# Rose Hip and *Lactobacillus plantarum* DSM 9843 Reduce Ischemia/Reperfusion Injury in the Mouse Colon

## Rose Hip & *Lactobacillus* in Colon Injury

Original Article

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## Abstract

**Purpose:** Ischaemia/reperfusion (I/R) of the colon is an inflammatory condition leading to tissue injury where reactive oxygen species play a central role. Rose hip is rich in biologically active polyphenols with antioxidative properties, which may be important in prevention of lipid peroxidation. *L. plantarum* DSM 9843 possesses enzymatic activity towards polyphenols. The objective of this study was to define the effect of oral administration of *L. plantarum* and rose hip in I/R injury.

**Results:** Administration of rose hip and *L. plantarum* significantly decreased MDA levels in caecum tissue and *Enterobacteriaceae* counts in caecum stool. A positive correlation between MDA levels and *Enterobacteriaceae* counts was found.

**Conclusions:** The results support a synergistic/additive role of rose hip and *L. plantarum* in reducing lipid peroxidation. Therefore rose hip and *L. plantarum* may be used as a pretreatment to tissue injuries, e.g. colonic surgery, organ transplantation and vascular surgery.

**Key words:** ischaemia, reperfusion, rose hip, polyphenols, lactobacilli.

## Introduction

The epithelial cells of the gastrointestinal mucosa form a physical barrier between the internal milieu of the body and the constituents of the gut luminal flora. The colonic mucosa harbors a huge bacterial load and plays a fundamental role in sustaining the physical barrier against translocation of potentially pathogenic bacteria and noxious substances from the luminal content (1). The commensal bacteria are harmless and even beneficial under normal circumstances, but may cause local or systemic inflammatory disease if the integrity of the host surface is disturbed (2). Ischaemia and reperfusion (I/R) of the intestinal tract disturbs the barrier function, causing sepsis (3) and ultimately multiple organ failure.

A rate-limiting step in the pathophysiology of I/R is the activation and recruitment of leukocytes (4,5). Activated leukocytes release toxic products, such as reactive oxygen species (ROS), proteases and vasoactive substances, which in turn cause tissue damage by lipid peroxidation and oxidation of enzymes, a massive protein oxidation, degradation and organ dysfunction (6,7).

The prevention of lipid peroxidation is an essential process in all aerobic organisms, as lipid peroxidation products can cause DNA damage (8). It is therefore of high interest to find antioxidant substances with the ability to scavenge ROS.

Among the various natural scavengers of these reactive molecule activities, polyphenols have received attention. These compounds, widely distributed in plants, constitute an important class of antioxidative natural substances (9). Rose hip possesses antioxidant properties which often have been attributed to vitamin C, known to be an effective

antioxidant. However, it was demonstrated that the antioxidant properties of rose hip are not due only to ascorbic acid, but also to polyphenols (10). Rose hip extract has also been shown to inhibit the chemotaxis and oxidative burst response of the human peripheral blood polymorphonuclear leucocytes *in vitro*, this effect was not due to vitamin C content of the extract (11).

It is well known that polyphenols can be toxic and bacteriostatic (12). Nevertheless, some bacteria are quite resistant to polyphenols and may degrade many phenolic compounds (13). Most *Lactobacillus* spp. are unable to degrade polyphenols, but strains of the closely related species *Lactobacillus plantarum, Lactobacillus pentosus* and *Lactobacillus paraplantarum* possess tannase activity (14) - tannins are naturally occurring water soluble polyphenols - and metabolise phenolic acids (15).

Lactobacilli constitute an integral part of the healthy gastrointestinal microecology and are involved in host metabolism offering nutritional and therapeutic benefits (16), having abilities to minimize the harmful effects of potentially pathogenic microbes (17) and stimulating immune response (18). In contrast, *Enterobacteriaceae* is a genetically close family which includes *Escherichia coli* and pathogenic taxa as *Shigella* and *Salmonella* and where even normally non-pathogenic taxa have a strong pro-inflammatory capacity due to bacterial lipopolysaccharide (LPS). LPS or endotoxin is present in almost all Gram-negative bacteria (19) and triggers the release of numerous inflammatory mediators such as ROS (20).

The objective of this study was to define the effect of oral supplementation of *L. plantarum* DSM 9843 and rose hip. It was hypothesized that the administered strain,

in the GI-tract, may convert the polyphenols of rose hip to compounds with anti-inflammatory capacity. Alternative actions may be an additive suppression of inflammation by immunological effects of bacteria and antioxidative effects of the polyphenols in rose hip. A mouse model was used to study I/R induced injury and the level of lactobacilli and *Enterobacteriaceae* in the caecum.

## Methods

### Animals

Male Balb/cJ mice (Taconic, Denmark), weighing approximately 20 g, were kept under standard laboratory conditions and maintained on 12 hours light and 12 hours dark cycle. They were acclimatized one week before use and were allowed free access to animal chow (nonradiated R3, [Lactamin, Sweden]) and tap water. All experimental procedures were performed in accordance with legislation on the protection of animals and were approved by the Committee of Ethics of animal experiments at Lund University.

### Experimental protocol

The animals were randomly divided into 6 groups of 9 animals in each and each animal was placed in its own cage with food dish. The cages were carefully separated so that contamination could not occur. After 7 days of acclimatisation the mice were fed experimental diet for 7 days. They were weighed after acclimatisation and before sacrifice. The feed was dissolved in water to soften the consistency (experimental diet) prior to the addition of supplementation. The mice were divided into the following groups and each group of animals was fed experimental diet and supplementation:
I/R-only group with ischaemia/reperfusion injury given supplementary carbohydrates, I/R-group given rose hip (Rh), I/R-group given *L. plantarum* and carbohydrates (Lp),

I/R-group given rose hip and *L. plantarum* (Rh+Lp), I/R-group given vitamin C and carbohydrates (Vit C), and a No-I/R group without I/R injury but given supplementary carbohydrates. 1 ml suspension of *L. plantarum* DSM 9843 (109 CFU/mouse), rose hip powder (1.6 g/mouse) or vitamin C (0.014 g/mouse) was added to the experimental diet in the above mentioned groups each day.

Supplementary carbohydrates (fructose and glucose, 0.65 g of each/mouse) were added to the groups indicated above to compensate for the carbohydrate supply of rose hip.

**Bacterial strain**

The bacterial strain used in the experiment was *Lactobacillus plantarum* DSM 9843 (= strain 299v) which had been isolated from colon from a healthy person and has ability to colonise the human intestinal mucosa after oral administration (21,22).

**Rose hip**

Rose hip powder was kindly supplied by Skane Dairy, Malmö, Sweden, and contained 863 mg/100 g of natural vitamin C and 81.8 g/100 g of carbohydrates as analysed by Steins laboratory, Lund, Sweden.

**Anaesthetic and surgical preparation**

The mice were anaesthesised with 7.5 mg Ketamine (Ketalar® 50 mg/ml, [Pfizer, UK]) and 2.5 mg Xylazine (Narcoxyl® 20 mg/ml, [Veterinaria AG, Schweiz]) per 100 g body weight by intraperitoneal injection. The animals were placed on a warming pad (37$^{o}$C) for maintenance of body temperature. A midline abdominal incision was made. Surgery was performed with careful attention to sterile technique. The superior mesenteric artery was identified and occluded using a vessel clamp, resulting in ischaemia of small intestine and colon. 1.0 ml Dulbecco´s PBS (Dulbecco´s phosphate buffered saline, [Sigma]), (pH 7.2-7.3), was given into the peritoneal cavity for fluid resuscitation. The artery was occluded for 30 minutes then the clamp was removed. The tissue was observed for immediate reperfusion. Next the abdomen was closed using a running Vicryl 4-0 suture (Johnson & Johnson, USA). The animal was allowed to awake from anaesthesia and was removed from the warming pad and placed back into the cage. After 240 minutes, the animal was given anaesthesia again (23) and tissue and stool samples were obtained in the following order and placed in preweighed tubes: caecum stool sample and caecum tissue sample for MDA586 TM (colorimetric assay for lipid peroxidation); samples for bacterial evaluation were weighed and collected in sterile tubes containing 3 ml of freezing media and frozen immediately at –70ºC for later evaluation. Samples for MDA586 TM were rinsed in ice cold Dulbecco´s PBS, weighed, homogenized, alliquoted, and then frozen immediately at –70ºC for later evaluation. Caecum was cut by its anatomical borders, i.e. the ampoule of colon and the rotund sac.

**Histopathological study**

Seven to eight millimetre samples of caecum were obtained and placed in phosphate buffered 4 per cent formaldehyde, fixated over night and dehydrated. Paraffin-embedded samples were cut and studied under light microscopy after staining with H & E (Hematoxylin-Eosin). At least eight samples from each group were analysed.

**Determination of intestinal microflora**

The samples were placed in ultrasonic bath for 5 minutes and swirled for 2 minutes. Viable cell samples (1 ml) were serially diluted in dilution liquid (sodium chloride [Merck], 8.5 g/l; Bacteriological peptone [Oxoid, Unipath LTD, Basingstoke, Hampshire, England], 1 g/l; Tween 80 [Merck], 1 g/l; L – Cystine hydrochloride monohydrate [Merck], 0.2 g/l) and 0.1 ml of the samples from the appropriate dilutions were spread plated. Viable counts were obtained from Rogosa agar (Oxoid) that was incubated anaerobically at 37oC for 72 hours (lactobacilli counts) and from violet redbile-glucose agar VRBG (Oxoid) that was incubated aerobically at 37oC for 24 hours (*Enterobacteriaceae* counts).

## Lipid peroxidation

Caecal content of malondialdehyde (MDA) was determined as an index of lipid peroxidation, using MDA 586TM (Oxis International Inc. Portland, Oregon, USA), a colorimetric assay designed to quantify MDA. Caecal tissues were collected, weighed, rinsed in ice cold Dulbecco´s PBS and homogenised in 1 ml Dulbecco´s PBS with 5 mM butylated hydroxytoluene. After homogenisation the samples were centrifuged at 4000 x g for 10 minutes at 4oC. The supernatants were stored at –70oC until determination. Lipid peroxidation was estimated by adding an aliquot (200 µl) of the supernatant to a reaction mixture containing 640 µl of N-methyl-2-phelindole, 10 µl probucol and 150 µl of 12 M hydrochloric acid. The samples were then incubated in a water bath for 60 minutes at 45oC and centrifuged at 10 000 x g for 10 minutes at 4oC. The absorbance of the supernatant was measured by spectrophotometry at 586 nm. Tetramethoxypropane (TMOP) was used as a MDA standard because MDA is not stable. TMOP is hydrolysed during the acid incubation step at 45oC, which will generate MDA. The results were expressed as nmol MDA.

## Statistics

All values are presented as median (25-75 percentiles). All statistical analyses were conducted in SigmaStat version nr 2.0 (SPSS Inc.). Differences between all groups were evaluated by Kruskal-Wallis test. When comparing only two groups Mann-Whitney rank

sum test was used. The correlation between expectations of benefit was ascertained using the Pearson correlation coefficient. P-values $<0.05$ were considered significant.

## Results

### Characteristics of mice during study

All animals tolerated the products well and no adverse events were reported during the period of intake. The weight of mice at the beginning of the treatments was: 23.5 (22.5-24.5) g in I/R-only group, 24.0 (23.0-25.0) g in Rh group, 24.0 (23.0-24.5) g in Lp group, 24.0 (23.0-25.0) g in Rh+Lp group, 24.0 (22.8-25.0) g in Vit C group and 24.0 (23.0-24.3) g in No-I/R group. The weight of mice after one week of treatment and before sacrifice was: 27.0 (26.0-28.0) g in I/R-only group, 26.0 (26.0-28.0) g in Rh group, 26.0 (25.5-28.0) g in Lp group, 28.0 (26.0-28.0) g in Rh+Lp group, 26.0 (24.0- 26.5) g in Vit C group and 26.0 (26.0-28.0) g in No-I/R group.

### Rose hip and *L. plantarum* DSM 9843 in I/R-induced tissue injury

Reperfusion of ischaemic caecum caused an increase in the caecal MDA concentration (Fig. 1, Table 1). A statistically significant difference in MDA concentration was found between the I/R-only group compared to the No-I/R group (Fig. 1, Table 1). Pretreatment with rose hip and *L. plantarum* DSM 9843 significantly reduced the tissue MDA level after I/R injury compared to I/R-only group. A statistically significant difference was also found when the group supplemented with rose hip and *L. plantarum*

DSM 9843 in combination was compared with the groups that were supplemented with vitamin C or bacteria alone (Fig. 1, Table 1).

No statistically significant difference was found between the group that received only rose hip compared to the group that received bacteria and rose hip in combination, but notably the group that received bacteria and rose hip showed a decreased level of MDA in comparison to the group that received rose hip alone. Vitamin C (in crystalline form) had no effect in decreasing the concentration of MDA (Fig. 1, Table 1).

**Caecal bacterial counts**

Administration of the *L. plantarum* strain resulted in a small increase in total lactobacilli count in the group supplemented with bacteria and rose hip in combination. The mean value was 8.9 (8.4 – 9.1) log CFU/g of faeces compared to the other groups with mean values ranging from 8.3 (8.2 – 8.9) to 8.8 (8.5 – 9.0) log CFU/g of faeces. No statistical significance in changes of lactobacilli count was found.

The *Enterobacteriaceae* count decreased significantly in the group supplemented with bacteria and rose hip in combination compared to the I/R-only group, the group that received only bacteria and to the vitamin C group (Fig. 2). The difference between the rose hip alone group and the group with rose hip and bacteria in combination was not statistically significant.

The incidence of *Enterobacteriaceae* count where no colonies were detected at the lowest plate count dilution (< 0 CFU/g caecal content per animal) for the different groups were 0/8 for the I/R-only group, 0/9 for the No-I/R group, 1/8 for the *L. plantarum* group, 1/9

for the vitamin C group, 3/9 for the rose hip alone group and 4/9 for the combination of rose hip and *L. plantarum*. There was only a small difference in caecal *Enterobacteriaceae* count between I/R-only group and No-I/R group.

**Correlation analysis**

A significant linear relationship between the *Enterobacteriaceae* counts (log CFU/g) and the MDA values (nmol/g) was observed, with a correlation coefficient (Pearson) $r = 0.3$, ($p<0.05$).

**Caecum histology**

The histological evaluation in H & E shows differences between groups. A severe diffuse injury indicated by mucosal damage, edema and congestion in the I/R-only group and much milder changes in treatment groups and among them the less damage was found in the group treated with rose hip and *L. plantarum* in combination (Fig. 3A-B).

## Discussion

Pretreatment with rose hip and *L. plantarum* DSM 9843 significantly attenuated the generation of caecal MDA in response to I/R injury. The tissue MDA was increased after 30 minutes of ischaemia followed by 240 minutes of reperfusion, indicating a substantial yield of ROS-induced damage in this colonic model of I/R. These findings suggest that the combination of rose hip and *L. plantarum* DSM 9843 reduced the production of ROS in the colon.

*Lactobacillus* is an important part of the intestinal microflora and plays a significant role in colonization resistance (24). It has been shown that certain diseases and stress are associated with reduction of the intestinal *Lactobacillus* population (25,26) and supplementation with these bacteria improves the overall condition, showed both in experimental and clinical studies (27,28). Oral administration of *L. plantarum* DSM 9843, with known probiotic properties (29), and rose hip in combination is accompanied by a significant decrease in the number of *Enterobacteriaceae.* This must be regarded as beneficial because the family *Enterobacteriaceae*, in general, is pro-inflammatory and also includes many potentially pathogens. The beneficial effect might be mediated by the high antimicrobial effect from polyphenols of rose hip and from the supplemented *Lactobacillus* strain, which is known to inhibit the growth of various potentially pathogenic bacteria (22, 30).

Brown *et al.* demonstrated that administration of LPS caused an elevated level of lipid peroxidation, measured as the amount of MDA in colonic tissue (31). In the present study, we found a positive correlation between the level of MDA in caecal tissue and

*Enterobacteriaceae* counts in caecal stool, which indicates an association.

It was shown that administration of *Lactobacillus* improved mucosal protein as well as DNA- and RNA-content in colon of methotrexate-induced enterocolitis rats (32). Intestinal lactobacilli may even play a role in maintaining gastrointestinal epithelial proliferation and function (33).

To evaluate the antioxidative effect of the vitamin C in rose hip, equal levels of vitamin C contained in rose hip was administrated to one of the groups. Pretreatment with vitamin C did not decrease either the production of MDA or the level of *Enterobacteriaceae*, which might use vitamin C as carbon- and energy source. This effect is not attained from the vitamin C naturally occurring in rose hip, maybe since the polyphenols in rose hip are bacteriostatic and inhibit bacterial growth. *L. plantarum* DSM 9843, on the contrary, possesses tannase activity (17). Thus, these bacteria are able to grow in environments containing polyphenols.

The results obtained from experiments on conventional animals may not be applicable to the human gut ecosystem, because the microflora of these animals differs in composition from that of humans (34). The lactobacilli is a major group in the intestinal microbiota of mice (35), which is not the case in humans where lactobacilli regularly are present but in a smaller proportion of the microflora (36, 37). The tannase activity of the lactobacilli naturally occurring in mice is not clarified. This might be an explanation why the results did not show significant difference between the group supplemented with rose hip and bacteria in combination, compared to the group supplemented with rose hip alone.

However, this must be further elucidated. In a clinical situation this difference between these two groups is expected to be more pronounced because lactobacilli is more prominent in mice than in humans.

In conclusion, our present study demonstrates that administration of *L. plantarum* DSM 9843 and rose hip in combination decreases the production of MDA in caecum in response to I/R injury and it also inhibits the growth of *Enterobacteriaceae*. Considering its safety (11), loss of adverse effects, low price and ease of administration, this indicates a role of rose hip and *L. plantarum* DSM 9843 in combination as a pretreatment to diminish lipid peroxidation and tissue injuries in fields of e.g. colonic surgery, organ transplantation and vascular surgery and in clinical conditions where I/R injury accounts.

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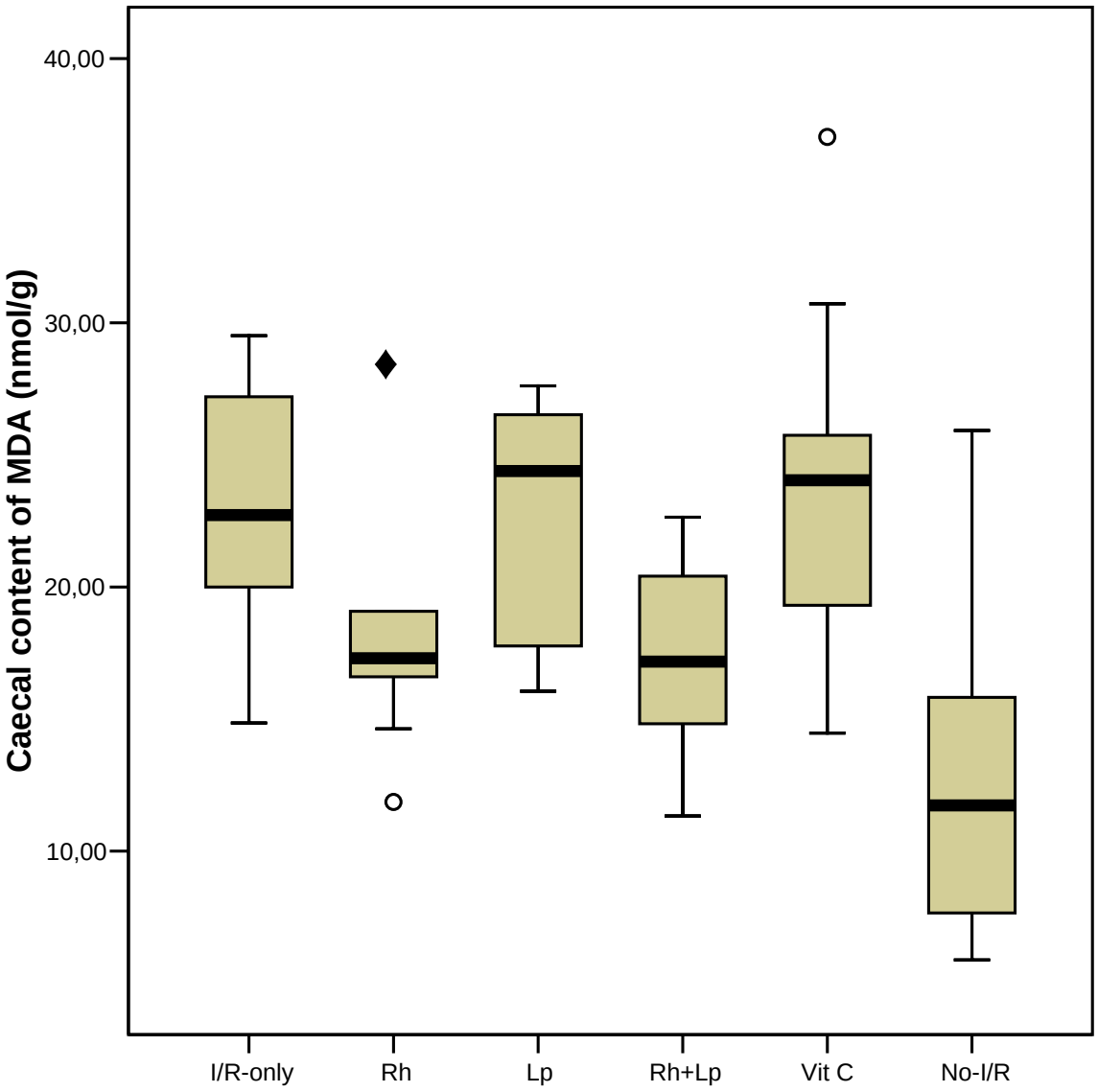
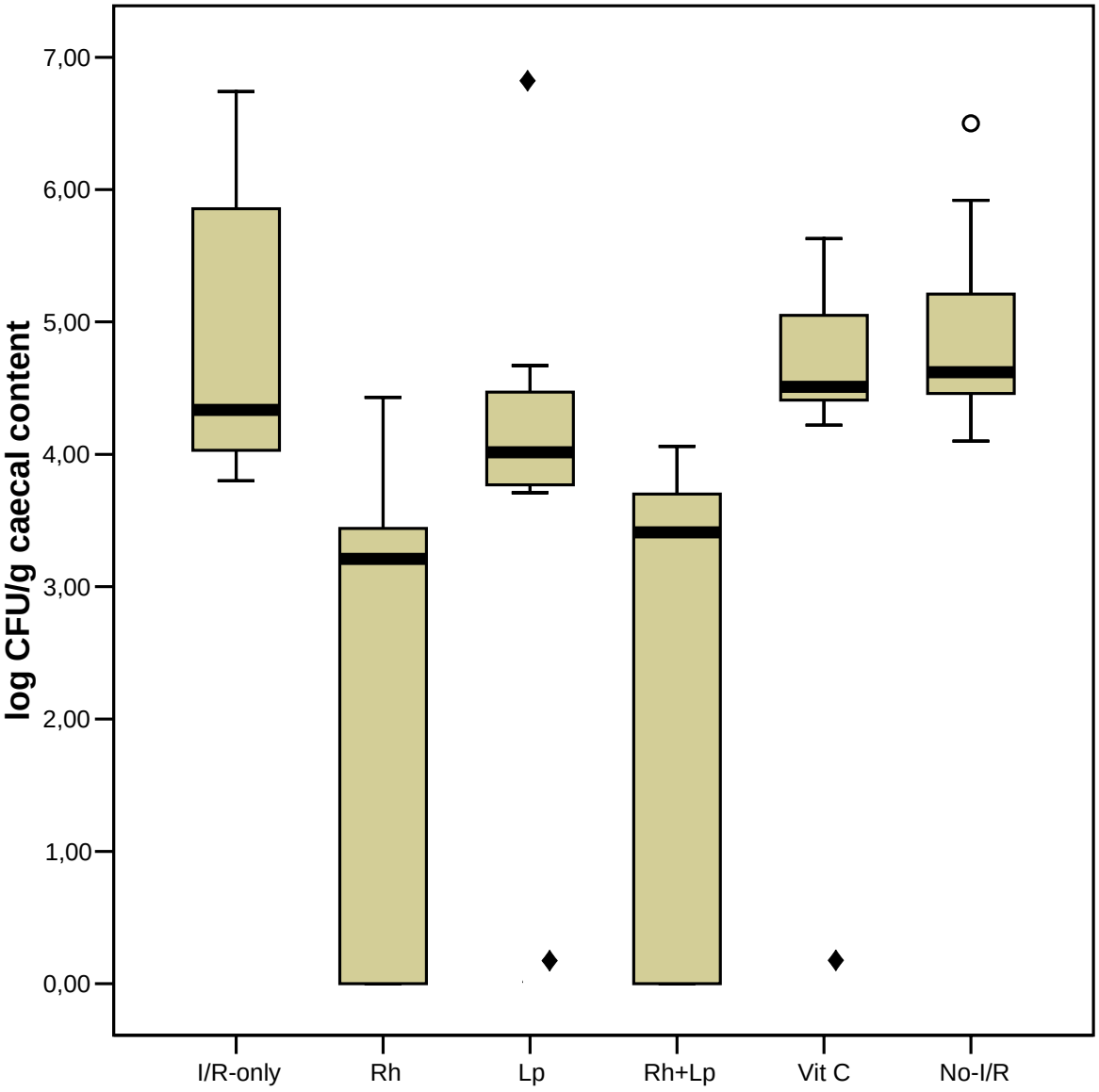

Caecal content of MDA (nmol/g)
40,00
30,00
20,00
10,00
I/R-only
Rh
Lp
Rh+Lp
Vit C
No-I/R


log CFU/g caecal content
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
I/R-only
Rh
Lp
Rh+Lp
Vit C
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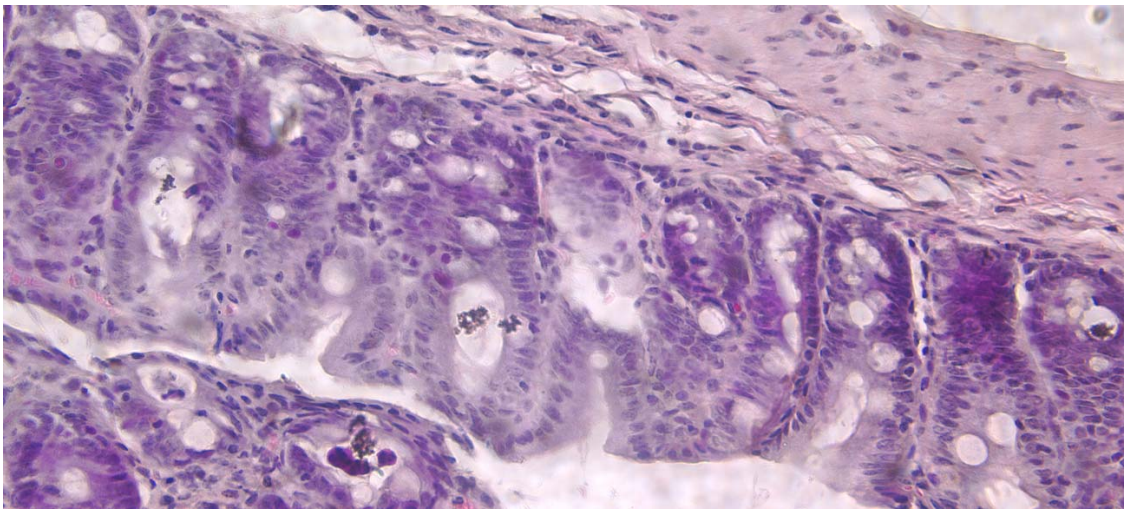

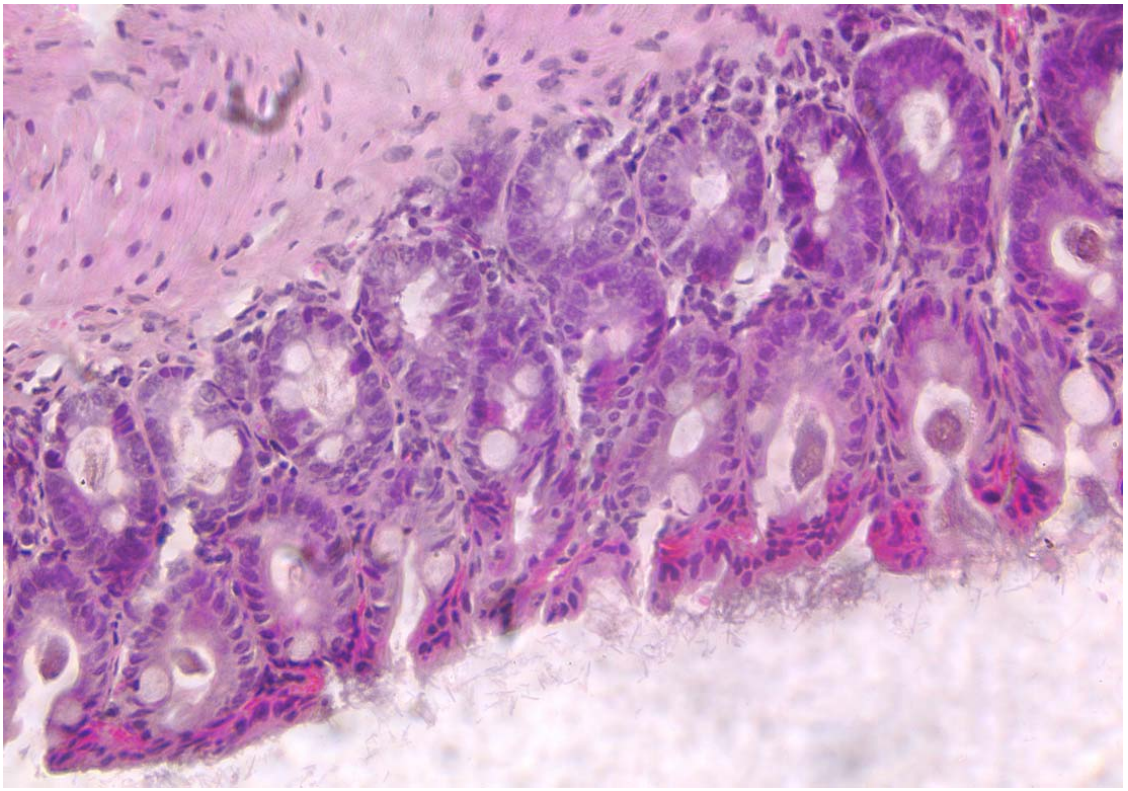

**Table 1. Caecal tissue content of MDA (nmol/g)**

**as median with range**

| Group | MDA (nmol/g) |
|---|---|
| I/R-only (n=8) | 22.7 * (20.0-27.2) |
| Rh (n=9) | 17.3 (16.1-21.7) |
| Lp (n=8) | 24.4 (17.8-26.5) |
| Rh+Lp (n=9) | 17.2 # Δ (14.6-20.6) |
| Vit C (n=9) | 24.0 (19.2-27.0) |
| No-I/R (n=9) | 11.7 (7.4-17.0) |

Abbreviations: I/R-only= ischaemia/reperfusion (I/R) injury and given supplementary carbohydrates; Rh= I/R injury and given rose hip; Lp=I/R injury and given *L. plantarum* and supplementary carbohydrates; Rh+Lp= I/R injury and given rose hip and *L. plantarum*; Vit C= I/R injury and given vitamin C and supplementary carbohydrates; No-I/R= without I/R injury but given supplementary carbohydrates.

* $P< 0.05$ *vs*. No-I/R Group

# $P< 0.05$ *vs*. I/R-only Group

. $P< 0.05$ *vs*. Lp Group and Vit.C Group

**Figure 1.** Levels of MDA (nmol/g) in the mouse caecum without injury and after I/R. The group supplemented with rose hip and *L. plantarum* in combination differed significantly from the I/R-only group, p=0.024; from the *L. plantarum* alone group, p=0.03; and from the vitamin C group, p=0.013. A statistically significant difference was found between I/R-only group compared with No-I/R group, p=0.011. Outliers (**O**) were cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box. Extremes (.) were cases with values more than 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range.

**Figure 2.** Caecal *Enterobacteriaceae* counts (log CFU/g content) from mice fed either experimental diet alone or supplemented with vitamin C or *L. plantarum* DSM 9843 and/or rose hip. The control group and the groups given *L. plantarum* alone and vitamin C significantly differed from the group treated with rose hip and lactobacilli in combination (p=0.002 vs control, p=0.004 vs vit C and p=0.024 vs bacteria). Between

I/R-only group and No-I/R group there was only a small difference in caecal *Enterobacteriaceae* count. Outliers (**O**) were cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box. Extremes (.) were cases with values more than 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range.

**Figure 3.** Caecum histology: **A**. I/R-only group is showing mucosal damage, congestion and edema. **B.** The group supplemented with rose hip and *L. plantarum* in combination is showing the most conservative shape of mucosa and the highest regenerative action.