticus* (IP Ingredients GmbH, Süderlügum, Germany) and incubated for 2 d at 28 °C. After filtration, the whey (pH 3.9) was directly used in fermentation batches. *Mercurialis* herbal parts were processed in 2021 according to official procedure GHP no. 34 c (GHP = German Homoeopathic Pharmacopoeia, [17]), comprising comminution of the herb (1.0 kg) in water (750 g), addition of whey (500 g), fermentation (7 days), filtration and storage for 180 days. For a detailed process description see *Sauer* et al. 2022 [20].

2.5. D-/L-Lactic Acid (LA) Quantitation

LA was determined enzymatically via a reaction with lactate dehydrogenase (LDH, without differentiation of enantionmers) by using a commercial assay kit (Enzytec™ Liquid *D-/L*-Lactic acid, R-Biopharm AG, Darmstadt, Germany). The produced NADH was quantitated spectrophotometrically at 340 nm (reaction principle:

D-/L-lactate + NAD^+ - LDH ⟶ Pyruvate + NADH + H^+. The recovery rate for *D-/L*-LA amounted to 101.13% (SD 0.03) utilizing the Enzytec™ Liquid multi-acid standard high (R-Biopharm AG). Prior to analysis the aqueous samples or reference standard were diluted with water (1:50 or 1:100 (*v*/*v*)).

2.6. HPLC(DAD)-ESI-MS^n Analysis of Secondary Metabolites

LC-MS analyses were performed on an Agilent 1200 HPLC system (Agilent Technologies Inc., Palo Alto, CA, USA), equipped with a binary pump, a micro vacuum degasser, an autosampler, a thermostatic column compartment and a UV-VIS diode array detector. The LC system was coupled to an HCTultra ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with an ESI source operating in the negative ionization mode, applying the following parameters: capillary voltage: +4000 V, dry gas (N_2) flow: 9.00 L/min with a capillary temperature of 365 °C; nebulizer pressure: 50 psi. Full scan mass spectra (mass range *m*/*z* 50–1300 Da) of HPLC eluates were recorded during chromatographic separation yielding $[M-H]^-$ ions. To obtain structural information, collision-induced dissociation (CID) experiments were performed. MS^n data were acquired in the auto MS/MS mode. The instruments were controlled by Agilent Chemstation and EsquireControl software (V7.1). A Kinetex® core-shell C18 100 Å reversed-phase column (2.6 µm particle size, 150 × 2.1 mm i.d., Phenomenex Ltd., Aschaffenburg, Germany) was used for chromatographic separation at 25 °C and a flow rate of 0.21 mL/min. UV detection of the constituents was performed at wavelengths of 200–600 nm. The mobile phase consisted of $HCOOH/H_2O$, 0.1/99.9 (*v*/*v*; eluent A) and MeCN (eluent B). The injection volume of each sample was 10 µL, and the gradient applied was as follows: 0–8 min, 0–10% B; 8–20 min, 10% B; 20–51 min, 10–23% B; 51–70 min, 23–60% B; 70–80 min, 60–100% B; 80–85 min, 100% B; 85–90 min, 100–0% B; 90–100 min, 0% B. Samples were centrifuged for 10 min at 18.213 rcf (12.700 rpm) prior to injection into the HPLC system.

2.7. GC-MS Analysis of Low Molecular Metabolites (<350 Dalton)

Samples of aqueous extracts (AE) and of whey-inoculated fermentation (WF, 25 mL each) were evaporated to dryness with a vacuum rotoevaporator. Water residues were removed by distillation with toluene (3 × 50 mL) and the residues were further dried 1 h at low pressure (p < 5 mbar, T = 45 °C). For derivatization (silylation) samples of the dry residues (approx. 20 mg) were suspended in DMF and treated with BSTFA (300 µL). After incubation at 105 °C (15 min) samples were injected into the GC-MS system. GC-MS analyses were performed with a PerkinElmer Clarus 500 gas chromatograph with split injection (split ratio 30:1, injection volume 1.0 µL), coupled to a single quadrupole mass detector. The column used was a Zebron ZB-5 MS capillary column (60 m × 0.25 mm i. d. × 0.25 µm film thickness, 5% phenylpolysiloxane and 95% dimethylpolysiloxane coating; Phenomenex, Torrance, USA). Carrier gas: helium at a flow rate of 1 mL/min. The injector used was a PSS (temperature-programmed split/splitless injector, temperature: 250 °C). The temperature program for the column oven was 100 to 320 °C with a linear gradient of 4 °C/min and a final hold time of 30 min. The mass spectrometer was run in electron ionization (EI) mode (70 eV). The software Turbomass (v. 5.4.2, PerkinElmer Inc., MA, USA) was used for data acquisition and processing.

2.8. Cell Culture of THP-1 NFκB-eGFP Reporter Cells

The THP-1 NFκB-eGFP human acute monocytic leukemia cell line (cat. # SCC223) was purchased from Merck (Darmstadt, Germany). It is characterized by the fusion of eGFP to the NFκB response element. This allows flow cytometric quantification of the NFκB activity. Cells were cultured in an RPMI medium (Fisher Scientific, Reinach, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (Bioconcept, Allschwil, Switzerland), 2 mM L-glutamine (Sigma Aldrich, Buchs SG, Switzerland), 100 U/mL penicillin (Sigma Aldrich) and 100 U/mL streptomycin (Sigma Aldrich) in T75 flasks at a cell density of 0.5–1.0 × 10^6/mL.

2.9. NFκB Activity Assay

THP-1 NFκB-eGFP reporter cells (5 × 10^5 cells/mL) were stimulated with either lipopolysaccharides (LPS, 100 ng/mL; Sigma Aldrich, Buchs SG, Switzerland), or medium (unstimulated control), dexamethasone (10^{-5} M; Sigma Aldrich) or the test samples at various concentrations (1, 3, 10, 30, 100, 300, 1000 µg/mL) for 24 h. A FACS buffer of 50 µL was added, 130 µL of the cell suspension was transferred to a 96-well plate with a U-bottom and the green fluorescent protein (GFP) expression (NFκB activity) was measured with a CytoFLEX flow cytometer (Beckmann Coulter International Sa, Nyon, Switzerland).

2.10. Analysis of Cytokines

THP-1 NFκB-eGFP reporter cells (5 × 10^5 cells/mL) were stimulated with LPS (1 µg/mL; except for the unstimulated control) and incubated with serum-free medium (unstimulated, stimulated), dexamethasone (10^{-5} M; Sigma Aldrich) or the test substances at various concentrations (1, 3, 10, 30, 100, 300, 1000 µg/mL) for 24 h. Then, supernatants were harvested and frozen at −80 °C. Cytokine content was determined using LEGENDplex™ according to the manufacturer's instructions (BioLegend, San Diego, CA, USA).

2.11. Viability Assay

THP-1 NFκB-eGFP reporter cells (5 × 10^5 cells/mL) were incubated for 24 h with medium (unstimulated), dexamethasone (10^{-5} M; Sigma Aldrich, Buchs SG, Switzerland) or test samples at various concentrations (1, 3, 10, 30, 100, 300, 1000 µg/mL). Then, cells were washed with PBS and resuspended in 100 µL WST medium (RPMI 1640 without phenol red, supplemented with 10% (v/v) heat-inactivated fetal calf serum (Bioconcept, Allschwil, Switzerland), 2 mM L-glutamine (Sigma Aldrich), 100 U/mL penicillin (Sigma Aldrich), 100 U/mL streptomycin (Sigma Aldrich) and 10% (v/v) WST-1 reagent (Sigma Aldrich). Cells were incubated for 120 additional minutes and the colorimetric measure-

ment was performed on a microplate reader (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland).

2.12. Micronucleus Test

The in vitro mammalian cell micronucleus test was performed in accordance with the OECD 487 guideline [21], without use of an exogenous source of metabolic activation. Flow cytometry was employed as a micronucleus scoring procedure. To begin with, THP-1 cells were seeded at an initial density of 1.5×10^4 cells per 100 µL and well (F-bottom 96-well plate; Sarstedt AG, Nümbrecht, Germany). After 24 h of precultivation, the cells were submitted to sample treatment, i.e., exposure to the extracts for 48 h, ensuring 1.5–2 normal cell cycle lengths. Addition of 50 µL of extract solutions led to the desired concentrations in the final volume (150 µL). Previously, the extracts (WF1, WF2 and WF3) were pre-adjusted according to their initial dry weight to volume ratio by dilution with culture medium, to reach 1000 µg/mL at the highest concentration level, followed by semi-logarithmic dilutions down to 1 µg/mL. Following 48 h extract exposure, cells were harvested by centrifugation and then submitted to a stepwise lysis and staining protocol, according to [22].

Per sample and replicate 5000 diploid nuclei were counted as a reference value in order to allow a normalization of the occurrence of micro- and hyponuclei. Extranuclear DNA-bodies (non-apoptotic) were classified into two types according to a potential clastogenic or aneugenic mode of action, that were detected by size discrimination of the induced micronuclei (micro- and hyponuclei, respectively). This allowed the concurrent assessment of micronucleus induction and a genotoxic mode of action [23]. In order to demonstrate assay performance and proficiency, a positive control substance (vinblastine sulphate, 1.25 nM, Cayman Chemicals, Ann Arbor, MI, USA) was always included. As the micronucleus mutagenicity assay assumes that sufficient treated cells have undergone mitosis during exposure and in order to ensure that the treatments were conducted at appropriate levels of toxicity, accompanying cell viability was assessed (see above).

2.13. Luminescent Bacteria Toxicity Test

The luminescent bacteria test was chosen for determining the acute toxicity potential of extract samples (WF1, WF2 and WF3) against the marine bacterium *Vibrio fischeri* (*Aliivibrio fischeri*, strain NRRL B-11177, dsm 7151, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and was performed in accordance with ISO 11348 guidelines [24]. After 30 min of contact time between bacteria and the respective extracts, bioluminescence was measured in a microplate reader (Tecan Infinite M200). Validation of the assay was ensured by complying to the specified toxicity threshold concentrations for positive control chemicals ($ZnSO_4$ and 2,4-dichlorophenol).

2.14. Statistical Analysis

Statistical data analysis was performed using the PRISM software (version 9.3.1; GraphPad Software, Inc., San Diego, CA, USA). The Shapiro–Wilk test was applied to check for normal distribution of the data. A multiple group comparison, using Brown–Forsythe and Welch ANOVA for non-normally distributed data, and standard ANOVA for normally distributed data in combination with the Dunnett's T3 post-hoc test, was performed. Statistical significance was considered for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

For the bacteria luminescence inhibition test, the half-maximum inhibitory responses (IC_{50}) induced by the extracts were determined by means of nonlinear regression (Probit analysis).

3. Results and Discussion

3.1. pH Change and Lactic Acid Formation in Fermentation Samples Inoculated with Lactobacteria (LBF) and Whey (WF)

The fermentation of *M. perennis* herbal material was investigated in two separate experiments: the first experiment was performed by inoculation with isolated Lactobacteria strains (*Lactobacillus plantarum* and *Pediococcus pentosaceus*) and sampled over a fermentation period of 7 d and the second by the addition of whey, sampled after 180 d. The latter experiment was performed according to the official protocol of the German Homoeopathic Pharmacopoeia, GHP no. 34 c [17,20].

Freshly prepared aqueous extracts (AE) of *M. perennis* herbal parts (plant material:water ratio = 1:5, *w*/*w*) displayed pH values between 6.5 and 7.0. In the first experiment, the sample was inoculated with *L. plantarum* and *P. pentosaceus* in order to facilitate fermentation [25,26]. Both bacterial strains have recently been isolated from fermented batches of *M. perennis* [18]. In these LBF the pH dropped rapidly from 7.0 to 3.8 within 3 days (Figure 1A) and then remained virtually unchanged until day 7 (pH 3.9). Simultaneously, the LA concentration increased from 0.0 g/L (day 0) to 3.4 g/L (day 3) and remained constant until day 7 (Figure 1A).

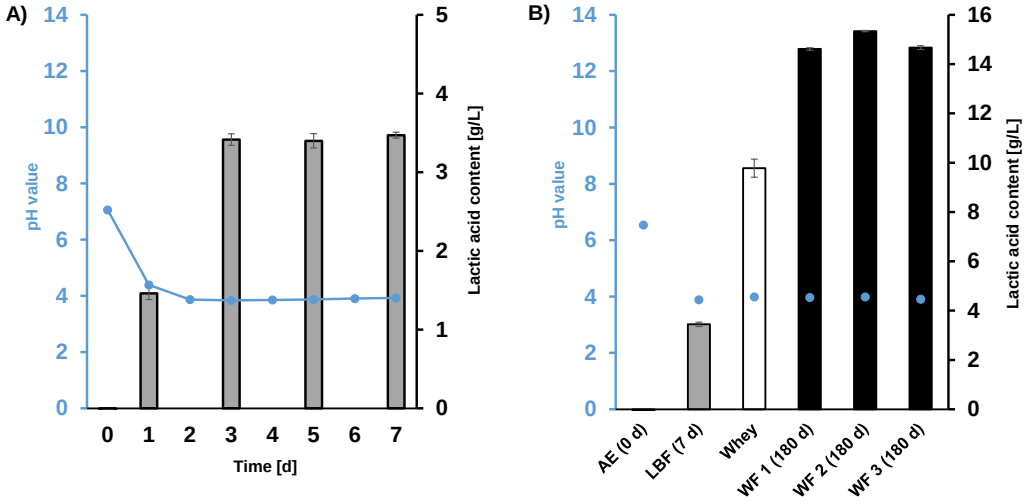

Figure 1. (**A**) pH decline (blue curve) and increase in LA concentration (grey bars) in a Lactobacteria-inoculated fermentation (LBF) of *M. perennis*, with two LAB strains (*L. plantarum* and *P. pentosaceus*; *n* = 3, measured in triplicate). (**B**) pH values (blue dots) and lactic acid concentrations in AE, LBF and WF samples (bars, measured in triplicate). AE = aqueous extract, LBF = Lactobacteria-inoculated fermentation; WF = whey-inoculated fermentation.

In the second experiment, whey-inoculated fermentation (WF) of *M. perennis* (according to GHP procedure no. 34 c [17]) was investigated. Deviating from the LBF samples, the WF differed in the composition of the fermented materials (plant material : whey : water ratio = 100 : 50 : 75, *w*/*w*/*w*) and the fermentation time (180 d). Supplementation of whey is an interesting option to facilitate LA production upon medicinal plant fermentation [20], setting up optimum starting conditions for Lactobacteria microflora by providing high lactose contents. Thus, the WF approach reached a 4.3–4.5 fold higher LA concentration (14.6–15.3 g/L, respectively, Figure 1B) compared to LBF without whey.

3.2. Characterization of Depsides, Flavonoid Glycosides and Fermentation Metabolites in AE, LBF and WF Samples by LC-MS^n

Several classes of natural compounds have been identified in *M. perennis*, i.e., alkaloid constituents [27–29], sterols, terpenes, carotenoids, *n*-alkylresorcinols [28,30], depsides and flavonoid glycosides [31]. To assign a broad spectrum of high- and low-molecular

metabolites, both aqueous unfermented (AE) and fermented extracts (LBF and WF) were comprehensively analyzed in the current study by LC-MS^n and GC-MS.

First, the AE of *M. perennis* was investigated by LC-MS^n (negative ionization mode) to monitor all water extractable constituents. In the base peak chromatogram (BPC) of these extracts (Figure 2A) a multitude of peaks were detected, being mainly characterized as depsides, i.e., condensation products of substituted cinnamic acids together with glucaric, malic or 2-hydroxyglutaric acids. Several of them have already been described in previous studies [31–33]. Glucaric acid depsides with caffeic, *p*-coumaric and ferulic acids (isomers of **9***, **12*** and **13***, respectively; for structures see Figure 3) were assigned based on their MS fragmentation and distinct UV spectra (Table S1, Figure S2A, Supplementary Materials) in a retention time range between 8.5 and 21.0 min (Figure 2A).

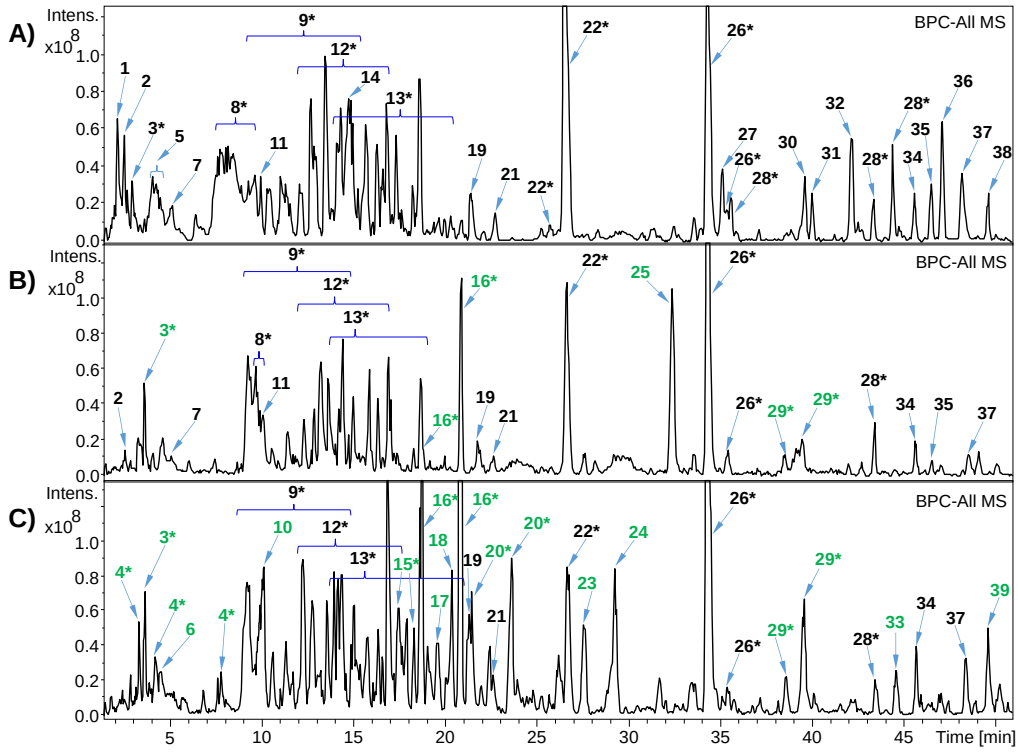

Figure 2. HPLC-(DAD)/ESI-MS^n base peak chromatograms (BPC) of aqueous (AE), Lactobacteria (LBF) and whey-inoculated (WF) fermentation extracts obtained from herbal parts of *M. perennis*. (**A**) BPC of AE; (**B**) BPC of LBF after 7 d; (**C**) BPC of WF after 180 d (according to GHP procedure no. 34 c). The peak numbering corresponds to the structural formulas illustrated in Figure 3. For compound assignment see Table S1 (Supplementary Materials). Compounds with green numbers were merely detected in LBF or WF extracts. Isomers are marked with an asterisk (*).

Since many signals were overlapped in a retention time range between 7 and 25 min, extracted ion chromatograms (EIC) were generated (Figure 4). Accordingly, EIC of $[M\text{-}H]^-$ ions at *m*/*z* 371, 355 and 385 revealed numerous isomers of **9***, **12*** and **13***, respectively (Figure 4B–D). These signals refer to constitutional isomers and diastereomers of the latter [33].

Furthermore, two intense peaks of the main depsides, phaseolic acid (*trans*-caffeoyl malate, **22***) and mercurialis acid (*trans*-caffeoyl 2-hydroxyglutarate, **26***), were recorded at t_R 26.5 and 34.3 min, respectively (Figure 2A). Moreover, novel depsides were also detected in unfermented *Mercurialis* extracts. For instance, at t_R 39.6, 45.7 and 49.6 min three ferulic acid depsides, i.e., feruloyl malate (**30**), feruloyl 2-hydroxyglutarate (**34**) and feruloyl syringate (**38**), were assigned based on their ESI-MS fragmentation patterns (Table S1, Figure S2C, Supplementary Materials). These depsides have not been described for *M. perennis* so far. In addition, another novel depside of dihydro-ferulic acid was detected at t_R

48.4 min (**37**, Figures 2A and 3). A neutral loss of 80 Da from the $[M-H]^-$ ion at m/z 453 in the collision-induced dissociation (CID) experiments yielded a fragment ion at m/z 373, which points to a sulfuric acid residue (HSO_3), (Supplementary Materials, Figure S2C). A subsequent loss of 178 Da upon further fragmentation resulted in an ion at m/z 195, indicating the molecule consisted of two dihydro-ferulic acid moieties. Therefore, **37** was tentatively assigned to a bis-dihydro-feruloyl sulfate. The structure of **37** was supported by a HR-MS experiment (Figure S3G).

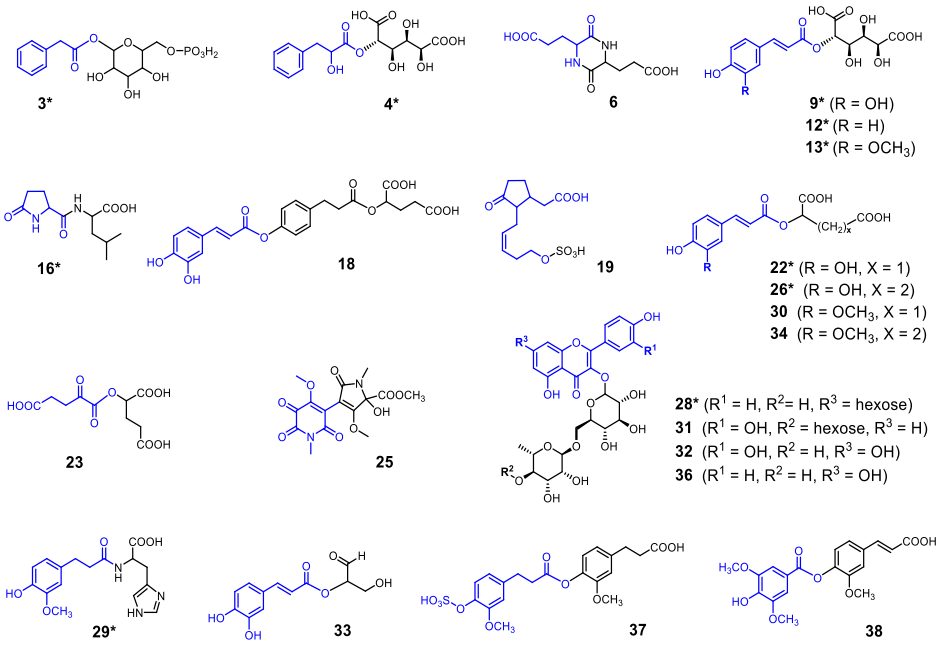

Figure 3. Chemical structures of depsides, carboxylic esters and flavonoid glycosides (and others) identified in non-fermented and fermented extracts of *M. perennis*. For better comprehensibility of structures one acyl moiety of each structure is drawn in blue color. Isomers are marked with an asterisk (*).

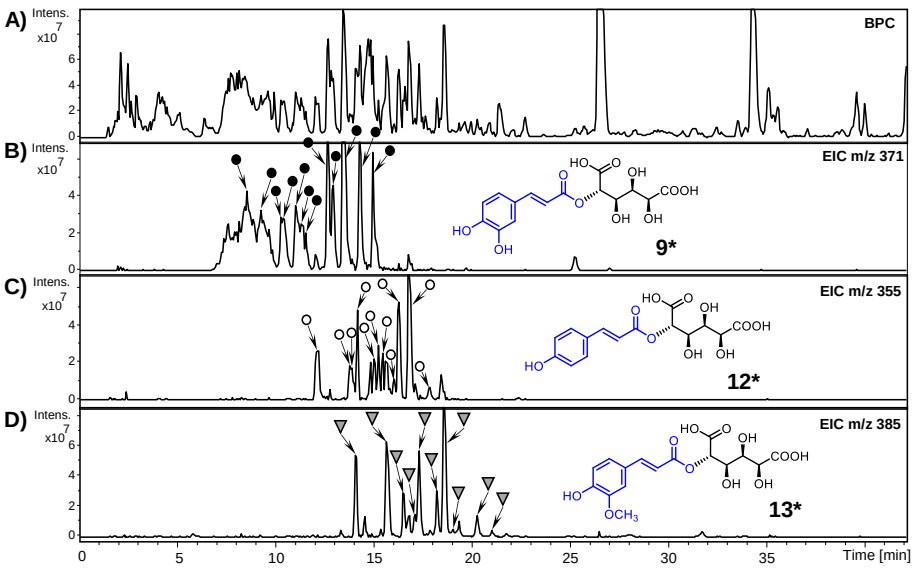

Figure 4. Detection of cinnamoyl glucarates by HPLC-(DAD)/ESI-MS^n in the AE of *M. perennis* herbal parts. (**A**) Base peak chromatogram (BPC). Extracted ion chromatograms (EIC) of $[M-H]^-$ ions at m/z 371, 355 and 385 showing caffeoyl glucarate isomers **9*** (**B**), *p*-coumaroyl glucarate isomers **12*** (**C**) and feruloyl glucarate isomers **13*** (**D**), respectively.

Besides the depsides, six known kaempferol and quercetin glycosides (Figure 3) were detected in the BPC of the AE between t_R 35.0 and 47.2 min (Figure 2A), which have formerly been described for *M. perennis* ethanolic extracts [31]. Moreover, by comparison of MS data with the literature [34] 12-hydroxyjasmonate sulfate **19** (Figure 3 and Figure S3D, Table S1) was identified (at t_R 21.3 min) for the first time in the AE of *M. perennis*.

When evaluating the fermented samples (LBF and WF) it became apparent that several of the depsides and flavonoids were partly degraded to different extents (Figure 2B,C), while at the same time novel metabolites were formed. For instance, in the BPC of LBF samples, three novel signals **16*** (two isomers at t_R 18.8 and 20.8 min) and **25** (t_R 32.4 min) appeared. The mass spectrum of **16*** exhibited the pseudomolecular ions $[2M\text{-}H]^-$ and $[M\text{-}H]^-$ at m/z 483 and 241 (Figure S2B). A subsequent release of CO_2 (44 Da) upon CID, yielding a fragment ion at m/z 197, indicated the presence of a carboxylic acid. By comparison with the literature data [35,36] **16*** was assigned to 5-oxoprolyl-leucine. For the assignment of compound **25**, high resolution MS data were recorded in HR-ESIpos/neg-MS experiments (for details see Supplementary Materials, Figure S3E,F). The corresponding $[M\text{-}H]^-$ and $[M+H]^+$ ions exhibited m/z ratios at 367.07855 (calcd for $C_{15}H_{15}O_9N_2^-$, m/z 367.07830) and 369.09274 (calcd for $C_{15}H_{17}O_9N_2^+$, m/z 369.09286) accordingly. The molecular formula $C_{15}H_{16}O_9N_2$ deduced from these results implies nine units of unsaturation (double bonds or ring systems). Based on these data **25** was tentatively allocated to a novel hermidin alkaloid artefact (see Figure 3, Table S1). An ethylester derivative of **25** has recently been identified from hydroalcoholic extracts of *M. perennis* [29].

A more complex composition was found for the 180 d WF samples. Several novel constituents were detected such as compound **3*** (phenylacetic acid hexoside phosphate), caffeic acid (**17**) and again isomers of **16*** (Figure 2C). Moreover, the depsides **4*** (glucaroyl phenyllactate isomers), **18** (caffeoyl *p*-dihydro-coumaroyl 2-hydroxyglutarate), **23** (2-ketoglutaroyl 2-hydroxyglutarate) and **33** (caffeoyl glycerinaldehyde) were tentatively assigned (Figure 3, Table S1). The latter were probably formed by pH-dependent acyl migration [37] and condensation of carboxylic functions from other depsides. Apart from these constituents, a few N-containing metabolites (**6**, **10**, **15***, **20***, **29***, **39**) were also detected, which showed characteristic even-numbered m/z ratios of their $[M\text{-}H]^-$ ions. These compounds are probably formed upon reaction of whey peptides with depsides or from alkaloid artefacts. Some of them could not be further characterized, except compounds **6** and **29***, one of which was tentatively identified as glutamic acid diketopiperazine (**6**, t_R 4.5 min) due to the loss of a $[\text{glutamic acid-}H_2O]^-$ moiety (129 Da) from the $[M\text{-}H]^-$ ion at m/z 257 and the other as condensation product of dihydro-ferulic acid and histidine (**29***, two isomers at t_R 38.6 an 39.6 min, Figures 2C and 3, Table S1). Accordingly, the mass spectrum of **29*** exhibited the elimination of a histidine moiety (155 Da) from the $[M\text{-}H]^-$ ion at m/z 332, yielding a fragment at m/z 177 (Figure S2B). The latter corresponds to a dihydro-feruloyl unit. In addition, the UV absorption maximum at 274 nm, which is similar to the chromophore of free histidine [38], corroborates the structural assignment of the isomers of compound **29***.

3.3. GC-MS Assignment of Low Molecular Weight Compounds in AE and WF Samples

For analyzing low molecular weight constituents, aqueous and a whey-inoculated fermented extract (AE and WF, respectively) were dried in vacuo, silylated with BSTFA and then investigated by GC-MS (Figure 5). Compound assignment was based on comparison of the respective mass spectra with the NIST database. At first glance, sugar constituents (fructose, galactose, glucose, sucrose) were assigned via MS and comparison with reference compounds (Figure 5C). In the total ion chromatograms (TICs) of the unfermented *M. perennis* water extract (AE) the monosaccharides fructose, glucose and *myo*-inositol were detected, together with the disacharides sucrose and melibiose, at t_R 28.8, 30.8, 35.5, 46.6 and 54.8 min, respectively (Figure 5A).

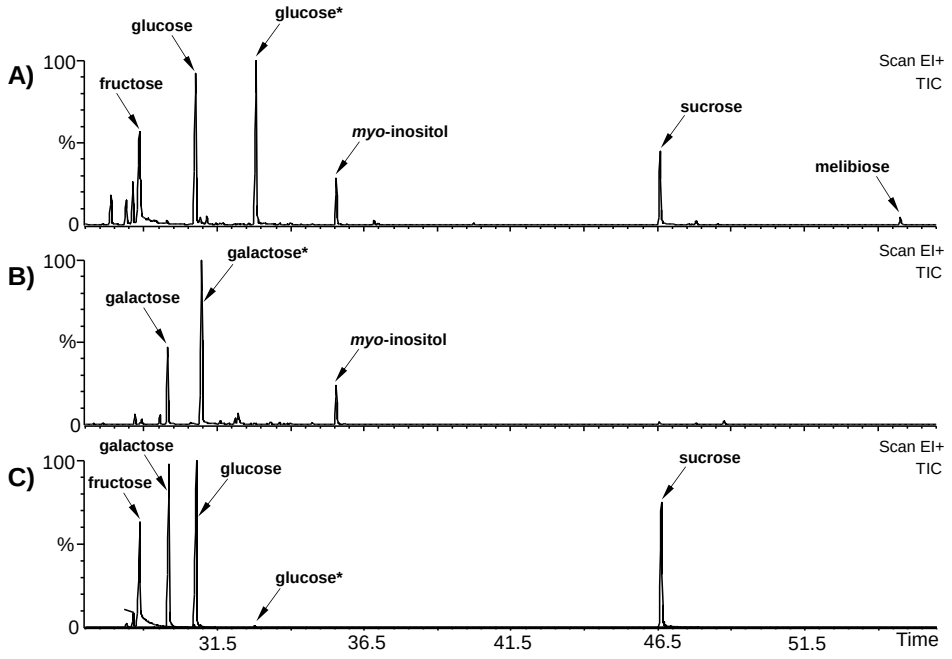

Figure 5. GC-MS sections of AE (**A**) and WF extracts (**B**) and reference compounds (**C**) after silylation. Asterisks mark the furanose form of the respective monosaccharides (beside pyranose form).

In the WF samples, fructose, glucose, sucrose and melibiose were almost completely metabolized while galactose was formed (Figure 5B). The latter constitutes a microbial degradation product, which is released from melibiose or lactose (the latter originate from whey). Galactose is obviously accumulated in the fermentate, since only certain Lactobacteria species (LAB) are able to further metabolize this monosaccharide [39,40]. Interestingly, *myo*-inositol, which may originate from phytic acid (inositol hexaphosphate) [41,42], was also essentially not converted upon fermentation.

Moreover, considerable amounts of lactic acid (LA) were detected in the TIC together with free amino acids, which were mainly analyzed in the WF extract (see Supplementary Materials, Figure S4). Remarkably, lower amounts of biogenic amines, such as putrescine (1,4-diaminobutane) and cadaverin (1,5-diaminopentane) as well as 4-aminobutyric acid could also be detected as decarboxylation products of their specific precursor amino acids ornithine, lysine and glutamic acid, respectively (Figure S4B). It is known from the literature that these amines may be produced by the action of *Lactobacillus* decarboxylases [43–45]. This is also corroborated by the fact that they were only detected in fermented, but not in unfermented extracts. Moreover, 5-oxoproline (pyroglutamic acid) was assigned as a further distinctive Lactobacteria marker metabolite [46], apparently formed from glutamic acid due to microbial enzymatic dehydration [47]. It has been reported in the literature that 5-oxoproline exhibits similar antimicrobial activity like LA and inhibits the growth of many spoilage bacteria, e.g., of the *Enterobacter* and *Pseudomonas* families [47]. Furthermore, 5-oxoproline-containing dipeptides such as compound **16***, assigned in the previous chapter, produced by *L. plantarum* have recently been shown to exhibit immunomodulatory activity in vivo [48].

3.4. Stimulation of NFκB Activity of THP-1 Cells by Treatment with AE, LBF and WF Extracts

To study the immunomodulating effects of *M. perennis* extracts, THP-1 cells were utilized as a model for human monocytes [49]. THP-1 cells were formerly applied, e.g., to screen diverse plant extracts regarding anti-inflammatory activity [50–52].

Proinflammatory mediator expression is regulated via molecular pathways including MAP kinases and NF-κB signaling. The binding of the transcription factor NFκB to the response element is essential for the transcription of different proinflammatory genes [2,3]. In

this study, the influence of the extracts on NFκB activity was investigated using an immune reporter cell line (THP-1), characterized by the fusion of GFP to the NFκB response element.

First, the effect of extracts on the cell viability of THP-1 cells was determined by a WST assay measuring the mitochondrial activity. Cell viability was significantly diminished in cells treated with AE, LBF and WF1 extracts at the highest concentration (1000 µg/mL), indicating a cytotoxic effect (see Supplementary Materials, Figure S5). For whey, as well as the WF2 and WF3 extracts, no effect on the viability of THP-1 cells could be detected in the entire concentration range investigated (Figure S5).

For all extracts, a concentration-dependent increase in NFκB activity was observed at the lower concentrations (1–100 µg/mL for AE and LBF, 1–300 µg/mL for whey and WF1, WF2 and WF3, respectively) (Figure 6). The decrease in NFκB activity at the higher concentrations (1000 µg/mL) is due to the cytotoxic effects of the *M. perennis* extracts. However, it can be assumed that a toxicity-related impact on the function of the cells already occurs at somewhat lower concentrations. No differences in activity were found between the AE, LBF and WF samples, indicating that fermentation has no influence on the ingredients causing NFκB activation.

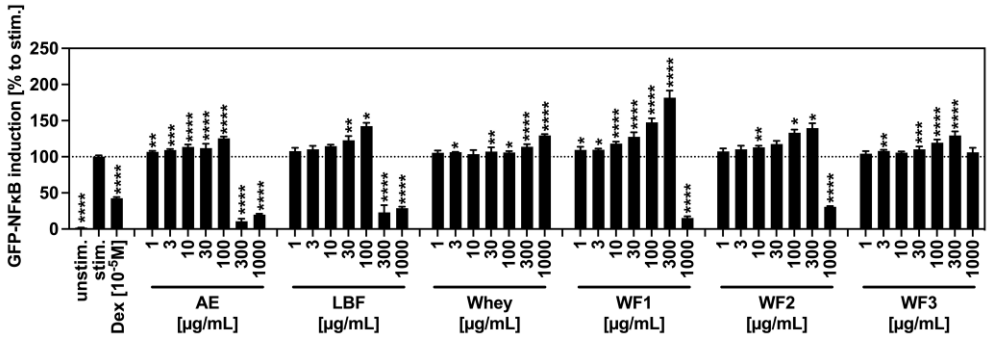

Figure 6. Effects of extracts on NFκB activity of THP-1 NFκB immune reporter cells. THP-1 cells were stimulated with LPS (100 mg/mL) and incubated for 24 h with medium (unstimulated, stimulated), dexamethasone (Dex) or extracts. GFP expression was quantified by flow cytometry. The percentage of cells was compared, normalized to the stimulated control and presented as mean ± standard deviation. $n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.5. Effect of Extracts on Cytokine Expression

The secretion of cytokines is one of the important functions of activated immune cells to fulfil its function. For further characterization of the extracts, their impact on the proinflammatory cytokine expression of LPS-stimulated THP-1 cells was investigated (Figure 7). Cytokine expression was moderately affected in the concentration range of 1–100 µg/mL and clearly exceeded the LPS-stimulated control only in the case of LBF (IL-6) and whey (IL-6, IL-1β, TNFα). At higher concentrations (1000 µg/mL and 300 µg/mL, respectively), a strong increase in cytokine secretion was detected in some cases, which, however, was also based on cytotoxic effects. In contrast to the extracts obtained from *M. perennis*, whey showed a clear concentration-dependent effect on all cytokines in the whole concentration range. An immunostimulatory effect of whey protein extracts on primary peripheral blood mononuclear cells has already been described in the literature [53–55], while the accumulation of the cytokines IL-10, IL-1β, IL-8, IL-6 and TNFα was observed.

In our study a tendency towards a concentration-dependent stimulating effect on cytokine expression by the *M. perennis* extracts was registered in the case of IL-8, IL-6 (AE and LBF only) and IL-1β (AE only). The TNFα expression remained unaffected by treatment with the extracts. IL-1β, IL-6 and TNFα are known as endogen pyrogens that can induce fever or a local inflammation, but also have an impact on the coagulation cascade and activation of other immune cells. IL-8 acts on migration and proliferation of keratinocytes and fibroblasts and has a chemotactic effect on granulocytes, all relevant in wound healing.

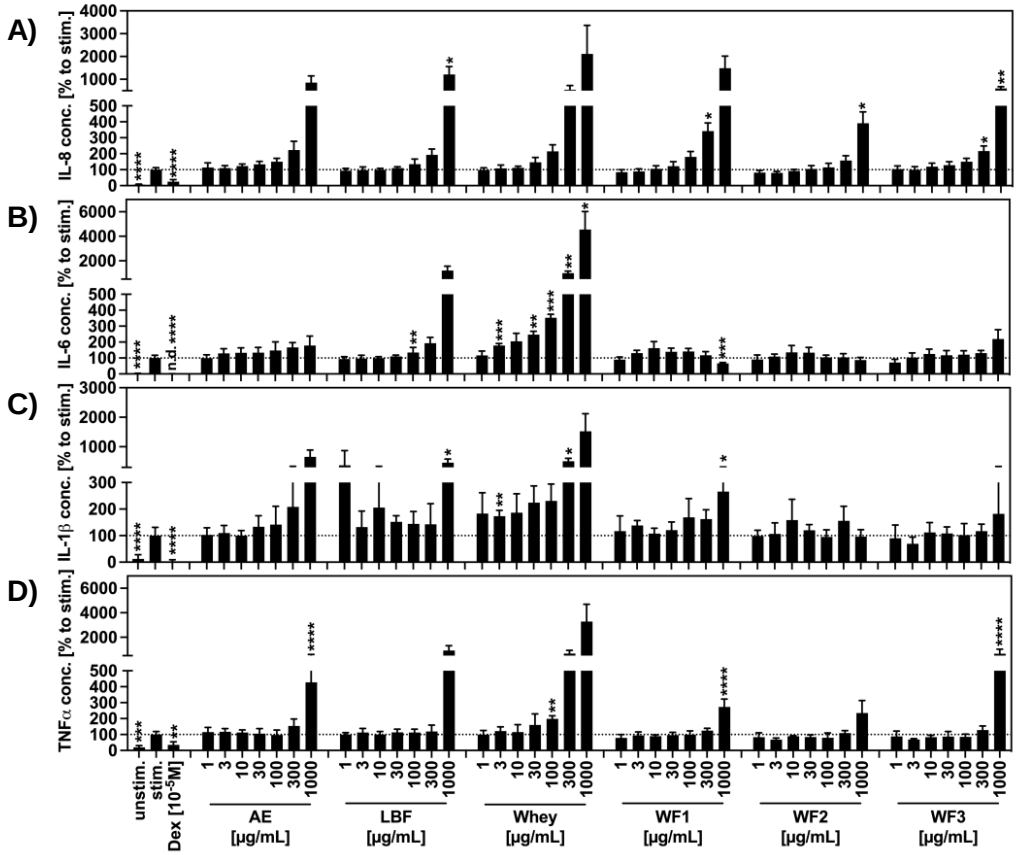

Figure 7. Effects of extracts on cytokines of THP-1 NFκB-eGFP reporter cells. THP-1 cells were stimulated with LPS (1 µg/mL) and incubated for 24 h with serum-free medium (unstimulated, stimulated), dexamethasone (Dex; 10^{-5} M) or extracts. The levels of IL-8 (**A**), IL-6 (**B**), IL-1β (**C**) and TNFα (**D**) in the supernatants were determined via the LEGENDplex™ (flow cytometry). Results are presented as mean ± standard deviation compared to stimulated control (stim.). $n = 4$; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Endotoxins, which may occur as autolysis products of Gram(-) bacteria [56] can mimic immunostimulating effects of fermented plant extracts. Hence, they must be taken into account when interpreting the results. Since in the present study no or only small amounts of endotoxins were detected in the samples tested, as evidenced by the *Limulus* amoebocyte lysate (LAL) assay (Table S2, Supplementary Materials), the observed immunostimulatory effect in vitro was not associated with endotoxins (bacterial LPS).

3.6. Bacterial Toxicity: Inhibition of Bioluminescence Induced by WF Extracts in V. fischeri

The marine Gram-negative bacterium *Vibrio fischeri* (strain NRRL B-11177) is widely used as a test organism for screening the aquatic toxicity of compounds and environmental samples. However, this nontarget organism test has been also discussed as an in vitro tool to support toxicology studies of xenobiotics, due to its sensitivity and predictive power. The rationale behind its implementation is based on the fact, that *V. fisheri* toxicity data have a good correlation with in vivo data of human toxicity [57]. Furthermore, this assay might give an indication for an antibacterial potential of an extract. In this study, the extracts WF 1–3 were evaluated against *V. fischeri* based on the bioluminescence read-out as a (anti-bacterial) toxicity endpoint. As expected, varying extract-specific bacterial toxicity was found, based on IC_{50} values (WF1 > WF2 > WF3), but far from physiologically and systemically relevant concentrations (Figure 8).

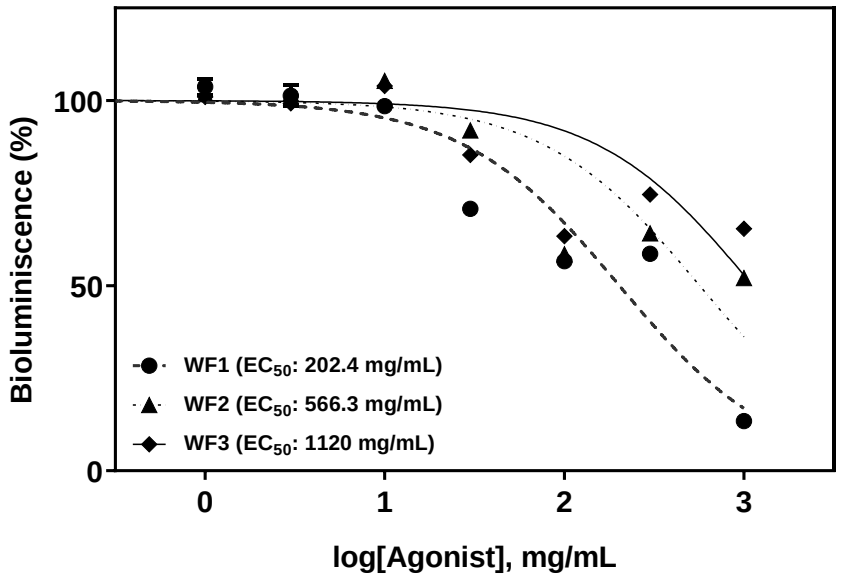

Figure 8. Toxicity of WF 1–3 on *V. fischeri* after 30 min of acute exposure as determined by bioluminescence. Data represent biological triplicates, each comprising technical quadruplicates.

3.7. Genotoxic Potential: In Vitro Micronucleus Test

WF extracts from *M. perennis*, processed according to GHP procedure no. 34 c, are used in pharmaceutical products for dermal applications or in ophthalmic drugs. To evaluate the mutagenic potential of the WF extracts these were tested in an AMES test and found to be non-mutagenic (data not shown) [58]. For further evaluation of genotoxic potential in the human system, the in vitro micronucleus test conducted with THP-1 monocytic cells was performed.

Exposure to a genotoxic test substance may result in damages to chromosomes or spindle apparatus of cells. During regular cell division, this type of damage can create a smaller micro- or hyponucleus, apart from the main nucleus. Therefore, the micronucleus test is an in vitro method that provides a comprehensive basis for investigating chromosome damage, because both aneugens and clastogens can be detected in cells that have undergone cell division during or after exposure to the test sample. Micronuclei represent damages that have been transmitted to daughter cells and in either case, the changes may not be compatible with cell survival.

As confirmed by the viability assessment (see above), none of the tested WF extract concentrations had the capability of producing artifactual positive responses by concurrent excessive cytotoxicity (Figure 9). Therefore, as the acceptability criteria for the assay were fulfilled (according to OECD 487; [21]), it can be emphasised that none of the test concentrations of WF1–3 exhibited a statistically significant increase in micro- and hyponuclei frequency, compared with the control background.

However, a visible, though non-significant decrease compared to the background control, as observed in WF1, was probably associated with reduced proliferation rather than specific chemopreventive or anti-genotoxic properties. Thus, the WF extracts were clearly negative, being unable to induce chromosome breaks (clastogenicity) and/or gain or loss micronuclei and hyponuclei (aneugenicity). Significant induction of both micro- and hyponuclei, i.e., chromosome breakage and whole chromosome loss, respectively, was however achieved by the subtoxic treatment of THP-1 cells with vinblastine sulphate (positive control, PC), demonstrating the sensitivity of the test system (Figure 9).

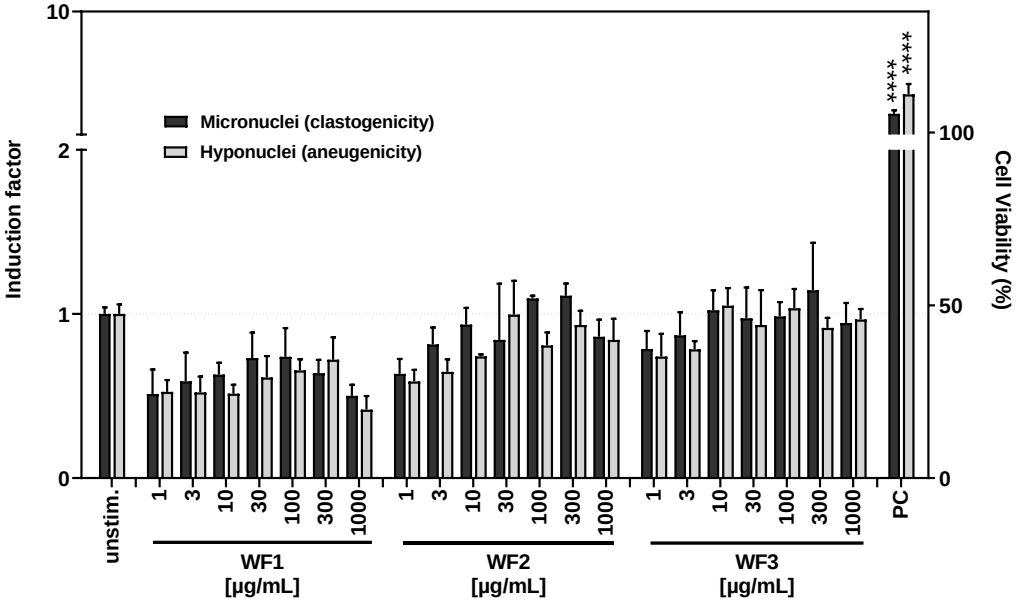

Figure 9. In vitro micronucleus test (genotoxicity) of WF extracts. Induction of micro- and hyponucleated cells was assessed by flow cytometric analysis and displayed as induction factor (IF) in comparison to the unstimulated control (IF = 1). PC: vinblastine sulphate (1.25 nM). Values represent mean ± SEM of three independent experiments (n = 3); **** $p < 0.0001$.

4. Conclusions

In the study presented here, the lacto-fermentation of the medicinal plant *M. perennis* was investigated with respect to its specific metabolite profile and the evaluation of immunomodulatory activity. Lacto-fermentation provides enzymes facilitating aqueous extraction by digestion of the plant cellular matrix and goes along with LA formation, which preserves the extracts by pH decrease and antimicrobial effects.

Fermentation of *M. perennis* by inoculation with either two Lactobacteria strains (LBF) or whey (WF) yielded extracts whose microbial stability was caused by low pH (3.9) and high LA concentration (maximum 15.6 g/L). Analysis of the compound profiles of LBF and WF extracts by LC-MS^n and GC-MS revealed a partial degradation of plant depsides and flavonoid glycosides. This went along with the formation of several novel compounds via fermentative conversion. Among others, novel substituted cinnamic acid depsides, but also nitrogen-containing metabolites, i.e., amino acid derivatives, biogenic amines, alkaloid artefacts, etc., could be tentatively assigned. However, some of the latter remain unknown and their exact structural assignment requires further studies. All extracts (AE, LBF and WF) enhanced the NFκB activity of LPS-stimulated THP-1 NFκB reporter cells in a concentration-dependent manner, while differences between unfermented (AE) and fermented (LBF or WF) extracts were not observed. Accordingly, bioactive constituents were not substantially affected by LA fermentation. Simultaneously, cytokine secretion was detected (most evidently for IL-8) for AE, LBF and WF in some cases, although this effect was partly superimposed by cytotoxicity (at 1000 µg/mL). However, whey exhibited a strong immunostimulating effect for all cytokines (IL-8, IL-6, IL-1β and TNFα). Further investigations are needed to understand potential correlations between the constituents of the fermented extracts and the observed immunomodulating activity. Conclusions about the contribution of the observed effects to specific phases of the healing process cannot be drawn yet. More complex in vitro models have to be applied to gain further understanding about the impact on other involved cell types and their interaction.

Finally, the toxicity and genotoxicity of the WF extracts was assessed in a *V. fisheri* assay and an in vitro micronucleus test with THP-1 cells, respectively. No toxic or mutagenic effects could be determined which supports the safety of WF extracts for their use, e.g., in dermal pharmaceutical remedies. The results of the study presented here significantly

expand our knowledge on the technology of producing LA-fermented medicinal plant extracts and their biological activity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9020190/s1, Figure S1: HPLC-MS^n fingerprints (BPC = base peak chromatograms) of *M. perennis* extracts. Figure S2A-C: Selections of MS^n(ESIneg) mass spectra and UV spectra of individual peaks, detected in the HPLC-DAD-MS^n(ESI^-) chromatograms of the none-fermented (AE) and fermented extracts (LBF, WF) of *M. perennis*. Table S1: Tentative assignment of polyphenolic constituents (depsides and flavonoids) in the water extract and lactic acid fermented extract, obtained from herbal parts of *M. perennis*, according HPLC-DAD-ESI(negative)-MS^n data. Method: HR-ESI-HPLC-MS analyses. Figure S3A: Base peak chromatogram of a Lactobacteria inoculated fermentation extract (LBF) of *M. perennis* with extracted ion chromatogram (mass scan on m/z 243; HR-ESI-MS of the peak (t_R 8.2 min) in the positive mode, with calculated masses and proposed molecular formula for metabolite **16*** (isomer 1). Figure S3B: Base peak chromatogram of a Lactobacteria inoculated fermentation extract (LBF) of *M. perennis* with extracted ion chromatogram (mass scan on m/z 243; HR-ESI-MS of the peak (t_R 9.2 min) in the positive mode, with calculated masses and proposed molecular formula for metabolite **16*** (isomer 2). Figure S3C: Structure annotation from FISh scoring node on MS^2 spectrum of **16***. The fragmentation was simulated with the Compound Discoverer software, Version 3.3 of Thermo Scientific. Figure S3D: Base peak chromatogram of a Lactobacteria inoculated fermentation extract (LBF) of *M. perennis* with extracted ion chromatogram (mass scan on m/z 305; HR-ESI-MS of the peak (t_R 8.0 min) in the negative mode, with calculated masses and proposed molecular formula for metabolite **19**. Figure S3E: Base peak chromatogram of a Lactobacteria inoculated fermentation extract (LBF) of *M. perennis* with extracted ion chromatogram (mass scan on m/z 369; HR-ESI-MS of the peak (t_R 12.3 min) in the positive mode, with calculated masses and proposed molecular formula for metabolite **25**. Figure S3F: Structure annotation from FISh scoring node on MS^2 spectrum of **25**. The fragmentation was simulated with the Compound Discoverer software, Version 3.3 of Thermo Scientific. Figure S3G: Base peak chromatogram of a Lactobacteria inoculated fermentation extract (LBF) of *M. perennis* with extracted ion chromatogram (mass scan on m/z 453; HR-ESI-MS of the peak (t_R 15.7 min) in the negative mode, with calculated masses and proposed molecular formula for metabolite **37**. Figure S3H: Structure annotation from FISh scoring node on MS^2 spectrum of **37**. The fragmentation was simulated with the Compound Discoverer software, Version 3.3 of Thermo Scientific. Figure S4: GC-MS total ion chromatogram sections of a WF extract, obtained after a HAB procedure, showing low molecular constituents (TMS derivatization). Method: Viability assay. Figure S5: Effects of test substances on cell viability of THP-1 NFκB reporter cells determined by WST assay. Method: Determination of evaporation residues (dry weight) of liquid extracts. Method: Endotoxin (LAL Limulus) assay. Table S2: Chemical and physical parameters of tested samples.

Author Contributions: Design of the study: P.L., I.Z., C.T., D.R.K., F.C.S. and C.G.; preparation of extracts, fermentation experiments: P.L. and I.Z.; data acquisition: P.L. and L.K.M.; HR-MS experiments and data interpretation: I.K. and P.L.; cell culture experiments, data equisition and interpretation: A.M.Z.-K., M.G.-K., S.N. and C.G.; toxicity (*V. fisheri*) and genotoxicity tests: M.G.-K.; evaluation of the data and preparation of the manuscript: P.L., I.Z., L.K.M., D.R.K., F.C.S., A.M.Z.-K., M.G.-K. and C.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: P.L., I.Z., L.K.M., C.T., F.C.S. and D.R.K. are employed at WALA Heilmittel GmbH. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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