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Red Yeast Rice or Lovastatin? A Comparative Evaluation of Safety and Efficacy Through a Multifaceted Approach

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ABSTRACT

The increasing use of red yeast rice (RYR) as a natural supplement to manage blood cholesterol levels is driven by its active compound, monacolin K (MK), which is chemically identical to the statin drug lovastatin (LOV). Despite its growing popularity, concerns persists regarding the safety and efficacy of RYR compared to pure statins. This study aimed to evaluate the phytochemical composition, pharmacological effects, and safety profile of various RYR samples in comparison with LOV. RYR samples with different MK content were analyzed using HPLC-DAD to quantify monacolins and other bioactive compounds. The inhibitory activity on HMG-CoA reductase was assessed through an enzymatic assay, while pharmacokinetic properties were predicted using in vitro simulated digestion and *in silico* models. In vitro cytotoxicity was evaluated in intestinal, hepatic, renal, and skeletal muscle cell models. Additionally, the transcriptional levels of muscle damage-related target genes were evaluated by qRT-PCR in skeletal muscle cells treated with a selection of RYR samples. Significant variability in the phytochemical composition of RYR samples was observed, particularly in the content of secondary monacolins, triterpenes, and polyphenols. The RYR phytocomplex exhibited superior inhibition of HMG-CoA reductase activity compared to isolated LOV, suggesting synergistic effects between secondary monacolins and other compounds. Molecular insights revealed that RYR samples had a lower impact on muscle cells than LOV, as reflected also by cell viability. These findings suggest that RYR could serve as a safe alternative to purified statins. However, further research is needed to fully elucidate the mechanisms behind the synergistic activity of the phytocomplex and to firmly establish the clinical efficacy of this natural product.

1 | Introduction

The market of red yeast rice (RYR) is steadily developing worldwide, fostered by robust scientific evidence on anti-cholesterolemic activity and by the consumers interest for botanical products, deemed safer and with reduced adverse effects compared to prescribed drugs (Bertuccioli et al. 2021; Garcia-Alvarez et al. 2014; Zhu et al. 2019). However, despite the

Abbreviations: ATG, autophagy; DeMK, dehydromonacolin K; DiMK, dihydromonacolin K; Fbxo32, Muscle Atrophy F-Box Protein 32; HMG-CoAR, 3-hydroxy-3-methylglutaryl-coenzyme reductase; LOVA, lovastatin hydroxy acid form; LOV, lovastatin; LOVL, lovastatin lactone form; MCK, muscle creatine kinase; MJ, monacolin J; MK, monacolin K; MKA, monacolin K hydroxy acid form; MKL, monacolin K lactone form; ML, monacolin L; MX, monacolin X; MyoD, myoblast determination protein 1; RYRs, red yeast rice samples; Trim63, Tripartite Motif-Containing Protein 63.

large literature available on the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme reductase (HMG-CoAR) exerted by monacolin K (MK), the lovastatin (LOV) equivalent molecule present in RYR, multiple question marks surround RYR, in particular regarding the combined effects of its complex phytochemical profile.

RYR phytocomplex comprises substances produced by *Monascus purpureus* Went. during its metabolic activity on the white rice substrate, namely carbohydrates (in particular, starch, >70%), proteins (15%), amino acids, phytosterols, triterpenoids, and polyphenols (Avula et al. 2014; Ma et al. 2000). RYR is also enriched in natural orange, yellow and red pigments produced by the esterification of azaphilone with β-ketoacid and most relevantly, several characteristic polyketides defined monacolins (Avula et al. 2014; Song et al. 2019) RYR mainly contains MK, but further monacolins are also present. Those in detectable quantities include MK derivatives such as dihydro, dehydro- and ester-MK derivatives and other secondary monacolins, including monacolin J, L, M and X, as well as compactin (Li et al. 2004), also known as mevastatin, the first statin identified (Endo, Kuroda, and Tanzawa 1976). RYR monacolins exist in two interconvertible forms, namely the lactone and the hydroxy acid form. Depending on the fermentation process and drying parameter, the ratio between the lactone and hydroxy acid forms may vary substantially but, due to low pH, a preferential lactone form is normally observed (Yuan et al. 2023).

In many countries within and outside Europe, supplements containing RYR are mostly used in primary prevention for cardiovascular health, according to therapeutic indications, in particular when statin-based pharmacotherapy is not advisable (Banach et al. 2022, 2019; Cicero et al. 2023, 2021, 2019; Garcia-Alvarez et al. 2014).

Clinical studies and meta-analyses have suggested that both RYR and statins are effective in lowering cholesterol levels (Laffin et al. 2023; Liu et al. 2006; Morze et al. 2020; Osadnik et al. 2022). This evidence was aready sufficiently clear more than a decade ago and led the European Food Safety Authority (EFSA) in 2011 to approve a health claim for the consumption of 10 mg/day of RYR-derived MK, stating its role in maintaining normal blood LDL-cholesterol concentrations (EFSA Panel on Dietetic Products and Nutrition and Allergies (NDA) 2011). However, in 2018, the EFSA raised concerns about the safety of RYR in food supplements due to reported side effects, particularly those affecting skeletal muscles, similar to those associated with LOV (Younes et al. 2018).

While some recent research suggests that RYR might be associated with fewer or less severe muscle-related side effects compared to statins, the evidence remains inconclusive (Becker et al. 2009; Fogacci et al. 2019; Halbert et al. 2010). The limited understanding of the differences in bioactivity and adverse effects between LOV, MK, and RYR further complicates safety assessments. This challenge is exacerbated by the heterogeneity of marketed RYR products, which have been linked to the onset of side effects (Banach et al. 2022). The mechanisms underlying myalgias and muscle toxicity associated with both natural and synthetic statins remain unclear, lacking a well-defined biological and molecular basis (Bouitbir et al. 2016; Päivä et al. 2005).

Given these unresolved safety concerns, in 2022, the European Commission issued a new regulation (Reg. (EU) 468/2022) that classified RYR as a substance with restricted use, setting a maximum daily intake of monacolins at less than 3 mg. Furthermore, on July 29th, 2024, the health claim associated with RYR was revoked (Commission Regulation (EU) 2024/241).

Undoubtedly, a deeper understanding is currently needed to properly address not only the safety of RYR, but also the pharmacokinetic and pharmacodynamic features of the phytocomplex.

In fact, he literature provide scarce studies comparing the pharmacokinetic features and biological activity of statins and RYR (Halbert et al. 2010; Ong and Aziz 2016; Xu et al. 2022). In addition, extensive cellular and molecular studies covering the biological activity of RYR as a phytocomplex are lacking (Patakova 2013). To date, some in vitro investigation highlighted the anti-inflammatory and antioxidant activity of RYR yellow and red pigments (Dhale, Javagal, and Puttananjaiah 2018; Lai et al. 2021), secondary monacolins, mainly recording the good docking score, and the potential HMG-CoAR inhibitory activity of compactin and MK derivatives was observed (Righetti et al. 2022).

Therefore, this study aimed at providing new insights on the efficacy and safety, in terms of biological activity, of RYR phytocomplex in comparison with LOV. To achieve this goal, the research work was conducted following four specific objective-related steps. First, a deep chemical analysis was performed to address the phytochemical profile of a wide range of raw RYR commercial products with different contents in MK and secondary monacolins through a validated HPLC-DAD method. Secondly, the pharmacological efficacy of RYR and LOV was investigated by a cell-free enzymatic assay for HMG-CoAR.

Moreover, a pharmacokinetics and bioaccessibility comparison among the different RYR samples (RYRs) and LOV was considered by exploiting an integrated in vitro simulated digestion and *in silico* ADME prediction. Finally, in vitro cytotoxicity of RYRs and LOV was evaluated in different cell models such as intestinal (Caco-2), hepatic (HepG2), renal (Hek293), and skeletal muscle (C2C12) cells. To compare the dynamics of side effects on skeletal muscle, the transcriptional effects of selected RYRs were finally evaluated, in comparison with LOV, on important factors regulating the processes of myogenesis (*MyoD*), autophagy (*Atg5*, *Atg7*) and muscle atrophy (*Fbxo32* and *Trim63*) as well as on muscle-related damage factors like muscle creatine kinase (MCK) and the cytokine IL-6.

2 | Material and Methods

2.1 | Chemicals and Sample Selection

Twelve commercial-grade raw RYRs, produced in China and used as ingredient for food-supplement formulations, were collected upon our request from different supplier through the major European companies located in Italy, Germany, Spain, and San Marino Republic. Samples were grouped according to the declared MK content, in order to evaluate differences related to distinct profiles: four samples having MKtot 1.5% ca., four

samples with MKtot 3% ca. and four samples with MKtot 5%. (Table 1). Two products with negligeable MK content were also tested, specifically supplied for this study and not available in the market.

LOV (purity 99.5%, PubChem CID:53232) (Merck, Milan, Italy) was used as reference standard.

Ultrapure water was produced with a reverse osmosis system and reprocessed with a Millipore device. RYRs and LOV were solubilized in ethanol 80% v/v (10 mg/mL) (Merck Sigma-Aldrich KGaA, Darmstadt, Germany) and sonicated in an ultrasound bath for 2 h.

2.2 | Phytochemical Analysis

RYRs were profiled by using a HPLC-DAD method. The HPLC-DAD instrument was a Shimadzu Prominence LC 2030 3D equipped with a Bondapak RP C18, 10 μm, 125 Å, 3.9 × 300 mm column (Waters Corporation, Milford, Massachusetts, USA). Ultrapure water +0.1% v/v formic acid (A) and acetonitrile +0.1% v/v formic acid (B) were used as mobile phase. Elution program was B 35% at 0 min., 75% at 20 min., then 75% up to 29 min, and 35% at 35 min. Flow rate: 1.0 mL/min. The identification and quantification of monacolins was performed at 237 nm. Injected volume 10 μL (10 mg/mL in ethanol 80% v/v).

MK lactone and hydroxy acid (MKL and MKA) were identified and quantified by means of the standard curve constructed with reference standard mevinolin, in lactone and hydroxy acid forms (purity ≥ 96%; Merck Sigma-Aldrich KGaA, Darmstadt, Germany). Secondary monacolins were identified according to UV–VIS spectra and according to previously published data (Righetti et al. 2022, 2021); secondary monacolins were quantified as mevinolin. The total polyphenols were determined by the Folin–Ciocalteu colorimetric assay as previously described (Biagi et al. 2019); Folin–Ciocalteu reagent and Na_2CO_3 were purchased from Merck.

For the quantification of triterpenes, the acid vanillin method was used; glacial acetic acid, vanillin and perchloric acid ($HClO_4$) were purchased from Merck (Vaccaro et al. 2023).

TABLE 1 | Red yeast rice samples based on the declared monacolin K content expressed in % (sum of lactone, MKL, and hydroxy acid, MKA, forms).

No. sample	Total monacolin K content (declared)
A0	< 0.15%
B0	< 0.15%
A1.5	1.5%
B1.5	1.5%
C1.5	1.5%
D1.5	1.5%
A3	3%
B3	3%
C3	3%
D3	3%
A5	5%
B5	5%
C5	5%
D5	5%

2.3 | Monacolins Conversion in Hydroxy Acid Form

The intestinal metabolism of MK, and consequently of LOV, includes the conversion of the lactone in the hydroxy acid form. This conversion is essential to exert the biological activity of this compound in inhibiting the HMG-CoAR enzyme activity.

To obtain the conversion of MKL into the biologically active MKA, RYRs, and LOV solubilization was performed with ethanol 80% v/v added of NaOH 0.1 M (pH 11) (ethanol:NaOH 0.1 M (5:1)). The hydrolysis reaction was incubated overnight at room temperature. The complete conversion was monitored by the HPLC-DAD method above described. The comparison analysis demonstrated a very similar conversion time for all RYRs and LOV with an efficiency > 95%.

2.4 | In Vitro Bioaccessibility of Monacolin K

The chemical stability and bioaccessibility rate of the MK contained in RYRs and LOV was assessed by an in vitro simulated digestion as previously described (Governa et al. 2022) with some modifications.

Based on the phytochemical results, selected RYRs within each concentration group (namely B1.5, D1.5, A3, D3, A5, C5, D5) were tested. All the samples were dissolved in ethanol 80% v/v at the final concentration of 20 mg/mL. Briefly, 1 mL of RYRs and LOV solutions were diluted in 9 mL of simulated gastric juice, containing pepsin from porcine gastric mucosa (300 UI/mL, Merck) and NaCl (10 mg/mL). The pH of the solution was adjusted to 1.2 using HCl. Samples were incubated for 2 h at 37°C with shaking. Then, pancreatin from porcine pancreas (activity equivalent to 4 × U.S.P., 10 mg/mL, Merck) and bile salts mixture (20 mg/mL, Merck) were added, and the pH was increased to 8.5 by adding $NaHCO_3$ (25 mg/mL, Sodalco S.p.A., Milan, Italy) to simulate the intestinal environment where physiologically MK, as well as LOV, are converted in hydroxy acid form. Intestinal digestion was carried out for 2 h at 37°C with shaking. Samples were then filtered and immediately used for further analysis. As a control condition, the differential influence of enzymatic degradation, solubility, and intestinal pH was evaluated incubating the samples at equal weight in a solution of $NaHCO_3$ at pH = 8.5 for 4 h at 37°C. At the end of the simulated digestion, samples were analyzed by HPLC-DAD to quantify LOV, MK, and other monacolins. Given the lack of standards for secondary monacolins in hydroxy acid form, only a qualitative analysis of the bioaccessibility rate to intestinal absorption of secondary

monacolins was performed. The relative gastrointestinal stability was calculated as the % recovery of the sample after digestion, compared to the starting amount of sample used.

2.5 | Computational Simulation of Intestinal Absorption

To evaluate the predictable pharmacokinetics of MK and other RYR monacolins a computational investigation was performed by using SwissADME platform (http://www.swissadme.ch/). Molecules were imported from PubChem (https://pubchem.ncbi.nlm.nih.gov/), using SMILES codes (Daina, Michielin, and Zoete 2017), or manually drawn.

2.6 | Enzymatic Assay for the Inhibition of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase (HMG-CoAR) Activity

To evaluate the effects of RYR and LOV on HMG-CoAR activity, the *HMG-CoA Reductase Assay Kit* was used (CS1090, Merck) following the manufacturer's instructions. An internal positive control (pravastatin) and non-statin controls (β-sitosterol and hyperoside, Merck) were used. The assay was performed on LOV and RYRs (Table 1) in hydroxy acid active form and in native lactone inactive form. First, we validated the in vitro cell-free assay testing the activity of LOV at different concentrations ranging from 10 ng/mL to 2 μg/mL, both in lactone (LOVL) and hydroxy acid (LOVA) form, compared to the positive (pravastatin) (Table 2). As declared by the manufacturer, pravastatin exerted HMG-CoAR inhibition > 50% at the provided concentration, while LOVA measured an IC_{50} of 74 ng/mL ca. for LOVA, in accordance with previous data (85 ng/mL) (Perchellet et al. 2009).

RYRs were tested at six different concentrations ranging from 50 to 5 μg/mL. The test is based on a spectrophotometric measurement of the absorbance at 340 nm, corresponding to the wavelenght of maximum absorbance of NADPH that undergoes oxidation by the catalytic subunit of HMG-CoAR, in the presence of the HMG-CoA substrate.

All the experiments were conducted in triplicate and the IC_{50} was calculated for each sample.

The absorbance kinetics at 340 nm was read with an interval of 5 min for 20 min using a Victor Nivo 3S plate reader (PerkinElmer, Waltham, Massachusetts, USA). One unit will convert 1.0 μmol of NADPH to $NADP^+$/minute at 37°C. The specific activity is defined as μmol/min/mg-protein (Units/mg-protein).

The activity of the samples, and therefore their ability to inhibit the HMG-CoAR enzyme, is evaluated using the formula as follow:

$$\text{Units/mgP} = \frac{\left(\Delta A_{340}/\text{min}_{\text{sample}} - \Delta A_{340}/\text{min}_{\text{blank}}\right) \times \text{TV}}{12.44 \times \text{V} \times 0.6 \times \text{LP}}$$

where $12.44 = \varepsilon^{nM}$- the extinction coefficient for NADPH at 340 nm is 6.22 $mM^{-1}cm^{-1}$;12.44 represents the 2 NADPH consumed in the reaction; TV = total volume of the reaction (mL); V = volume of enzyme used in the assay (mL); 0.6 Enzyme concentration in mg-protein (mgP)/mL (0.50–0.70 mgP/mL); LP = Light path (cm).

2.7 | Cell Lines and Treatment

Mouse skeletal muscle cell line (C2C12, ATCC #CRL-1772), human hepatic cell line (HepG2, ATCC #HB-8065), human colorectal adenocarcinoma cell line (Caco-2, ATCC #HTB-37) and human embryonic kidney cell line (Hek293, ATCC #CRL-1573) were grown in DMEM High Glucose Medium (Merck KGaA, Darmstadt, Germany) supplemented with 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% FBS (Gibco, Thermo Fisher Scientific, Whaltam, Massachusetts, USA). Cells were grown at 37°C and 5% CO_2 with saturating humidity. Cells under passage 20 were used for all experiments. For cell treatment, RYRs and LOV were dissolved at the concentration of 10 mg/mL (stock solutions) in ethanol 80% v/v added of NaOH 0.1 M (ethanol 80% v/v:NaOH 0.1 M 5:1) to obtain the active hydroxy acid form. The control group received the vehicle (ethanol 80% v/v:NaOH 0.1 M 5:1).

2.8 | Cell Viability Assay

Cell viability was tested using Cell Counting kit-8 (CCK-8, Merck) as previously described (Pressi et al. 2022, 2023). Briefly, HepG2, Hek293, Caco2, and C2C12 cells (5×10^4) were cultured in 96-well plates. After 24 h, cells were treated with RYRs (Table 1) or LOV at different concentrations, respectively (RYRs: 20, 50, 100 μg/mL; LOV: 1, 5, 10, 50, 100 μg/mL) for 24 h, then CCK-8 solution was added to each well and incubated at 37°C, 5% CO_2 for 60 min. Cell viability was calculated by measuring the absorbance at 450 nm. Three independent replicates were performed.

2.9 | Interleukin-6 (IL-6) Dosage

C2C12 cells were seeded into 24-well plates (1×10^5 cells/well) and cultured for 24 h. The cells were treated for 24 h with LOV (1 μg/mL) or selected RYRs in order to consider products with different MK content. To compare the effects of LOV and RYR, proper dilutions were made starting from comprehensive, actual content of MKs in samples as determined by HPLC, obtaining

TABLE 2 | IC_{50} values of the inhibition activity of the enzyme HMG-CoA reductase of lovastatin in the lactone (LOVL) and hydroxy acid (LOVA) forms, measured by cell-free in vitro assay. Data are expressed in ng/mL ± standard deviation.

Sample	IC_{50} (ng/mL)
Lovastatin lactone form (LOVL)	1457.07 ± 139.34
Lovastatin hydroxy acid form (LOVA)	74.09 ± 8.22

samples providing the same content of total MK (equivalent to 1 μg/mL of MKtot) (Table S1).

IL-6 production was evaluated in both C2C12 cell lysate and medium by non-competitive sandwich ELISA (BMS603-2, Biolegend e-Bioscience DX Diagnostic, San Diego, California, USA) according to the manufacturer's instructions, as previously reported (Rigillo et al. 2019). Absorbance was measured at 450 nm using a Victor Nivo 3S plate reader (PerkinElmer). Two independent experiments were performed, and each sample was dosed in triplicate.

2.10 | Total RNA Extraction, Reverse Transcription, and Real-Time PCR

C2C12 cells were seeded in 12-well plates (3×10^5 cells/well) and cultured for 24 h. Cells were treated for 24 h either with LOV (1 μg/mL) or RYRs at the same relative content of total MKs as described above (Table S1). RNA extraction and DNAse treatment were performed as previously described (Belluti et al. 2018; Ciani et al. 2024) using Ripospin II mini-Kit (GeneAll, Seoul, Korea), according to the manufacturer's protocol. For cDNA synthesis, 500 ng of RNA was reverse transcribed with PrimeScript RT Reagent Kit (Takara Bio Inc., Kusatsu, Japan). Quantitative RealTime PCR (RT-qPCR) was performed in CFX connect thermocycler (Bio-Rad Laboratories, California, USA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, California, USA) and specific forward and reverse primers at a final concentration of 300 nM (Table 3). Cycle threshold (Cq) value was determined by the CFX maestro software (Bio-Rad Laboratories, CA), mRNA expression was calculated with the ΔΔCt method with Ribosomal Protein L27 (*Rpl27*) as endogenous control.

2.11 | Statistical Analysis

Data are presented as mean ± standard deviation (SD). For the analysis of correlation between bioaccessibility rate and RYR phytocomplex constituent content, one-tailed Pearson test was used.

For gene expression analysis, data were firstly analyzed for normality assumption using Kolmogorov–Smirnov one-sample test for normality: all targets displayed a normal distribution. To study the transcriptional effects of RYR and LOV in muscle cells, we performed a one-way analysis of variance (ANOVA) for the main effect of the treatment, followed by Tukey's post hoc tests for multiple comparisons.

No exclusion criteria were pre-determined, and possible outliers were identified before statistical analysis using the boxplot tool in SPSS. The statistical significance was considered at $p < 0.05$. Statistical analyses were conducted using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, New York, USA). Graphs were created using GraphPad Prism version 10 for Microsoft (GraphPad Software, San Diego, California, USA).

3 | Results

3.1 | Phytochemical Analysis of RYRs

3.1.1 | Identification and Quantification of Monacolins in Red Yeast Rice Samples

The HPLC-DAD method resulted reliable (Table 4) and allowed a proper separation of monacolins in RYRs.

TABLE 3 | Transcript and sequence of each primer used in quantitative real-time PCR.

Target	NCBI GenBank	Sequence
Rpl27	*NM_011289.3*	FW: AAGCCGTCATCGTGAAGAACA
		RV: CTTGATCTTGGATCGCTTGGC
Interleukin (IL)-6	*NM_031168*	FW: CTTCACAAGTCGGAGGCTTA
		RV: CAAGTGCATCATCGTTGTTC
Fbxo32	*NM_026346*	FW: CGGGTACACACACAGATGGA
		RV: TGACTGAACATTGGGCACAC
Trim63	*NM_001369245*	FW: AGGCAGAGATTGGCAGGAT
		RV: CCATTTCATTGTGTCGTTGC
Atg5	*NM_053069*	FW: CAGGAGCTGTCACAGGATTTAT
		RV: GTGACCAACGTAACAGCAAAG
Atg7	*NM_001253717.2*	FW: TCCTGAGAGCATCCCTCTAAT
		RV: GGCTCGACACAGATCATCATAG
MyoD	*NM_010866.2*	FW: GCTACGACACCGCCTACTAC
		RV: GAGATGCGCTCCACTATGCT
Mck	*NM_007710.2*	FW: CTTGTGTGGGTGAACGAGGA
		RV: CGTTCCACATGAAGGGGTGA

As expected, the main peak at the retention time (RT) = 17.4 min was attributable to MK lactone form (MKL), while the RT relative to MK hydroxy acid form (MKA) referred to MKL was 0.83 (Figure 1) (Ajdari et al. 2011; Li, Liu, and Wang 2005). MKL and MKA were identified and quantified by using mevinolin as external standard.

To date, no other analytical standards for quantification are commercially available for the main secondary monacolins besides MK, therefore the identification and subsequent quantification of secondary monacolins (Msec) were made by comparing the sequence of the RTs reported in literature and UV spectra (Li et al. 2004; Ma et al. 2000; Righetti et al. 2022) and by expressing results as mevinolin. In all samples, dehydromonacolin K (DHMK) and monacolin L (lactone) (ML), resulted the most abundant among Msec. Monacolin M lactone (MM), monacolin L as hydroxy acid (MLA), compactin, monacolin X lactone (MX) and dihydromonacolin K (DiHMK) were also found in variable amounts (> 0.05%) depending on different samples.

Total monacolin content of the 14 tested samples is summarized in Table 4, described in terms of MKL, MKA and secondary monacolins (Msec), expressed as the sum of MM, MLA, ML, MX, compactin, DiHMK and DHMK I. In each of the four-cluster considered, the declared content of total MK, as a sum of MKA and MKL, resulted sufficiently compliant with the actual content, in accordance with Vitiello et al. (Vitiello et al. 2023) and differently from what reported by Righetti et al. (Righetti et al. 2022) for products sold via web market.

More precisely, the content in MK declared by producers, referred to the sum of MKL and MKA, resulted slightly lower (−5%) than declared content in 2 of 12 samples, whereas we observed a maximum discrepancy between actual and declared content of +20% (A0 and B0 were excluded). We observed instead a high variability in Msec content, according to previous data (Righetti et al. 2022). The average of Msec content of tested RYRs was 23% of total monacolins (A0 and B0 were excluded), with the highest content recorded for D1.5 (3%), while 8 samples out of 12 displayed 20%–30% of Msec, and 2 samples the 15%–20% of Msec relative to total monacolins. Sample D5 had only 3% of Msec calculated as total monacolins, an exception worthy to be taken into account.

The MKL and MKA relative ratio showed a very large range and only in three samples the MKA:MKtot ratio was > 0.30, typical of not refined RYR (Lachenmeier et al. 2012; Ma et al. 2000) (Table 5). This analysis could confirm that the fermentation, drying and refining process may lead to a pH decrease producing a high rate of MKL; indeed, we noticed that in six out of eight samples with MKtot content 1.5% or 3% ca., the MKA:MKtot ratio was greater than 0.15, whereas samples with 5% of MKtot ca. showed MKA:MKtot ratio less than 0.15 (Table 5).

TABLE 4 | HPLC-DAD parameters validated for the quantification of monacolin K in red yeast rice samples.

Reference standard	mevinolin
Linearity range	0.1–10 μg in column
R^2	0.999
Equation	y = 2837.000 + 49.926

3.1.2 | Analysis of Polyphenol and Triterpenes Content

An average content of 0.92% was found for total polyphenols, ranging from 0.41% (C5) to 1.33% (C1) (Table 6). A valid correlation between polyphenols content and MK or Msec was not observed, but the ratio polyphenols:MK was higher in RYRs with low or medium MK content (Table 6). Interestingly, sample D5

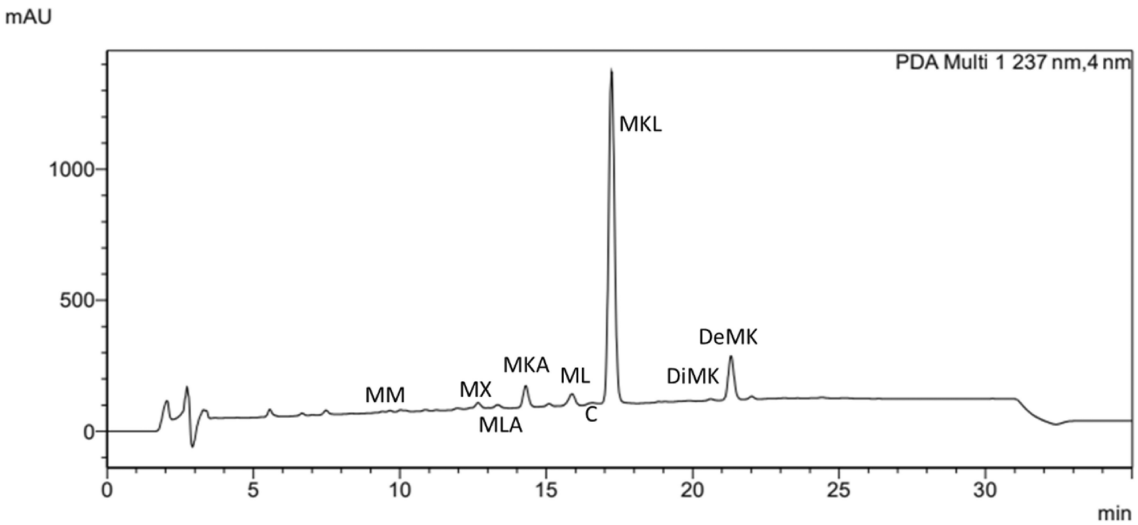


FIGURE 1 | HPLC-DAD chromatogram recorded at 237 nm (sample A5). The main peak is represented by monacolin K in the lactone form (MKL). Monacolin K in acid form at RT = 14.30 min (MKA). Secondary monacolins identified according to their rRT (MKL relative RT) are represented in the following order: MM = monacolin M (rRT = 0.54) MX = monacolin X (rRT = 0.74); MLA = monacolin L hydroxy acid form; ML = monacolin L (rRT = 0.92); C = compactin (rRT = 0.97); MK = monacolin K (RT = 17.4); DiMK = dihydromonacolin K (rRT = 1.19); DeMK = dehydromonacolin K (rRT = 1.24).

TABLE 5 | Monacolins quantification in red yeast rice samples.

RYR sample	MKtot (MKL + MKA) % *w/w*	MKL % *w/w*	MKA % *w/w*	Msec % *w/w*	MKA:MKtot
A0	0.05 ± 0.01	0.04 ± 0.01	0.01 ± 0.01	0.50 ± 0.01	0.20
B0	0.10 ± 0.01	0.08 ± 0.02	0.02 ± 0.01	0.60 ± 0.01	0.20
A1.5	1.42 ± 0.05	0.90 ± 0.04	0.52 ± 0.03	0.41 ± 0.02	0.36
B1.5	1.55 ± 0.04	1.31 ± 0.04	0.24 ± 0.02	0.64 ± 0.04	0.15
C1.5	1.70 ± 0.07	1.39 ± 0.06	0.31 ± 0.06	0.74 ± 0.06	0.18
D1.5	1.80 ± 0.05	1.50 ± 0.04	0.30 ± 0.01	1.04 ± 0.03	0.16
A3	2.85 ± 0.09	1.45 ± 0.06	1.40 ± 0.06	0.90 ± 0.06	0.49
B3	3.15 ± 0.09	3.03 ± 0.09	0.12 ± 0.01	0.95 ± 0.02	0.04
C3	3.22 ± 0.07	2.06 ± 0.05	1.16 ± 0.03	0.81 ± 0.05	0.36
D3	3.50 ± 0.11	3.40 ± 0.10	0.10 ± 0.01	1.03 ± 0.03	0.03
A5	5.03 ± 0.13	4.30 ± 0.12	0.73 ± 0.02	2.04 ± 0.06	0.14
B5	5.09 ± 0.12	4.58 ± 0.11	0.51 ± 0.02	0.97 ± 0.04	0.10
C5	5.13 ± 0.10	4.37 ± 0.08	0.76 ± 0.04	1.23 ± 0.05	0.15
D5	5.25 ± 0.16	4.90 ± 0.21	0.35 ± 0.07	0.17 ± 0.01	0.06

Note: Data are expressed as % *w/w* ± standard deviation.
Abbreviations: MKA = hydroxy acid form of monacolin K; MKL = lactone form of monacolin K; MKtot = sum of MKL and MKA; Mtot = sum of MKL and MKA.

TABLE 6 | Total polyphenols and triterpenes quantification in red yeast rice samples. Data are expressed as % *w/w* ± standard deviation.

RYR Sample	MKtot % *w/w*	Total polyphenols % *w/w*	Total triterpenes % *w/w*
A0	0.05 ± 0.01	0.62 ± 0.16	0.19 ± 0.18
B0	0.10 ± 0.01	0.67 ± 0.02	0.30 ± 0.14
A1.5	1.42 ± 0.05	1.33 ± 0.07	2.71 ± 0.21
B1.5	1.55 ± 0.04	0.63 ± 0.07	2.35 ± 0.20
C1.5	1.70 ± 0.07	0.68 ± 0.06	2.89 ± 0.25
D1.5	1.80 ± 0.05	0.85 ± 0.01	4.62 ± 0.15
A3	2.85 ± 0.09	1.18 ± 0.07	4.71 ± 0.35
B3	3.15 ± 0.09	1.09 ± 0.15	6.00 ± 1.35
C3	3.22 ± 0.07	1.05 ± 0.08	4.41 ± 0.39
D3	3.50 ± 0.11	0.92 ± 0.16	7.57 ± 0.26
A5	5.03 ± 0.13	0.93 ± 0.01	4.84 ± 1.49
B5	5.09 ± 0.12	1.19 ± 0.09	4.08 ± 0.34
C5	5.13 ± 0.10	1.31 ± 0.11	4.56 ± 0.40
D5	5.25 ± 0.16	0.41 ± 0.05	1.56 ± 0.08

showed a different profile compared to other samples, with the lowest content of total polyphenols. Despite being free of MK, the content of total polyphenols in samples A0 and B0 was comparable to those with MKtot content of 1.5%, suggesting a low interference of the post-fermentative process on the content of this class of secondary metabolites.

Triterpenes were found in relevant concentration in all samples (Table 6) with the exception of A0, B0, where a separation process was used, and highlighting the unique phytocomplex of D5.

Among samples with 1.5% and 5% MKtot (A5, B5, C5), the total triterpenes content showed limited variability, while, among

samples with MKtot 3% the content of total triterpenes resulted both higher on average and more variable (Table 6).

Overall, our screening added some interesting insights on the topic of heterogeneity of RYRs, suggesting that while some issues may have been somehow resolved in terms of MKtot, differences in the phytocomplex may be present, potentially leading to consequences that should be investigated. At the same time, this screening allowed us to better understand different profiles of RYR undergoing the subsequent determination of bioactivities.

Seven samples showed a content of MKtot of ±5% in agreement with label statements, while in 5 samples the variance was not fully compliant with pharmaceutical Good Manufacturing Practices requirements (95%–105%) content of active constituent. These findings furtherly support the need of analytical attention to RYR to standardize product formulation with a precise MK dosage.

We also confirmed the relevant content of Msec in all RYRs, as on average they constitute more than 20% (but with samples exceeding 30%) of total monacolins, being MLL and DHMK the most represented in all samples. Then, we found that RYR phytocomplex is enriched in biologically active secondary metabolites such as polyphenols, known to exert antioxidant activity (Bertelli et al. 2021) and triterpenes that have also a role in cholesterol absorption (Machaba et al. 2014).

Finally, we found that, in some RYRs the phytocomplex could be almost completely lost likely due to the post-fermentative processes employed to concentrate MK, as observed for D5.

3.2 | Bioaccessibility of Monacolin K and Lovastatin

One of the most common synergistic mechanisms differentiating the use of a botanical from a pure compound involves the modulation of bioaccessibility induced by co-occuring substances, that may hamper or facilitate the process. The pharmacokinetic differences between RYR phytocomplex and pure LOV were therefore evaluated by means of an in vitro model of simulated digestion. Based on the phytochemical profile observed, we addressed the bioaccessibility on a selection of RYRs: two representative RYRs with MKtot 1.5% and 3% ca., and three samples at 5% of MKtot were chosen because of the different phytocomplex's characteristics highlighted within the cluster. MK-free samples were excluded from this analysis (Table 7).

Through this model, the pharmacokinetic study of single Msec was difficult and unreliable due to their low concentration after digestion, therefore only the fate of MK was monitored.

Under the conditions created during the simulated digestion, both pure LOV and pure MK underwent a rapid and stable conversion into the hydroxy acid form: more than 90% of LOV and MK were found in their biologically active form after the simulated digestion (Figure S1). The bioaccessibility rate of LOV resulted 65.23% ± 18.45 (expressed as percentage of post-digestion amount compared to the pre-digestion one), a medium rate basically affected by the poor solubility of LOV in digestive environment. Indeed, by replicating the simulated digestion without enzymes, but only changing the pH, we obtained a comparable LOV recovery. Once RYRs were evaluated, different bioaccessibility rates were noticed. We observed that bioaccessibility in three out of four samples with 1.5% and 3% of MKtot content exceeded 80%, whereas only one in the 5% cluster barely reached this result (Table 7). Currently we are not able to explain the reasons behind the bioaccessibility rate of B1.5 lower than expected. To better understand the contribution of RYRs constituents in determining bioaccessibility, we examined the correlation between bioaccessibility rate and the different components content. The Pearson correlation analysis displayed that RYR bioaccessibility was significantly correlated with triterpenes content (one-tailed Pearson test: $r = 0.8013$, $R^2 = 0.6421$, $p = 0.015$) and with (polyphenols+triterpenes)/MKtot ratio ($r = 0.8562$, $R^2 = 0.7331$, $p = 0.070$) (Figure 2), while no significant correlations were observed when comparing bioaccesibility rate with MKtot, Msec, and polyphenols content respectively (MKtot: $r = -0.4909$, $R^2$0.24; Msec: $r = 0.1386$, $R^2 < 0.10$; polyphenols: $r = 0.4196$, $R^2 = 0.54$). These results suggest an important role of the phytocomplex in the solubility and bioaccessibility of RYR. The phytocomplex's role in improving RYR's bioavailability was confirmed by D5, the sample displaying the least rich phytocomplex, which had the lowest recovery after simulated digestion, worsen than only LOV (Table 6). These differences may have relevant relapses in both research and clinical use, as RYRs with different phytocomplexes may determine different pharmacokinetics and thus different effects both in vitro and in vivo. For instance, the differences in MKtot content of samples D3 and D5 may be almost leveled by their completely different bioaccessibility, which is relatable to their overall phytochemical profile.

TABLE 7 | Bioaccessibility rate of monacolin K contained in red yeast rice (RYR) sample with different content of MK or lovastatin (LOV) measured by in vitro model of gastrointestinal simulated digestion followed by HPLC-DAD analysis. Data are expressed as % of recovered MK after test compared to initial concentration.

RYR sample	Bioaccessible MK (% versus initial concentration)
B1.5	73.65 ± 2.25
D1.5	89.62 ± 10.24
A3	84.52 ± 3.20
D3	> 95
A5	68.67 ± 5.51
C5	80.08 ± 4.14
D5	62.43 ± 7.02
LOV	65.23 ± 18.45

3.3 | *In Silico* Prediction of Pharmacokinetics and Intestinal Absorption of Monacolins

The SwissADME platform is a freely web accessible tool which proved to be instrumental for an early assessment of the pharmacokinetic properties of molecules.

The study of MK in active form (MKA) showed a high intestinal absorption (Figure 3). Another important pharmacokinetic parameter is represented by the P-glycoprotein for which MK was found to be a plausible substrate (Figure 3). Concerning the predicted interaction with cytochromes, MK could plausibly be an inhibitor of CYP3A4 as already confirmed by some experimental-based evidence (Chen et al. 2012; Fung et al. 2012) DHMK and DiHMK were predicted to share the most important pharmacokinetic features with MK. As regards Msec, compactin and monacolin X, were completely comparable to MK in terms of pharmacokinetic parameters. Instead, monacolin L differed not being predicted as an inhibitor of CYP3A4.

Overall, in vitro simulated digestion and computational pharmacokinetic predictions suggested that intestinal bioaccessibility is one of the most important feature that determines statins bioavailability and that RYR phytocomplex could act in a synergistic way to improve MK absorption.

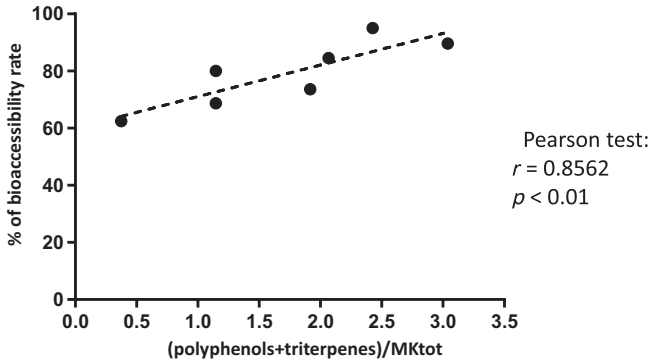
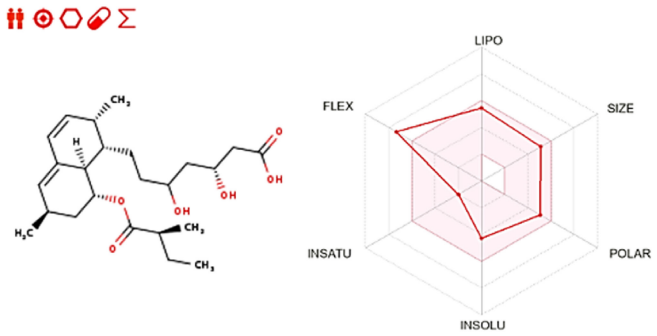


FIGURE 2 | Correlation analysis between (polyphenols+triterpenes)/MKtot ratio and the bioaccessibility rate. Data were analyzed by one-tailed Pearson test: $r = 0.8562$, $R^2 = 0.7331$, $p = 0.0070$.

3.4 | Inhibition of HMG-CoA Reductase Activity

One of the aims of this research was to evaluate and compare the efficacy of LOV and different RYRs in the specific function of inhibiting the HMG-CoA reductase activity. In fact, to the best of our knowledge, this was the first comparison between LOV and an extensive array of RYRs with different and detailed chemical profiles.

A preliminary test showed that the inhibitory activity of RYRs was markedly higher in samples subjected to conversion of MKL to MKA. For this reason, all tested samples were converted in the active form and then tested.



SMILES CC[C@@H](C(=O)O[C@@H]1C[C@@H](C)C=C2[C@H]1[C@@H](CCC(C[C@H](CC(=O)O)O)O)[C@H](C=C2)C)C

Physicochemical Properties	
Formula	C24H38O6
Molecular weight	422.55 g/mol
Num. heavy atoms	30
Num. arom. heavy atoms	0
Fraction Csp3	0.75
Num. rotatable bonds	11
Num. H-bond acceptors	6
Num. H-bond donors	3
Molar Refractivity	117.69
TPSA	104.06 Å²
Lipophilicity	
Log $P_{o/w}$ (iLOGP)	3.26
Log $P_{o/w}$ (XLOGP3)	4.04
Log $P_{o/w}$ (WLOGP)	3.72
Log $P_{o/w}$ (MLOGP)	2.75
Log $P_{o/w}$ (SILICOS-IT)	3.16
Consensus Log $P_{o/w}$	3.39

Water Solubility	
Log *S* (ESOL)	-4.28
Solubility	2.22e-02 mg/ml ; 5.26e-05 mol/l
Class	Moderately soluble
Log *S* (Ali)	-5.93
Solubility	4.98e-04 mg/ml ; 1.18e-06 mol/l
Class	Moderately soluble
Log *S* (SILICOS-IT)	-2.32
Solubility	2.02e+00 mg/ml ; 4.79e-03 mol/l
Class	Soluble
Pharmacokinetics	
GI absorption	High
BBB permeant	No
P-gp substrate	Yes
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	Yes
Log K_p (skin permeation)	-6.01 cm/s
Druglikeness	
Lipinski	Yes; 0 violation
Ghose	Yes
Veber	No; 1 violation: Rotors>10
Egan	Yes
Muegge	Yes
Bioavailability Score	0.56
Medicinal Chemistry	
PAINS	0 alert
Brenk	0 alert
Leadlikeness	No; 3 violations: MW>350, Rotors>7, XLOGP3>3.5
Synthetic accessibility	5.89

FIGURE 3 | *In silico* prediction of the pharmacokinetic properties of monacolin K hydroxy acid by using the SwissADME platform (http://www.swissadme.ch).

As expected, A0 and B0 (MKtot < 0.12% and Msec < 0.62%) were those with the highest IC_{50} value: 3512.22 and 2502.30 ng/mL respectively, confirming that the HMG-CoAR inhibitory activity was strongly related to the MK content (Table 8). Analyzing RYRs clusters with different MK content (Table 8), results suggested a relationship between MK content and enzymatic inhibitory activity despite a large variability between samples within clusters was recorded, especially in the cluster of RYRs with MK 1.5%, for which we measured both the best and the worst activity among all samples. A less pronounced difference in the inhibitory activity was observed within clusters with MK 3% and 5% (Table 8). In the attempt of understanding the role of other constituents of RYR phytocomplex, we also took into account in this work the activity of β-sitosterol as representative triterpene of RYR: the IC_{50} obtained was > 10 μg/mL highlighting a very low inhibition of HMG-CoAR activity.

By comparing IC_{50} of LOVA and RYRs at the relative MK content, we found that MK, in RYR phytocomplex, was always more effective than the pure compound. Even in the worst case (C3), the inhibitory concentration related to its MK content (IC_{50} 47.45 ng/mL) was lower than LOVA (IC_{50} 72.88 ng/mL; Table 8); not considering A0 and B0 because of their large difference in chemical composition respect to other RYRs, in the best case (D1.5) we observed a strong decrease in IC_{50} (more than 10 folds) by comparing the relative MK content in the sample with LOVA (Table 8). Moreover, by observing the mean value of inhibitory activity of different clusters of RYR, according to the relative content of MK, it is possible to gain a more understandable interpretation of the results. Indeed, we found small differences in the mean value of IC_{50} based on MK content of RYR clusters, demonstrating two crucial points: MK plays the major role in inhibiting HMG-CoAR activity in all RYRs, but a synergistic effect of RYR matrix was clearly observed allowing us to suppose that Msec (but not sterols) may act in reducing cholesterol levels.

This behavior must be considered during both the design of clinical trials and overall investigations, leading to suggest that a precise phytochemical profiling must be performed each time a RYR sample is evaluated.

3.5 | In Vitro Cytotoxicity of RYR and Lovastatin

A further aim of this work was to compare the cytotoxicity of RYR to LOV by using cell in vitro models taking into account differences in the phytochemical profile of samples. To estimate the impact of RYRs toward different organs, we used several cell lines miming the route of absorption, distribution, and excretion of RYR and LOV. Specifically, intestinal (Caco2), hepatic (HepG2), kidney (Hek293), and muscle (C2C12) cell lines were used. The cells were treated for 24 h with RYRs and LOV, both in hydroxy acid active form, at the concentrations of 10, 25, 50, 100, and 200 μg/mL.

By means of cell viability assay (CCK-8), the IC_{50} values were calculated indicating the concentration of RYR and LOVA capable of inhibiting cell viability by 50%.

The analysis performed on Caco2, HepG2 and Hek293 showed that RYR and LOVA did have a very low impact on cell viability (Table 9); indeed, the IC_{50} values for most RYRs tested resulted over 200 μg/mL. The sole exception regarded C5 that showed a lowest IC_{50} (120.48 ± 30.12 μg/mL) in Hek293 cells.

Further analyses were carried out on the skeletal muscle cells, recognized as the target of adverse effects of statins. Cell viability test on C2C12 myoblasts confirmed the myotoxicity of LOVA that showed an IC_{50} of 38.21 ± 5.41 μg/mL (Table 9). Conversely, RYRs demonstrated a low impact on muscle cell viability; indeed, most samples did not reach the 50% of mortality at the maximum tested concentrations, and all samples showed IC_{50} higher than 100 μg/mL. We are aware that the differences in muscle cells viability recorded for LOV and RYRs do not allow us to draw any conclusion on RYR safety: we just considered these tests as a confirmation of the specific muscle impact of statins as well as a starting point to choose non-toxic concentrations of LOV and RYRs with the same MK content to be used in molecular investigation.

3.6 | Transcriptional Regulation of Muscle-Related Markers

With the aim to evaluate the RYR biological safety and better understanding the molecular mechanism underlying the statin-induced myotoxicity, we explored the transcriptional effects of selected RYRs in comparison with LOVA in modulating the expression of several target genes primarily involved in muscle function and metabolism. Considering the unavoidable heterogeneity in the phytochemical profile, but also the effects on HMG-CoAR, and the bioaccessibility mainly related to MK content, we decided to test one representative sample per cluster including one MK-free RYR product, for the subsequent molecular analysis. Furthermore, considering the different profile of the D5 sample within the cluster at MKtot 5%, we evaluated its possible different impact at molecular level.

In particular, we conducted a gene expression analysis of MyoD, a key regulator of skeletal myogenesis that directs contractile protein synthesis and muscle metabolism, and of muscle creatine kinase (MCK), known to be an indirect marker of muscle damage. The myoblasts C2C12 were treated for 24 h with LOVA (1 μg/mL) or RYRs at the equivalent MK concentration, corresponding to 1 μg/mL of MK; we also studied the prototype A0 (80 μg/mL), to better define the impact of the sole RYR matrix without MK at very high concentration. The mRNA levels of the selected targets were measured by RT-qPCR.

Statistical analysis showed that LOVA significantly downregulated *MyoD* transcriptional levels compared to untreated cells ($p < 0.01$ vs. CTRL) [one-way ANOVA: $F(6,18) = 5.732$; $p = 0.0018$] (Figure 4A). Differently, all the RYRs tested did not alter the mRNA levels of *MyoD* with the exception of the sample D5, which significantly upregulated the transcription of *MyoD* with respect to control ones ($p < 0.05$ vs. CTRL) (Figure 4A). The emerged effect of RYR with highest MK content in increasing the transcriptional levels of the myogenic factor MyoD is undoubtedly interesting and deserves further investigation.

TABLE 8 | IC_{50} values relative to the inhibition activity of the enzyme HMG-CoA reductase measured through a cell-free in vitro test. Table shows IC_{50} of single samples and mean values of the different RYR clusters (0, 1.5, 3, 5% of MK content) calculated as sample weight and relative to MK content. All data are compared to LOV in the hydroxy acid (LOVA) or lactone (LOVL) forms. Data are expressed as ng/mL ± standard deviation (SD).

RYR Sample	MKtot % *w/w*	IC_{50} ng/mL (sample weight)	IC_{50} ng/mL (equivalent MK)	Mean IC_{50} ng/mL (sample weight)	Mean IC_{50} ng/mL (equivalent MK)
A0	0.05 ± 0.01	3512.22 ± 188.02	1.76	3007.26 ± 714.12	2.26
B0	0.10 ± 0.01	2502.30 ± 189.21	2.50		
A1.5	1.42 ± 0.05	2607.76 ± 426.47	37.03	1376.38 ± 1086.72	22.27
B1.5	1.55 ± 0.04	1962.77 ± 308.78	30.42		
C1.5	1.70 ± 0.07	591.11 ± 100.75	10.05		
D1.5	1.80 ± 0.05	343.86 ± 54.55	6.19		
A3	2.85 ± 0.09	1532.31 ± 237.09	43.67	1009.29 ± 572.19	32.09
B3	3.15 ± 0.09	464.91 ± 78.20	14.64		
C3	3.22 ± 0.07	1473.89 ± 214.74	47.45		
D3	3.50 ± 0.11	566.04 ± 111.21	19.81		
A5	5.03 ± 0.13	682.79 ± 98.20	34.34	561.28 ± 175.01	28.77
B5	5.09 ± 0.12	738.27 ± 102.55	37.58		
C5	5.13 ± 0.10	436.93 ± 70.04	22.41		
D5	5.25 ± 0.16	387.11 ± 50.01	20.32		
LOVL		1248.06 ± 351.25	1248.06		1248.06
LOVA		72.88 ± 28.02	72.88		72.88

Regarding *Mck*, the treatment with LOVA significantly induced gene expression of about 52-fold compared to control cells ($p < 0.0001$ vs. CTRL) [$F(6,17) = 22.34$; $p < 0.0001$]. No effects on *Mck* gene expression were induced by treatment with all the RYRs examined compared to control cells (Figure 4B). The reduction of *MyoD* transcriptional levels and the increase of those of *Mck* suggested a negative impact of treatment with LOVA on muscle cells unlike RYRs.

3.7 | IL-6 Production in Muscle Cells

After analyzing two specific muscle-related markers, our attention shifted on myokine IL-6, a pleiotropic cytokine with different functions aimed to maintain muscle homeostasis. We decided to investigate the transcriptional and post-translation effects of a 24 h treatment with LOVA (1 μg/mL) or RYRs (MK 1 μg/mL) in myoblasts C2C12.

The RT-qPCR analysis highlighted that cell exposure to LOVA was able to upregulate of about 2-fold the transcriptional levels of *Il-6* compared to controls [$F(6,17) = 11.23$; $p < 0.0001$]. Interestingly, *IL-6* mRNA levels were not altered by exposure to any of the RYRs with respect to untreated cells (Figure 5A).

After 24 h of treatment, an ELISA assay was used to measure the levels of IL-6 produced by C2C12 cells. One-way ANOVA revealed that LOVA significantly increased IL-6 levels compared to controls as well as the RYRs A0, A5, and D5 [$F(6,14) = 25,60$; $p < 0.0001$] (Figure 5B). In particular, sample D5 induced a very similar response in IL-6 release in C2C12 cells if compared to LOVA ($p < 0.001$ vs. CTRL), whereas A5 produced a weaker myokine release ($p < 0.05$ vs. CTRL) (Figure 5B). Considering that D5 and A5 differ in Msec, polyphenols and triterpenes (Table 6), we may postulate that the enriched phytocomplex of RYR provided by sample A5 had a protective role in reducing the pro-inflammatory effect elicited by MK (=LOV).

Samples D1.5 and B3 did not stimulate IL-6 release in muscle cells even after a prolonged stimulus, thus suggesting the importance of a balanced polyphenols/MK and triterpenes/MK ratios to reduce the stressor effects of MK (Figure 5B).

Sample A0, devoid of MK, used at very high concentration, exerted a strong release of IL-6 compared to controls ($p < 0.0001$ vs. CTRL) (Figure 5B), thus confirming that the RYR phytocomplex also produced a stress effect if unproperly used.

In general, within the RYRs-treated cells, an increase in IL-6 levels related to the MK content was observed, asserting the importance of a well-balanced phytocomplex. Overall, the dosage of myokine IL-6 clearly demonstrated both the impact of LOV at muscular level even at low, non-cytotoxic concentrations, and the lower impact of a complete RYR phytocomplex even when MK and LOV concentrations were the same.

TABLE 9 | CCK-8 cell viability test on intestinal Caco2, HepG2, Hek293, and C2C12 cells treated for 24 h with red yeast rice samples (RYR) and lovastatin (LOVA) at the concentration of 10, 25, 50, 100, 200 μg/mL. IC_{50} values are expressed in μg/mL ± standard deviation (SD).

RYR sample	Caco2 IC_{50} (μg/mL)	HepG2 IC_{50} (μg/mL)	Hek293 IC_{50} (μg/mL)	C2C12 IC_{50} (μg/mL)
A0	> 200	> 200	> 200	105.27 ± 26.62
B0	> 200	> 200	> 200	> 200
A1.5	> 200	> 200	> 200	> 200
B1.5	> 200	> 200	176.88 ± 39.02	> 200
C1.5	> 200	> 200	> 200	> 200
D1.5	> 200	> 200	> 200	> 200
A3	> 200	> 200	> 200	> 200
B3	> 200	> 200	> 200	146.96 ± 30.02
C3	> 200	> 200	> 200	> 200
D3	> 200	> 200	> 200	> 200
A5	> 200	> 200	> 200	> 200
B5	> 200	> 200	> 200	136.82 ± 19.18
C5	> 200	> 200	120.48 ± 30.12	108.17 ± 22.41
D5	> 200	> 200	> 200	> 200
LOVA	180.72 ± 45.25	> 200	186.04 ± 41.55	38.21 ± 5.41

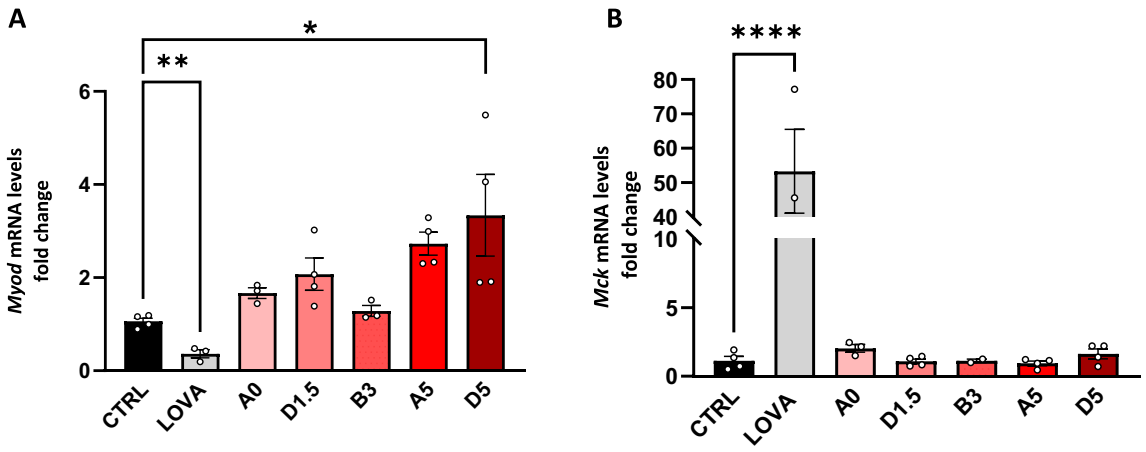


FIGURE 4 | Analysis of *MyoD* (A) and *Mck* (B) mRNA levels measured by RT-qPCR in C2C12 myoblasts treated for 24 h with lovastatin or red yeast rice samples containing MK 1 μg/mL. Each column represents mean ± S.E.M. Data were analyzed by one-way ANOVA followed by post hoc Tukey ($N = 3–4$): *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ versus CTRL.

3.8 | Transcriptional Regulation of Muscle Autophagy and Atrophy Specific Markers

To investigate the possible molecular mechanisms underlying the statin-induced damage on muscle cells and to evaluate the potential effect of RYR, we analyzed the expression levels of selected target genes well-known to be crucial markers of the alteration of muscle proteostasis. In particular, we explored the transcriptional effects induced by LOVA in comparison with RYRs on *Atg5* and *Atg7* genes, encoding essential effector enzymes in autophagy process, and on two main atrophy-related genes, *Fbxo32* and *Trim63*, encoding proteasome subunits (Atrogin-1 and Murf-1).

By means of RT-qPCR, we measured the mRNA levels of the selected genes in myoblasts C2C12 treated for 24 h with LOVA (1 μg/mL) or RYRs samples containing MK 1 μg/mL. No significant differences were found in the gene expression of *Atg5* in C2C12 cells after treatment with both LOVA or RYRs compared to untreated cells [$F(6,17) = 1.081$; $p = 0.412$] (Figure 6A). Differently, LOVA was able to significantly up-regulate *Atg7* mRNA levels of about 2.3-fold with respect to controls [$F(6,17) = 13.43$; $p < 0.0001$] (Figure 6B). Interestingly, no significant changes in *Atg7* transcriptional expression were induced by all RYRs tested on C2C12 cells compared to controls.

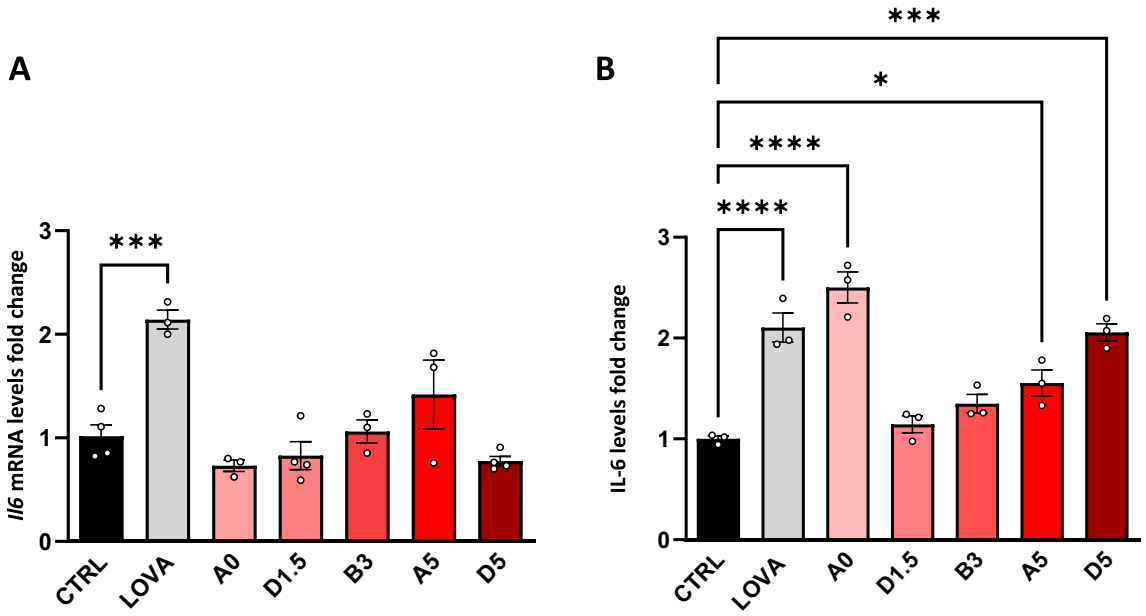


FIGURE 5 | RT-qPCR analysis of *Il6* mRNA levels (A) and IL-6 released protein (B) measured by ELISA assay in C2C12 myoblasts treated for 24 h with LOVA or RYRs at the equivalent MK concentration. Each column represents mean ± S.E.M. Data were analyzed by one-way ANOVA followed by post hoc Tukey ($N = 3–4$): *$p < 0.05$; ***$p < 0.001$, ****$p < 0.0001$ versus CTRL.

As concerns the expression levels of atrophy-related genes, no significant differences were revealed for *Fbxo32* after 24 h of treatment with LOVA or with RYRs samples [$F(6,17) = 0.995$; $p = 0.459$] (Figure 6C). On the contrary, an increase of about 2-fold of *Trim63* mRNA levels was induced by LOVA compared to untreated cells [$F(6,18) = 4.316$; $p = 0.0072$] (Figure 6D), whereas the exposure to all RYRs examined did not alter *Trim63* gene expression compared to control cells. The specificity of this effect is probably attributable to the participation of *Atg7* and *Atg5* in different stages of the autophagic process. Although initial studies showed that statins induced an increase in Atrogin-1 expression (*Fbxo32*) (Hanai et al. 2007; Sorrentino 2019), this data was not confirmed in later clinical studies (Mallinson et al. 2015), but the increase of Murf-1 gene (*Trim63*) was observed in statin-treated patients accordingly with our results (Camerino et al. 2017; Mallinson et al. 2015).

Gene expression data supported the capability of LOVA in inducing muscle cell damage through specific molecular mechanisms, but interestingly RYR appeared to be not involved in the activation of the same pathways, indicating that RYR had a less impactful activity on muscle cells, likely as a consequence of the synergistic effects provided by other constituents.

4 | Discussion

This work was conceived with the aim of providing new preclinical evidence on the differences between RYR as a combination of multiple, synergistic substances including MK, and its homolog LOV in pure form, thus seeking to clarify some major critical points affecting the regulation by the European Commission concerning the safety of use of RYR in food supplements (Reg. (EU) 468/2022) (Younes et al. 2018).

In all the analyses conducted in this work, from chemical characterization to biological investigation, the aim was to compare the phytocomplex of RYR with the pure statin, as a positive control. To emphasize and identify any potential synergistic effects of the entire RYR phytocomplex, the comparison with MK-free samples was essential, as it allowed us to validate our results and ensure scientific rigor.

The first phase of the study provided a comprehensive chemical characterization and quality assessment of raw RYR samples, chosen among those authorized and used as ingredients of food supplements available in the European marketplace. Far from the alarming findings related to the discrepancies in composition and monacolins content found in products sold by web (Righetti et al. 2022), our results showed a good agreement between label declarations and actual MK content, but also revealed large variations in Msec profile and in the relevant presence of triterpenes and polyphenols. These results led us to confirm that a full characterization of the phytocomplex is always advisable, each time that a RYR is evaluated, including a profiling of all monacolins produced by *M. purpureus*. Backing to MK, we hypothesized that the productive and drying process, as well as pH fluctuations, may affect MKA:MKL ratio that resulted very variable in tested samples and not related to other phytocomplex parameters.

In the second step of the study, we investigated differential pharmacokinetic aspects of RYR and LOV confirming a very good bioaccessibility rate of MK in most RYRs, better than that provided by pure LOV. After addressing the simulated digestion with and without digestive enzymes, we observed that the lipophilicity of RYRs enriched in MK and LOV may affect the intestinal bioaccessibility more than enzymatic degradation, in accordance with other evidence (Vitiello et al. 2023) and differently from Kraboun et al. (Kraboun et al. 2018). A positive role of the whole RYR phytocomplex in improving MK bioaccessibility is supported by the linear correlation observed between the bioaccessibility rate and the total amount

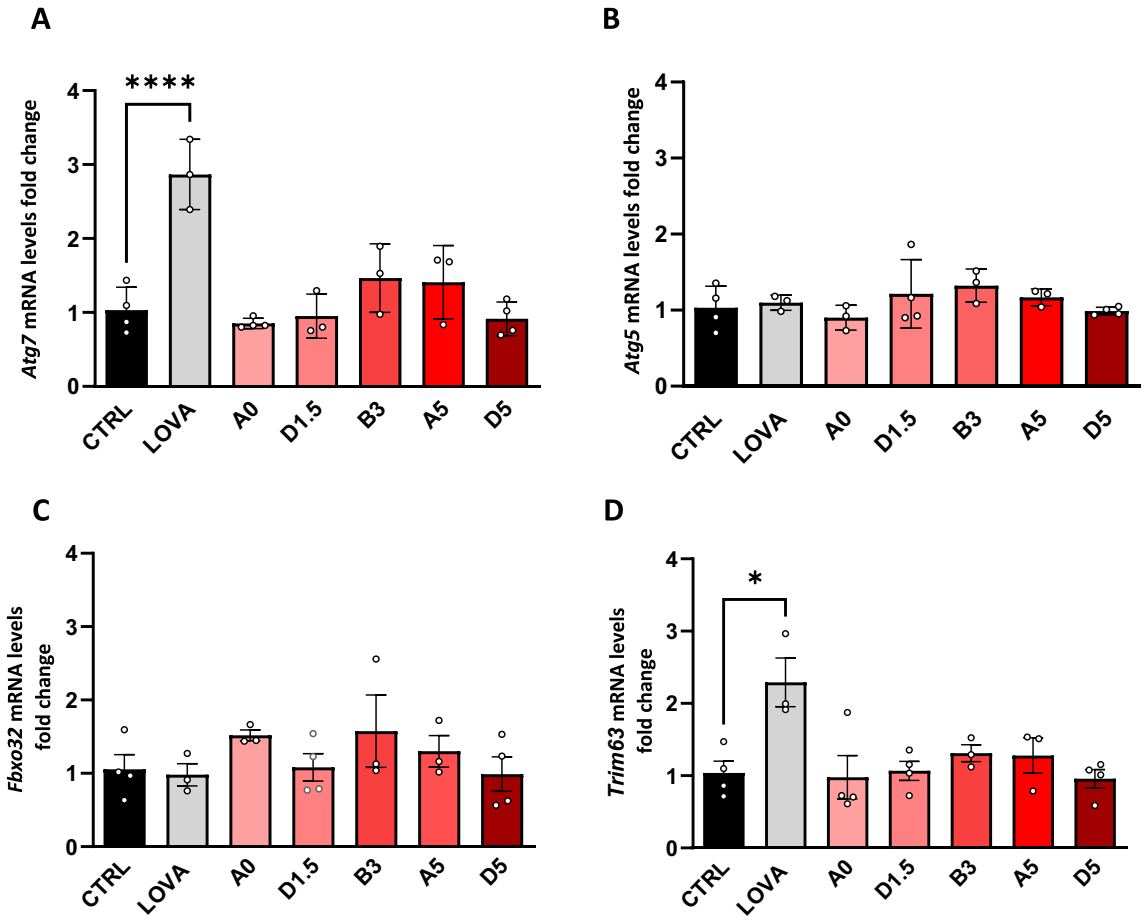


FIGURE 6 | Analysis of *Atg5* (A), *Atg7* (B), *Fbxo32* (C) and *Trim63* (D) mRNA levels measured by RT-qPCR in C2C12 myoblasts treated for 24 h with LOVA (1 μg/mL) or RYRs at the equivalent MK concentration. Each column represents mean ± S.E.M. Data were analyzed by one-way ANOVA followed by post hoc Tukey ($N = 3–4$): *$p < 0.05$, ****$p < 0.0001$ versus. CTRL.

of polyphenols and triterpenes in relation to MK content. In addition, the improved bioavailability of MK in RYR product, with respect to pure LOV, was previously observed and attributed to its higher dissolution rate and reduced crystallinity (Chen et al. 2013).

Despite the large variability observed among tested samples, even within the same cluster, we demonstrated that, all RYR phytocomplexes were more effective in limiting HMG-CoAR activity compared to pure LOV when comparing the MK content. This suggests a synergistic effect of other constituents in the phytocomplex, likely Msec, in line with recent docking studies (Righetti et al. 2021). On the other hand, the enzymatic test confirmed that MK plays the major role in inhibiting HMG-CoAR activity in all RYRs, as evidenced by its significant contribution if analyzing the clusters of RYRs. Although numerous clinical studies indicate that RYR is generally well tolerated, with no evidence of direct toxicity, to date there are still conflicting and unclear insights on the safety of RYR use.

To gather more information about the safety of RYR and considering that few studies reporting the possible mechanism underlying statin-induced myotoxicity, often unclear or conflicting, the last part of the research focused on a cellular and molecular investigation. Cell viability tests confirmed that skeletal muscle cells are mostly affected by LOV exposure, differently from liver, intestinal, and kidney cells, the main tissues involved in the metabolism of this molecule (du Souich, Roederer, and Dufour 2017; Lapi et al. 2008; Masters et al. 1995; Mohaupt et al. 2009; Tretter and Parinandi 2015). The negative impact of LOV on muscle cells, already at very low concentrations, was also supported by the increase of the transcriptional levels of *Mck*, one of the main markers of muscle injury (Brancaccio, Lippi, and Maffulli 2010), and the concurrent downregulation of *MyoD*, a key factor in muscle differentiation and regeneration (Yamamoto et al. 2018). Moreover, LOV exerted a pro-inflammatory effect by upregulating the myokine IL-6 both at the transcriptional and post-transcriptional level. The increase of *Mck* and IL-6 levels are characteristic diagnostic indexes of statin-induced muscle toxicity. It was demonstrated that this pleiotropic cytokine plays a pivotal role in the regulation of homeostatic processes at muscle level and its chronic persistence is associated with several pathophysiological conditions affecting muscle tissue, in particular decrease of muscle mass and muscle protein content, muscle waste, or slow the rate of muscle growth (Belizário et al. 2016; Haddad et al. 2005; Pelosi et al. 2021). Despite several evidence in vitro and in vivo showed the anti-inflammatory effects of statin treatment (Arnaud et al. 2005; Berthold et al. 2012; Sun et al. 2014), none explored the effect at the muscle level. To our knowledge, the sole study available on this topic is in accordance with our

findings, reporting the IL-6 increase production after C2C12 exposure to pravastatin (Cseri, Szentesi, and Csernoch 2021).

In terms of cytotoxic effects and, consequently on RYR safety, the most relevant evidence of our study highlighted the importance of RYR matrix on cell viability despite the same MK concentration used in treatments.

All RYRs demonstrated no effects on gene expression of *Mck* and of *MyoD* compared to LOV, with the exception of D5 sample that generates hypotheses for additional research. Interestingly, it was noted that none of RYRs modulated *IL-6* transcriptional levels and that RYR phytocomplex played a protective role in limiting IL-6 expression at post-translational level. Indeed, samples with a more balanced phytocomplex did not upregulate IL-6, differently the poorest was found very similar to LOV.

Finally, considering statin-induced myotoxicity, our data demonstrated a specific effect of LOV in upregulating only *Atg7* and *Trim63* gene expression after 24 h treatment, partially explaining some clinical findings reported in literature. In fact, although initial studies showed that statins induced an increase in Atrogin-1 (*Fbxo32*) expression (Cao et al. 2009; Hanai et al. 2007; Sorrentino 2019), this data was not confirmed in later clinical studies (Mallinson et al. 2015). Differently, the increase of gene expression of Murf-1 (*Trim63*) was observed in statin-treated patients accordingly with our results (Camerino et al. 2017; Mallinson et al. 2015). RYRs showed no activity in regulating these targets supporting the lower impact on muscle cells even in terms of cell viability observed.

Summarizing, RYR phytocomplex has two contrasting aspects that must be taken into account by researchers, clinicians and regulatory boards such as EFSA: the matrix may provide additional biological effects compared to isolated compounds, but it needs to be adequately characterized in terms of phytochemical profile and of biological activity of different compounds. In particular, our knowledge on synergistic effects provided by other constituents besides MK should be deepened, as it may represent a relevant key to mitigate side effects and provide adequate inhibition of HMG-CoA reductase.

5 | Limitations of the Study

This work aimed to provide a comprehensive, broad-spectrum analysis of RYR's effectiveness and safety compared to pure LOV, employing multiple approaches to ensure consistency and reproducibility of the results. However, we recognize certain limitations of the study. In order to evaluate the biological effects of RYRs on muscle, murine muscle cell line was used. In muscle-related basic research, there are limited in vitro cell models available, with a primary cell line being the only human option. Notably, the C2C12 cell line is the most widely used in vitro model in preclinical research for studying muscle development, regeneration, and pathological dysfunctions concerning skeletal muscle.

Moreover, our molecular analyses aimed to preliminarily assess the safety of RYR by analyzing its effects on the transcriptional level. Although aware of the limits in the evaluation of gene transcription, it represents one of the earliest cellular response to biological functional alterations thus useful to understand potential molecular mechanisms involved in cell process. These preliminary data pay the way for more in-depth research into the mechanisms of action underlying statin-induced muscle damage and RYR phytocomplex that are still not well-known and controversial.

6 | Conclusion

This study provides new findings concerning the in vitro efficacy and safety of RYR through a multidisciplinary approach and a reliable comparison between RYR and LOV. Data emerged from this study allow to ascertain that not all RYR products are identical and the RYR phytocomplex should be considered. Moreover, the right dosage and the intended use of RYR must be considered according to therapeutic indications: in fact, this fermented product could exploit the synergistic effect of the phytocomplex, in order to avoid the side effects referred to pure drugs and to guarantee effectiveness in the context of a non-pharmacological treatment.

Author Contributions

Giovanna Rigillo: conceptualization, data curation, formal analysis, investigation, writing – original draft. **Giulia Baini:** data curation, formal analysis, methodology, validation, writing – review and editing. **Renato Bruni:** visualization, writing – review and editing. **Giulia Puja:** resources, visualization, writing – review and editing. **Elisabetta Miraldi:** visualization. **Luca Pani:** resources, visualization, writing – review and editing. **Fabio Tascedda:** resources, visualization, writing – review and editing. **Marco Biagi:** conceptualization, data curation, project administration, supervision, writing – original draft.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.