Evaluation of the hepatoprotective effect of combination between fermented camel milk and *Rosmarinus officinalis* leaves extract against CCl₄ induced liver toxicity in mice

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Revised: 10 November 2018 / Accepted: 5 December 2018 / Published online: 5 January 2019
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Abstract The present study was conducted to evaluate the in vitro antioxidant activity of fermented camel milk with *Lactococcus lactis* subsp. *cremoris* (FCM-LLC) alone, *Rosmarinus officinalis* extract (R) alone and their combination and to investigate their hepatoprotective effects against CCl₄ liver damage in mice. The antioxidant activity in vitro of FCM-LLC supplemented with R exhibited the highest free radical scavenging and ferric reducing power activities. The results showed that the pretreatment with a combination of FCM-LLC and R significantly alleviated the increased levels of hepatic markers and the elevated lipid levels induced by CCl₄ in mice. Meanwhile, the enzymatic antioxidants activities (superoxide dismutase, glutathione peroxidase, and catalase) and GSH level in liver significantly were increased while the malondialdehyde level was significantly improved by pretreatment with FCM-LLC plus R. These data suggest that FCM-LLC in combination with *R. officinalis* extract possesses better antioxidant and hepatoprotective activity than FCM-LLC alone.

Keywords Carbon tetrachloride · Fermented camel milk · Hepatoprotection · *Lactococcus lactis* subsp. *cremoris* · *Rosmarinus officinalis*

Introduction

The liver is a crucial organ play an important role in the detoxification of diverse xenobiotics. Exposure of general population to environmental pollutants reduces the protective mechanisms of the liver and causes hepatic failure. Carbon tetrachloride (CCl₄), an environmental contaminant, acts as alterations in biochemical, hematologic and oxidative stress parameters. It has the greatest concern as a toxic agent to the liver tissue attributed to the trichloromethyl radical created through oxidative stress (ATSDR 1994). Liver injury is related to cellular necrosis, abnormal cell function, and reduction in glutathione level (GSH). Serum hepatic markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) are elevated reflecting hepatic structural alteration.

There has been an explosive tendency to use of antioxidant dietary supplements. Epidemiologic data has recommended that some vitamins, minerals, and other food constituents could protect the body against certain disease including cancer, obesity and the aging process (Marcason 2007). Several activities of the antioxidants have involved to inhibition of the cellular levels of reactive oxygen species, which are generated during the oxidative burst. Consequently, the utility of antioxidants in defending cellular components against oxidative stress is well established (Abdel-Wahhab et al. 2005).

Fermented milk has attracted the attention of several research studies and consumers. Furthermore, it was possible that consuming fermented milk products provides various health benefits such as its antipathogenic and antiinflammatory proprieties, which assists the defense systems against oxidative stress and treating liver diseases (Gigante et al. 2007).

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For human consumption, Lactic acid bacteria are widely used for the dairy products. Some strains of lactobacilli have been shown to modulate the risk of oxidative damage accumulated during ingestion (Abdel-Wahhab et al. 2005). *Lactococcus lactis* is one of the most frequent bacteria that is used in probiotic preparations of fermented dairy products (Ribereau-Gayon and Stonestreet 1968). The oral administration of lactic acid bacteria has also been revealed to attenuation liver damage induced by CCl₄ of mice (Güven et al. 2003), indicating that *L. lactis* can interfere with the prevention of liver damage. In addition, we have recently demonstrated that fermented camel milk by *Lactococcus lactis* subsp. *cremoris* had a potent cardioprotective and nephroprotective effects against CCl₄ (Hamed et al. 2017a, b, 2018a, b).

Moreover, there has been a growing interest in the antioxidant potential of aromatic herbals to prevent or treat hepatotoxicity. The antioxidant power of medicinal plants is principally attributed to the phytochemicals, mainly flavonoids, contained in their essential oils. Rosemary (*Rosmarinus officinalis* L.) is one of the common species of the family Lamiaceae, which grow in many parts of the world. Nevertheless, Rosemary and its constituents have a remedial potential in treating or preventing many physiological, biochemical and histopathological alterations with certain hepatotoxin including CCl₄ (Abu Taher Sagor et al. 2016). But until now, not enough studies were done previously on the hepatoprotective activities of fermented camel milk and rosemary extract against CCl₄ toxicity.

The current work was designed to explore the ameliorative effect of rosemary and fermented camel milk on CCl₄ induced toxicity on the liver of mice.

Methods and materials

Fermented milk preparation

Samples of fresh camel milk were collected from a local farm in November 2015 (Sfax, Tunisia). Samples were cooled immediately at 4 °C and then blended to obtain a homogenous sample followed by the pasteurization process. Fresh milk heated at 80 °C for 20 min followed by cooling at 43 °C as described by Moslehishad et al. (2013). Batch culture experiments were performed in the pasteurized fresh milk with *L. lactis* subsp. *cremoris* were carried out without mechanical agitation. Mixed cultures were inoculated with an equal volume of *L. lactis* subsp. *cremoris* 2% (v/v). The flasks were then incubated at 37 °C in an incubator set. After 24 h of fermentation, the samples were stored at 4 °C until used for the assays.

Rosmarinus officinalis extracts preparation

Fresh leaves of *R. officinalis* were collected from Oueslatia (Kairouan, Tunisia, latitude 35.84 and longitude 9.56). 10 g of *R. officinalis* were dissolved in water and the volume was made up to 100 ml. The air-dried leaf powder was extracted by maceration in shaking water at room temperature during 48 h. The filtrate was kept at 4 °C in the dark until a further analysis.

Animals

The assays of the present study were conducted on female Wistar mice (body weight of 29 ± 3 g) were purchased from the Central Pharmacy of Tunis. All mice were handled under standard laboratory conditions of a 12 h light–dark cycle and temperature around 22 °C. Animals were feed with a basic food (SNA–Sfax) and drinking water. The experimental protocol was approved by the Ethical Committee of the Faculty of Sciences of Sfax. All the experimental procedures were carried out in accordance with international guidelines for Care and use of living animals in scientific investigations (Council of European Communities 1986).

Reagents

All the reagents used were of analytical grade. Carbon tetrachloride was purchased from SD Fine Chemicals, Bhoisar, Mumbai, India. All chemicals used in this study were obtained from Sigma Chemical Co., (St. Louis, MO, USA).

In vitro study

Antioxidant activities

Total antioxidant activity Total antioxidant activity was determined by the method of Prieto et al. (1999). The ascorbic acid was used as a reference standard and the antioxidant activity of the samples was expressed as milligrams of ascorbic acid equivalents per mg dry weight.

DPPH radical scavenging activity The antioxidant activity of the samples determined in terms of hydrogen-donating or radical-scavenging ability, using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to the method of Son and Lewis (2002). The inhibition free radical DPPH was calculated in percent (I%) as follows: $I\% = 100 \times (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}$ where A control is the absorbance of the control reaction (containing all reagents except the test compound), A sample is the absorbance of the test compound. All

determinations were done in triplicate from 3 independent experiments.

Ferric reducing antioxidant power (FRAP) assay The FRAP procedure was modified from Benzie and Strain (1996). The reagent contained 0.83 mmol (TPTZ, 2,4,6-tripyridyl-*s*-triazine) and 1.67 mmol ferric chloride in 0.1 mol acetate buffer (pH 3.6). A proper amount of sample was mixed with 0.9 ml of reagent and incubated at 25 °C for 10 min. The reduction of Fe^{3+} -TPTZ to Fe^{2+} -TPTZ was determined at 595 nm. Trolox was used as standard, and triplicates were analyzed for each sample.

Experimental design

Fifty-six mice were randomly assigned to eight group (7 mice each): (C): control mice received distilled water and standard diet; group CCl_4 : (CCl_4 hepatotoxicity pathological model) was given a single dose of CCl_4 (10 ml/kg in 0.3% olive oil by intraperitoneal injection(i.p), (Huang et al. 2012) at day 14; Group FCM-LLC: daily administered by oral gavage of fermented camel milk by *L. lactis* subsp. *cremoris* (100 mg/kg/bw) during 15 days; group R: daily administered of *R. officinalis* (100 mg/kg/bw) during 15 days; group FCM-LLC + R: daily administered by oral gavage 0.4 ml (V/V) of *R. officinalis* and FCM-LLC; group FCM-LLC + CCl_4 : pre-treated with fermented camel milk and intoxicated with CCl_4 on the 14th day; group R + CCl_4 : pre-treated with *R. officinalis* and intoxicated with CCl_4 on the 14th day and group FCM-LLC + R + CCl_4 : pre-treated with the FCM-LLC and rosemary and intoxicated with CCl_4 on the 14th day. These doses were selected according to the dose effect and verified before adjustment of the experiment. During the experimental period, all animals were weighed and then sacrificed.

At the end of the experiment period, mice were sacrificed by decapitation after anesthesia by intra-abdominal injection with chloral hydrate. The plasma was obtained by centrifugation ($2500 \times g$, 15 min, 4 °C) and stored at -20 °C for biochemical assays. Livers tissues were removed; weighted and rinsed with a physiological saline solution. They were homogenized in (1:2, w/v) in 50 mmol/l phosphate buffer (pH 7.4). After centrifugation at 4 °C and $5000g$ for 25 min, the resulting supernatants were collected and kept at -20 °C until analyses. Some samples were fixed in 10% buffered formalin solution and embedded in paraffin for histological examination.

For the biochemical and the histological experiments, samples (liver tissue, plasma) were taken from seven mice in each group. All samples were analyzed in triplicate.

Biochemical assay

Liver damage biomarkers Plasmatic activities of transaminases, i.e. alanine aminotransferase (ALT) and aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and γ -Glutamyl transpeptidase (γ -GT) were measured by autoanalyzer (Erba xl 200, Mannheim, Germany) using Erba diagnostic kit.

Analysis of plasma lipids Plasma lipid parameters such as total cholesterol (T-Ch), triacylglycerol (TG), LDL-cholesterol (LDL-Ch) and high-density lipoprotein-cholesterol (HDL-C) levels were determined by were measured by autoanalyzer (Erba xl 200, Mannheim, Germany) using Erba diagnostic kit.

Protein quantification Protein contents were measured according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Lipid peroxidation The level of lipid peroxidation in heart was estimated by measuring the formation of thiobarbituric acid reactive substances (TBARS), according to the method of Yagi (1976). In brief, 0.5 ml of homogenate was treated with 2 ml (1:1:1 ratio) TBA–TCA–HCl reagent (thiobarbituric acid, 0.37%, 0.25 N HCl, 15% TCA) placed for 15 min in a water bath and cooled. The absorbance of the clear supernatant was measured against reference blank at 535 nm.

Antioxidant enzyme activities Superoxide dismutase (SOD) activity was estimated according to Beyer & Fridovich (1987). The developed blue color reaction was measured at 560 nm. Units of SOD activity were expressed in the amount of enzyme required to inhibit the reduction of NBT (4-nitro blue tetrazolium chloride) by 50% and the activity was expressed in U/mg protein.

Catalase (CAT) activity was assayed by H_2O_2 consumption, following Aebi (1984) method. An enzyme unit was defined as the amount of enzyme that catalyzes the release of one nmoles of H_2O_2 per min. Specific activity was calculated in terms of units per mg of protein. The assay was performed at 25 °C.

Glutathione peroxidase (GPx) activity was measured according to Flohe and Gunzler (1984) using hydrogen peroxide as a substrate and the reduced glutathione. The GPx activity was expressed as nmoles of GSH per mg of protein.

GSH content GSH in plasma was determined by the method of Ellman (1959) based on the development of a yellow color when 5,5-dithiobis-2 nitro benzoic acid(DTNB) was added to compounds containing

sulfhydryl groups. The absorbance was measured at 412 nm after 10 min. Glutathione content was expressed in $\mu\text{g}/\text{mg}$ protein.

Liver histology

Specimens from liver tissues were taken from all mice groups after scarification. The sections of the liver collected and fixed in Bouin solution and embedded in paraffin, cut at 2–5 μm slices and stained with hematoxylin and eosin for histological examination. Eight slices were prepared from each liver.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test as a post hoc test for comparison between groups [treated groups (CCl_4 vs (C)) and [(FCM-LLC + CCl_4 , R + CCl_4 , FCM-LLC + R + CCl_4) vs (CCl_4)]. All values were expressed in means $\pm$ SE. Differences were considered significant if $P < 0.05$.

Results

Antioxidant activities of fermented camel milk by *Lactococcus lactis* subsp. *cremoris* or Rosemary

Table 1 summarizes that the fermented milk by *L. lactis* subsp. *cremoris* and rosemary extract had a wide DPPH radical-scavenging activity ($\text{IC}_{50} = 0.83 \text{ mg}/\text{ml}$; $\text{IC}_{50} = 0.99 \text{ mg}/\text{ml}$, respectively).

In the present work, DPPH radical scavenging of FCM-LLC supplemented with different concentration of rosemary (10, 20, 40 and 50%) increased linearly with increasing the ratio of added Rosemary extract. This power was significantly increased with increasing the added ratio to 50% of *Rosemary* extracts to FCM-LLC. Thus, FCM-LLC + R (50%) exhibited strong antioxidant activity proved by the low IC_{50} values ($\text{IC}_{50} = 0.52 \text{ mg}/\text{ml}$).

A high ferrous ion chelating activity of FCM-LLC supplemented with rosemary as compared to FCM-LLC or rosemary was observed. Furthermore, the *Rosemary* extract was added to fermented milk to 50%, their metal chelating capacity significantly higher than FCM-LLC supplemented with different concentration of rosemary (10, 20, and 40%). FCM-LLC + R (50%) reveal a significant increase in their total antioxidant capacity (1.99 mg AAE/ml) as compared to FCM-LLC alone or R alone (Table 1).

Effects of fermented camel milk by *L. lactis* subsp. *cremoris* or/and Rosemary on livers biochemical parameters

Table 2 shows that CCl_4 had significantly raised serum AST, ALT, ALP, LDH and γ -GT level in mice liver compared to control (+ 75%; 2.12 fold; + 34%; + 76%; 1.09 fold, $P < 0.001$, respectively) (Table 2).

Treatment with FCM-LLC or *Rosemary* or combination of both had no effect per se on the hepatic biomarkers in mice.

After CCl_4 administration, FCM-LLC and *Rosemary* together was capable to retain these parameters closest to the normal level and has better performance than treatment with FCM-LLC or *rosemary*.

The results depicted by Table 3 illustrated that, in CCl_4 intoxicated mice, the serum lipid profile viz., TG, T-Ch and LDL was increased (+ 62%, + 48% + 76%, respectively) conversely, HDL-Ch level was significantly reduced (− 49%, $P < 0.001$) in comparison to the normal control.

Treatment with FCM-LLC or *rosemary* or combination of both did not cause any significant change in lipid profile in the liver.

After CCl_4 administration, all group showed a slight decrease of TG, TC and LDL-Ch levels ($P < 0.001$). However, these were more ameliorated in FCM-LLC and *rosemary* treated mice (1.13 fold; 85%, and 29.26%, respectively). Conversely, HDL-Ch level showed a significant decrease (− 91.9%, $P < 0.001$) compared to the CCl_4 group.

Estimation of lipid peroxidation in liver

TBARS level was revealed an increase in CCl_4 treated animals compared to control untreated mice (+ 1.51 fold, $P < 0.001$) (Table 3). Here again, the treatment of FCM-LLC, *Rosemary*, and their combination had no effect per se on the lipid peroxidation level in its offspring (data not shown). In the group that received FCM-LLC in combination with *rosemary* plus CCl_4 , the TBARS level was significantly decreased compared with other groups (Table 3).

Antioxidant enzyme and non enzyme activities

In the liver homogenates of CCl_4 -treated mice, antioxidant enzymes activities decreased significantly when compared to controls. In fact, CCl_4 administration to mice caused a strong decrease in hepatic SOD activity (− 30%) (Table 3). The pretreatment with FCM-LLC or *Rosemary* were lightly ameliorated the SOD activity compared to CCl_4 -treated group ($P < 0.05$). The combined treatment

Table 1 Antioxidant activities in the fermented camel milk with *Lactococcus lactis* subsp. *cremoris* or/and *Rosmarinus officinalis*

	DPPH ^a	FRAP ^b	AAT ^c
FCM-LLC	0.83 ± 0.02	4.76 ± 0.02	1.37 ± 0.05
R	0.99 ± 0.02	3.01 ± 0.04	0.39 ± 0.01
FCM-LLC + R (10%)	0.85 ± 0.04	4 ± 0.03	1.22 ± 0.02
FCM-LLC + R (20%)	0.82 ± 0.05	4.2 ± 0.04	1.56 ± 0.03*
FCM-LLC + R (40%)	0.81 ± 0.03*	5.1 ± 0.01*	1.74 ± 0.01*
FCM-LLC + R (50%)	0.52 ± 0.05**	5.56 ± 0.02**	1.99 ± 0.01**

Values represent the means ± SE of 3 replicates

^aDPPH scavenging activity (mg/mL)

^bFRAP (μg/mL)

^cTotal antioxidant capacity (mg AAE/g DW)

Values represent the means ± SE of 3 replicates: * $P \leq 0.05$; ** $P \leq 0.01$, compared to FCM-LLC alone

prevented the reduction of SOD activity since the mice were pretreated with FCM and rosemary extract.

Similarly, catalase activity decreased significantly in the liver of mice (− 83.72%, $P < 0.001$) upon CCl₄ exposure (Table 3). The pretreatment with FCM-LLC or *Rosemary* alleviated the CAT activity compared to CCl₄-treated group ($P < 0.05$). FCM-LLC with *Rosemary* kept hepatic catalase activity close to control value.

A huge decrease in GPx activity was found in the liver of the CCl₄ group (− 57.5%, $P < 0.01$). The pretreatment with FCM-LLC alone or *Rosemary* alone slightly ameliorated the GPx activity compared to CCl₄-treated group ($P < 0.05$). Here again, the addition of FCM-LLC combined with *Rosemary* of mice restored GPx activity, without reaching normal values (Table 3).

Intoxication by CCl₄ caused a significant decrease in GSH content compared with the negative control group (− 79%, $P < 0.001$). GSH level was less ameliorated with the pretreatment by FCM-LLC alone or *Rosemary* alone. Animals treated with CCl₄ along with probiotic FCM-LLC and rosemary respectively revealed a considerable increase (3 fold, $P < 0.001$) in GSH compared with the CCl₄ group (Table 3).

Effects of fermented camel milk by *Lactococcus lactis* subsp. *cremoris* or/and rosemary on hepatic tissues

The biochemical modifications mentioned above were connected with our histological studies (Fig. 1). In fact, the control group showed normal hepatic cells [Fig. 1(A, A')]. The administration of CCl₄ caused severe hepatocytes necrosis, inflammation and hepatocyte ballooning [Fig. 1(B, B')]. Pretreatment with FCM-LLC, *Rosemary* and their combination revealed entirely normal histological features (data not show).

Fermented camel milk treated group's illustrated mild inflammatory changes, with less severity than changes observed after CCl₄ administration [Fig. 1(C, C')]. However, that pretreatment with rosemary alone showed no significant histological changes compared to CCl₄ group [Fig. 1(D, D')]. Nevertheless, pretreatment with FCM-LLC and *Rosemary* markedly prevented liver inflammation and necrosis induced by CCl₄ [Fig. 1(E, E')].

Discussion

CCl₄ is a hepatotoxin that has been used to induce acute damage of liver and its hepatotoxicity is a due to the trichloromethyl (CCl₃) production during oxidative stress. Administration of CCl₄ in mice induces lipid peroxidation, protein alteration, and oxidative DNA damage (Soni et al. 2008). In the present study, the antioxidant activity of FCM-LLC and *Rosemary* was measured by assessing free radical scavenging and the antioxidant capacity assays, as well as hepato-preventive effect were evaluated in vivo using CCl₄-induced mice. Our results showed that FCM-LLC Combined with *R. Officinalis* extract displayed a noticeable radical scavenging and antioxidant activity in a dose-dependent manner. In the same context, Gad and Abd El-salam (2010) found that DPPH scavenging capacity and ferric reducing antioxidant power increased with a rise in the ratio of rosemary extracts added to fermented milk. The antioxidant property of the rosemary extract was associated with its total phenolic, polyphenol and tannins content which act synergistically to reduce free radical and ROS formation. Similarly, milk was also able to act as free radical scavengers. It has been found that milk contains the peptides deriving from milk proteins (Nishino et al. 2000). Our in vitro antioxidant study suggests that FCM-LLC plus

Table 2 Effect of fermented camel milk with *Lactococcus lactis* subsp. *cremoris*, *Rosmarinus officinalis* or their combination on serum biochemistry changes of control and mice treated with CCl₄ after 15 days of treatment

Groups(n = 8)	C	FCM-LLC	R	FCM-LLC + R	CCl ₄	CCl ₄ + R	CCl ₄ + FCM-LLC	CCl ₄ + FCM-LLC + R
¹ AST (U/L)	120 ± 12.5	119 ± 26	121 ± 16.1	132 ± 11	211 ± 13.8***	170 ± 20 [#]	130.64 ± 21 [#]	123.13 ± 13.5 ^{##}
² ALT (U/L)	6.41 ± 1.7	6.54 ± 2.3	6.09 ± 2	6.74 ± 1.1	20 ± 3.3***	14.93 ± 8.9 [#]	12.61 ± 2.1 [#]	9.03 ± 2.7 ^{##}
³ ALP (U/L)	79.00 ± 8.2	78.00 ± 4.6	67 ± 9.7	62 ± 4.2	106 ± 11*	90.00 ± 6.5 ^{##}	87 ± 7.3	81 ± 4.0 ^{##}
⁴ LDH (U/L)	2283 ± 112	2400 ± 75	2293 ± 182	2230 ± 118	4000 ± 108***	2969 ± 98 ^{##}	2566 ± 99 ^{##}	2290 ± 111 ^{##}
⁵ γ-GT (U/L)	1.10 ± 0.70	0.99 ± 0.3	1.12 ± 0.1	1.30 ± 0.5	2.30 ± 0.10***	1.80 ± 0.30 [#]	1.70 ± 0.20 ^{##}	1.28 ± 0.30 ^{##}
⁶ TG (mg/dL)	2.05 ± 0.18	2.88 ± 0.05	2.18 ± 0.17	2.17 ± 0.01	3.33 ± 0.23***	2.59 ± 0.08 [#]	2.9 ± 0.19 [#]	2.09 ± 0.13 [#]
⁷ T-Ch (mg/dL)	2.50 ± 0.17	2.77 ± 0.32	2.59 ± 0.12	2.33 ± 0.16	3.70 ± 0.35***	2.77 ± 0.20	2.81 ± 0.15	2.60 ± 0.35
⁸ HDL (mg/dL)	1.94 ± 0.15	1.99 ± 0.28	1.96 ± 0.02	2.00 ± 0.02	0.98 ± 0.09***	1.73 ± 0.22 ^{##}	1.55 ± 0.50 [#]	1.88 ± 0.33 [#]
⁹ LDL (mg/dL)	0.6 ± 0.03	0.69 ± 0.08	0.6 ± 0.07	0.57 ± 0.01	1.06 ± 0.10*	1.08 ± 0.15 [#]	0.94 ± 0.31 [#]	0.82 ± 0.01 ^{##}

C, control; (FCM-LLC), fermented camel milk with *Lactococcus lactis* subsp. *cremoris*; (R); *Rosmarinus officinalis*, (FMC-LLC + R), fermented camel milk with *Lactococcus lactis* subsp. *cremoris* supplemented with *Rosmarinus officinalis*; (CCl₄), carbon tetrachloride; (CCl₄ + R), mice pre-treated with *Rosmarinus officinalis* and intoxicated with CCl₄ at 14 day; (CCl₄ + FCM-LLC), mice pre-treated with fermented camel milk and intoxicated with CCl₄ at 14 day; (FCM-LLC + R + CCl₄); mice pre-treated with the FCM-LLC and rosemary and intoxicated with CCl₄ at 14 day

¹AST, Aspartate transaminase; ²ALT, Alanine transaminase; ³ALP, Alkaline phosphatases; ⁴LDH, lactate dehydrogenase; ⁵γ-GT, γ-Glutamyl transpeptidase; ⁶TG, Triglycerides; ⁷T-Ch, Total cholesterol; ⁸HDL-Ch, High density lipoproteins of cholesterol; ⁹LDL-Ch, Low density lipoproteins of cholesterol

Values are expressed as means ± SE of seven samples per group. One-way ANOVA followed by Fisher's protected least significant difference (PLSD) as a post hoc test for comparison between groups

Comparison between (CCl₄) versus control (C) group: * $P < .05$; ** $P < .01$; *** $P < .001$

Comparison between [FCM-LLC + CCl₄, R + CCl₄, FCM-LLC + R + CCl₄] groups versus (CCl₄) group: # $P < .05$; ## $P < .01$; ### $P < .001$

Table 3 Effect of fermented camel milk by *Lactococcus lactis* subsp. *cremoris* or/and rosemary on oxidative status and antioxidant system activity in mice liver

Parameters	Groups (n = 8)							
	C	FCM-LLC	R	FCM-LLC + R	CCl ₄	CCl ₄ + R	CCl ₄ + FCM-LLC	CCl ₄ + FCM-LLC + R
¹ TBARS	1.38 ± 0.09	1.36 ± 0.02	1.49 ± 0.03	1.47 ± 0.05	3.47 ± 0.50***	2.41 ± 0.01 [#]	1.72 ± 0.5 ^{##}	1.40 ± 0.08 ^{###}
² SOD	137.2 ± 11	136.4 ± 23**	146.12 ± 18	148.5 ± 11	94.95 ± 10**	104.42 ± 17	122.33 ± 54 [#]	135.25 ± 10 ^{###}
³ CAT	0.86 ± 0.03	0.99 ± 0.08	0.96 ± 0.16	1.04 ± 0.11	0.14 ± 0.09***	0.45 ± 0.07 ^{##}	0.66 ± 0.01 [#]	0.84 ± 0.02 ^{###}
⁴ GPx	4.52 ± 0.10	4.6 ± 0.50	4.06 ± 0.70	4.31 ± 0.40	1.92 ± 0.20**	2.70 ± 0.50	2.99 ± 0.30 [#]	4.02 ± 0.45 ^{###}
⁵ GSH	0.77 ± 0.002	0.72 ± 0.04	0.55 ± 0.014	0.63 ± 0.12	0.16 ± 0.03***	0.35 ± 0.01 [#]	0.42 ± 0.03 [#]	0.69 ± 0.02 ^{###}

C, control; (FCM-LLC), fermented camel milk with *Lactococcus lactis* subsp. *cremoris*; (R); *Rosmarinus officinalis*, (FCM-LLC + R), fermented camel milk with *Lactococcus lactis* subsp. *cremoris* supplemented with *Rosmarinus officinalis*; CCl₄, carbon tetrachloride; (CCl₄ + R), mice pre-treated with *Rosmarinus officinalis* and intoxicated with CCl₄ at 14 day; (CCl₄ + FCM-LLC), mice pre-treated with fermented camel milk and intoxicated with CCl₄ at 14 day; (FCM-LLC + R + CCl₄); mice pre-treated with the FCM-LLC and rosemary and intoxicated with CCl₄ at 14 day

¹TBARS, thiobarbituric acid reactive substances (nmol/mg protein); ²SOD, superoxide dismutase (U SOD/mg protein); ³CAT, catalase (nmol/mgprotein); ⁴GPx, glutathione peroxidase (nmol/mg protein); ⁵GSH, glutathione (μg/mg protein)

Values are expressed as means ± SE of seven samples per group. One-way ANOVA followed by Fisher's protected least significant difference (PLSD) as a post hoc test for comparison between groups

Comparison between (CCl₄) versus control (C) group: * $P < .05$; ** $P < .01$; *** $P < .001$

Comparison between [FCM-LLC + CCl₄, R + CCl₄, FCM-LLC + R + CCl₄] groups vs(CCl₄) group: # $P < .05$; ## $P < .01$; ### $P < .001$

R may exert a preventive effect against oxidative stress damage.

Lipid peroxidation has been generally used as a marker of oxidative injury in vivo associated with CCl₄ toxicity, which involves a free radical attack on polyunsaturated fatty acids. Free radical cause oxidative damage to cells leading to loss of membrane fluidity and elasticity. MDA was performed using method for monitoring lipid peroxidation, it's well known for the major reactive aldehyde resulting from lipid peroxidation (Janero 1990).

In the present study, the high level of MDA in the liver in CCl₄-treated mice suggests increased lipid peroxidation during tissue damage and imbalance between antioxidant defense mechanism and free radical production. This is consistent with previous studies on CCl₄ caused oxidative stress in liver tissue (Wunjuntuk et al. 2016). Interestingly, preventive treatment with FCM-LLC and R was markedly reducing the rise of TBARS compared to treatment with FCM-LLC alone and R alone. The decreased levels of lipid peroxidation prove that FCM-LLC in combination prevents oxidative injury of the hepatic membrane principally through radical scavenging activity and restore normal membrane integrity and function. Antioxidants defense systems are essential to prevent oxidative stress by limiting the development of reactive oxygen species (ROS). SOD, CAT, and GPx constitute a team, which provides a defense system against ROS (Venukumar and Latha 2002).

Our data showed a significant decrease in antioxidant enzymes activities following acute exposure to CCl₄. The perturbations of these enzymes can be explained by the high production of H₂O₂ and O₂ resulted by CCl₄ toxicity. Our results corroborated other previous studies which have demonstrated that the deficiency of the enzymatic antioxidant defense system is one of the causes of hepatotoxicity induced by CCl₄ (Abd El-Ghany et al. 2012).

The antioxidant activity of *Rosemary* extract might be attributed to its phytochemical constituents such as polyphenol, flavonoid and tannins (Abu Taher Sagor et al. 2016, Hamed et al. 2018a, b).

In addition, the antioxidant activity of FCM-LLC may be related to milk proteins, vitamins and beta-carotene. Several studies have reported that fermentation induces an increased level of beta-carotene (Panda and Ray 2007).

Our observations revealed that FCM-LLC and *Rosemary* supplementation ameliorated the impaired antioxidative defense system in mice livers, as indicated by the restoration of all antioxidants enzymes activities. Although our preliminary in vivo data confirmed the hepato-protective effect owing to the combined pretreatment by depletion of free-radical generation induced by CCl₄.

Hepatic GSH contributes to the detoxification of CCl₄, but involved in the regulation of intracellular redox homeostasis and remove free radicals and products of lipid

Fig. 1 Histological sections of liver in control and CCl₄ mice treated with rosemary extract or/ and *Lactococcus lactis* subsp. *cremoris* after 15 days. Sections were stained with hematoxylin–eosin (original magnification $\times 100$, $\times 400$). **A1, A2** Control mice; **B1, B2** CCl₄ mice; **C1, C2** CCl₄ mice treated with *Lactococcus lactis* subsp. *cremoris*; **D1, D2** CCl₄ mice treated with Rosemary, **E, E'** CCl₄ mice treated with Rosemary and *Lactococcus lactis* subsp. *cremoris*. Arrows indicate: $\rightarrow$ parenchyma dilatation, $\rightarrow$ cell necrosis, $\Rightarrow$ ballooning degeneration

peroxidation protecting in this way against the development of oxidative stress (Couto et al. 2016). Our results showed that administration of CCl_4 induced a significant decrease of GSH level in comparison with normal control. The degradation of GSH may be explained by the break of GSH cycle production by free radicals. On this basis, our study showed that FCM-LLC plus R reduces the cellular oxidative damage induced by CCl_4 by regulating the level of GSH. Moreover, it has been found that fermented milk increases GSH synthesis (Kumar et al. 2012). Thus, the combination fermented camel milk and *Rosemary* prevented oxidative damage linked to its capability to the free radical scavenging.

Liver transaminases such as AST, ALT, and ALP are the sensitive markers of the hepatocyte damage. The administration of a single dose of a CCl_4 led to liver injury followed by an increase of AST, ALT and ALP levels indicating the damage of the hepatic cells and cellular infiltrations, as reported by others (Liu et al. 2016). On the other hand, the administration of combined treatment attenuated the increased levels of the serum enzymes could be due to the β -carotene and phytochemical derivatives; which have been reported to decrease the severity of liver fibrosis induced by CCl_4 (Seifert et al. 1995).

Serum LDH and γ -GT levels are clear indicators of liver cell injury and a loss of the functional integrity of cell membranes. Similarly, our results revealed a high level of LDH and γ GT in CCl_4 treated group as compared to the control group. In fact, the oxidative tissue injury of hepatic membrane, after acute exposure to CCl_4 , caused changes in the molecular organization of lipids leading to liver cell damage and leakage of enzymes in cells (Wang et al. 2016). Thus, reduction in the levels of liver enzymes induced by FCM-LLC supplemented with *Rosemary* extract against CCl_4 built a continuous resistant-barrier with more preventive against the production of free radicals. Indeed, our study demonstrated that the co-administration of FCM-LLC and *Rosemary* before CCl_4 intoxication tended to preserve the normal cellular architecture of liver.

Under normal physiological conditions, the liver plays an important role in regulating lipid and lipoprotein metabolisms. Therefore, examination of plasma lipid and lipoprotein levels could be a useful tool for understanding hepatic pathophysiology. This study showed that CCl_4 induced liver metabolic disorders including lipid profiles perturbations, it is suggested that the lipid metabolism of mice was disorganized.

Pretreatment with FCM-LLC plus rosemary restored the lipid parameters indicating its ability to regenerate or protected hepatic cell membrane integrity.

This could be due FCM-LLC and R to possess hypocholesterolemic action, which was shown by low

triacylglycerol level combined with low LDL cholesterol and high HDL cholesterol. Similar findings from other experimental models could be done upon fermented milk supplementation showed a decline in LDL and total cholesterol and an increase in HDL concentrations. This effect may be related to the decrease of intestinal absorption of cholesterol and the enhancement of the catabolism of cholesterol to form bile acids (St-Onge et al. 2000) and the inhibition of cholesterol synthesis. Therefore, administration of this combined treatment can maintain a balance between fat synthesis and fat metabolism.

Previous biochemical findings are supported by histological examination. The hepatotoxicity of CCl_4 was confirmed in our study by the histological changes seen in the liver symptomized by ballooning degeneration, cell necrosis and centrilobular inflammatory infiltrate. This finding was confirmed by Fahmy et al. (2016), who found that CCl_4 -induced liver damage characterized by the lymphocyte infiltration in the central vein, fatty liver degeneration, as well as necrosis, ballooning degeneration, mitosis, calcification and fibrosis. It can be explained by the enhanced production of ROS the basis of necrotic process or alter the cell integrity. The oral administration of fermented camel milk showed partial protection against hepatic damage with less severity than histopathological changes observed after CCl_4 administration. Based on these findings, we can suggest that FCM-LLC alone-treatment exhibited limited protection against CCl_4 toxicity, compared with the combined treatment. Liver histoarchitecture impairment, induced by CCl_4 , was remarkably reduced by FCM-LLC in combination with R confirming, consequently, the biochemical results.

Conclusion

The present study showed that CCl_4 -intoxication of mice led to liver injuries reflected in hepatic perturbations, oxidative damage, and histopathological alterations. Pretreatment with fermented camel milk with *L. lactis* subsp. *cremoris* and *R. officinalis* contributes to a significant alleviation of hepatic disorders. Our study provided that the antioxidant activity of FCM-LLC in combination with R is more effective than that of FCM-LLC alone or *Rosemary* alone. Our results suggested that pretreatment with FCM-LLC combined with *R. Officinalis* extract is beneficial to reduce liver injury induced by CCl_4 .

Acknowledgements This research was supported by the Tunisian Ministry of higher Education and Scientific Research.

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