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Anti-genotoxic activity of the mushroom *Lactarius vellereus* extract in bacteria and in mammalian cells *in vitro*

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In a previous study we screened a range of mushroom species growing in Slovenia for their anti-genotoxic potential and found *Lactarius vellereus* to be the most effective. In this study genotoxic and anti-genotoxic activities of methanol extracts of *Lactarius vellereus* (Fr.: Fr.) Fr. were evaluated in the bacterial reverse mutation test with *Salmonella typhimurium* TA98 and, in the mammalian cell test with human hepatoma (HepG2) cells, using the comet assay to measure DNA damage. The extract induced no mutations in *S. typhimurium* TA 98 and no DNA damage in HepG2 cells. Against the indirect acting mutagen 2-amino-3-methylimidazo(4,5-f)quinoline (IQ) the extract showed significant, dose dependent antimutagenic activity, while it did not counteract the direct acting mutagen 4-nitroquinoline oxide (4-NQO). The extract also exerted a protective effect against IQ induced genotoxicity in mammalian cells of human origin. Treatment of HepG2 cells with the *L. vellereus* extract (125–500 μg/ml) together with IQ, reduced the genotoxic effect of the latter in a dose dependent manner. Our findings show that a methanol extract of *L. vellereus* is highly protective against IQ induced DNA damage in human derived cells and *L. vellereus* can be considered as a natural source of anti-mutagens with potential pharmacological applications in cancer prevention.

1. Introduction

From epidemiological and laboratory studies it has become evident that a variety of dietary and medicinal phytochemicals have substantial cancer preventive properties (Dragsted et al. 1993). Most of these have been identified and isolated from plants, while mushrooms were, until recently, neglected as a source. *Basidiomycetes* have been used in folk medicine all over the world since ancient times (Mizuno et al. 1995). They show diverse beneficial physiological and therapeutic effects, such as immunomodulatory, lipid lowering, antithrombotic, antihypertensive, anti-inflammatory and antitumor activities (Chang 1996). It has been known for many years that selected species from higher basidiomycetes are effective against cancer of the stomach, oesophagus, lung, etc. (Wasser and Weis 1999).

Anticarcinogens may act to inhibit the initiation, promotion or progressive phases of the carcinogenic process (Kada et al. 1982). Anticarcinogenic activity at the level of the initiation phase may be due to a variety of mechanisms of inhibiting genotoxic effects, such as direct interaction with mutagens, inhibition of metabolic activation of indirect mutagens, induction of detoxification enzymes, interference with DNA repair mechanisms, etc. (De Flora et al. 1999; Birt et al. 2001). There are reports that certain higher mushrooms have anti-genotoxic activity against direct and indirect acting mutagens. The most extensively studied mushroom with respect to anti-genotoxic activity is *Agaricus blazei*. Its hexane and chloroform-methanol extracts inhibited benzo(a)pyrene induced mutagenesis (Osaki et al. 1994). Aqueous extracts inhibited methyl methane sulphonate induced DNA damage and formation of micronuclei in V79 cells *in vitro* (Rodriguez et al. 2001), and of cyclophosphamide induced micronuclei in bone marrow and peripheral blood cells in mice *in vivo* (Delmanto et al. 2001). The ethanol extract of *Cratarellus cornucopioides* inhibited the mutagenicity of indirect mutagens aflatoxin B1, benzo(a)pyrene, acridine half mustard (ICR-191) and 2-nitrofluorene, while it was not effective against 4-nitroquinoline-*N*-oxide, methyl methanesulfonate and *N*-methyl-*N*-nitro-*N*-nitrosourea in *Salmonella typhimurium* TM677 (Gruter et al. 1990). The mutagenicity of 2-nitrofluorene was also inhibited by ethanol extracts of *Agaricus abruptilus, Agaricus bisporus, Cantharellus cibarius, Lactarius lilacinus, Lyophyllum connatum* and *Xerocomus chrysenteron* (Gruter et al. 1990). Antimutagenic activity against benzo(a)pyrene induced mutagenicity in the Ames *Salmonella*/microsome assay was observed in the desert mushroom Al-faga (*Tiramaria pinoyi*) (Hannan et al. 1989). In our previous study we screened a range of different species of mushrooms growing in Slovenia for their anti-genotoxic potential. We found several species of the Russulaceae family that have anti-genotoxic activity, of which *Lactarius vellereus* was the most effective (Filipič et al. 2002).

Table 1: Mutagenicity of *Lactarius vellereus* methanol extract (Lv) in *Salmonella typhimurium* TA98 with and without metabolic activation (10% S9 mix)

Treatment	his$^+$ revertants/plate −S9 MIX	his$^+$ revertants/plate +S9 MIX
Control	22.0 ± 3.2	25.6 ± 3.7
Lv 125 µg/ml	22.2 ± 4.2	22.5 ± 3.1
Lv 250 µg/ml	26.3 ± 5.1	22.7 ± 7.8
Lv 500 µg/ml	29.8 ± 2.3	20.3 ± 8.2
Lv 750 µg/ml	21.8 ± 3.5*	26.9 ± 5.6
Lv 1000 µg/ml	25.3 ± 6.5*	28.1 ± 6.4

* toxic effects observed as reduced density of the background

In this study we have investigated the mutagenic and antimutagenic effects of methanol extracts of *Lactarius vellereus* in the bacterial reverse mutation test system with *Salmonella typhimurium* TA98. As model mutagens, against which antimutagenic activity of the extract has been investigated, we used a direct acting mutagen 4-nitroquinoline oxide (4-NQO) and an indirect acting food mutagen and carcinogen 2-amino-3-methylimidazo(4,5-f)quinoline (IQ). Genotoxicity and the protective effect of the extract against the IQ-induced genotoxic effect was further evaluated using a recently developed test system with metabolically competent human HepG2 hepatoma cells (Uhl et al. 2000). Genotoxic effects were measured with the comet assay, also called single cell gel electrophoresis (SCGE), which is a very sensitive method for detecting single and double DNA strand breaks as well as other types of DNA damage (e.g. alkali-labile sites, DNA-DNA and DNA-protein cross-links) and single strand breaks associated with incomplete excision repair at the level of single cells (Tice et al. 2000).

2. Investigations and results

2.1. Mutagenicity and antimutagenicity of L. vellereus Lv extract in Salmonella typhimurium TA98

The methanol extract, Lv, of *Lactarius vellereus* did not induce mutations in *S. typhimurium* TA98 at concentrations up to 1000 µg/plate (Table 1). When tested without metabolic activation at concentrations above 750 µg/plate the density of background lawn was slightly reduced, indicating that the extract inhibited growth of bacteria. No growth inhibition was detected when the extract was tested in the presence of S9 mixture, indicating that toxic components were metabolically transformed to non-toxic metabolites.

Table 2: Effect of *Lactarius vellereus* methanol extract (Lv) on 4-NQO and IQ induced *his*$^+$ revertants in *Salmonella typhimurium* TA98

Treatment	his$^+$ revertants/plate −S9 MIX	Reduction (%)	his$^+$ revertants/plate +S9 MIX	Reduction (%)
Control	19.9 ± 3.6		25.6 ± 1.0	
NQNO (1 µg/plate)	117.0 ± 4.2	0	not tested	
IQ (10 ng/plate)	not tested		665.0 ± 29.6	0
Lv 4 µg/ml	125.3 ± 9.6	0	not tested	
Lv 20 µg/ml	116.7 ± 12.7	0	not tested	
Lv 125 µg/ml	101.0 ± 17.1	16	not tested	
Lv 250 µg/ml	17.7 ± 4.2*	toxic	597.7 ± 28.6	11
Lv 500 µg/ml	0	toxic	478.0 ± 15.1	29
Lv 750 µg/ml	not tested		386.0 ± 35.6	44
Lv 1000 µg/ml	not tested		245.3 ± 66.0	66

* toxic effects observed as reduced density of the background

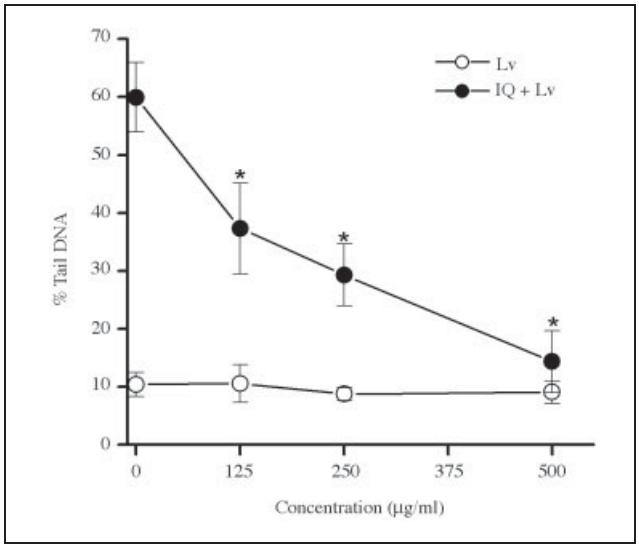

Fig. 1: Effect of methanol extract of *L. vellereus, Lv,* on IQ induced DNA damage. HepG2 cells were exposed to IQ (1 mM) in the presence or absence of different concentrations of Lv. Control cells were exposed to different concentrations of Lv and the solvent (1% DMSO). After 24 h, the cells were washed, harvested and analysed for DNA damage. Each experimental point represents the mean ± SD of medians of four cultures; 25 cells were analyzed from each culture. Asterisks indicate statistical significance ($P < 0.001$; Mann–Whitney U-test).

When tested for antimutagenic effect against 4-NQO, no significant reduction in the number of revertants was detected at concentrations from 4 to 125 µg/plate (Table 2). At concentrations of 500 and 1000 µg/plate the density of the background lawn was almost completely reduced, indicating that, in combination with 4-NQO, the extracts strongly inhibited the growth of bacteria.

The extract Lv reduced the number of IQ induced revertants in a dose dependent manner at concentrations from 125 to 1000 µg/plate (Table 2). Microscopic examination of the density of the background lawn on plates with bacteria treated with IQ alone or in combination with the extracts showed no differences, showing that the reduced number of the revertants was due to an antimutagenic effect and not to inhibition of bacterial growth. The concentration required to reduce the number of IQ induced His$^+$ revertants by 50% (EC_{50}) was 787 µg/plate.

2.2. Genotoxicity and anti-genotoxicity of L. vellereus Lv extract in HepG2 cells

No increase of % tail DNA was detected in human hepatoma HepG2 cells exposed to different doses of the Lv extract for 24 h, indicating that the extract did not induce DNA damage (Fig. 1). Exposure of HepG2 cell to 1 mM IQ induced extensive DNA damage in HepG2 cells (60% tail DNA). Addition of 125 µg/ml extract Lv during the exposure to IQ led to about 40% reduction of IQ induced DNA damage while, at the highest dose (500 µg/ml), IQ-induced DNA damage was reduced by about 70% (Fig. 1). The protective effect was statistically significant at all three doses applied. The concentration of Lv which reduced the IQ induced DNA damage by 50% (EC_{50}) was 156 µg/ml.

2.3. Effect of L. vellereus Lv extract on survival of HepG2 cells

The effect of the extracts, alone and in combination with IQ, on the viability of HepG2 cells was determined in the

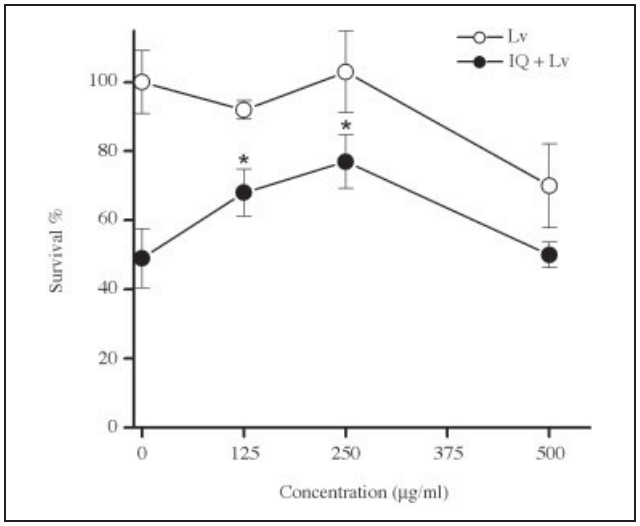

Fig. 2: Survival of HepG2 cells after exposure for 24 h to *L. vellereus* methanol extract, Lv, to IQ (1 mM) and to combined IQ and Lv. Cell survival was determined with MTT, and the data are expressed as % viable cells compared with untreated controls. The results are the means ± SD of six measurements. Asterisks indicate significant difference between cells exposed to IQ alone and in combination with Lv ($P < 0.01$; Student t-test).

MTT test which measures the conversion of MTT to insoluble formazan by the mitochondrial dehydrogenase enzymes of living cells. Exposure of the cells to the extract Lv under the same conditions as those used in the comet assay reduced their survival by only 30% at the highest dose employed (500 µg/ml) (Fig. 2). Survival of cells exposed to IQ alone was reduced by about 50%, compared to non-treated control cells. Treatment with IQ in the presence of 125 and 250 µg/ml of Lv led to cell survivals 40 and 60% higher than the survival of cells treated with IQ alone.

3. Discussion

Lactarius vellereus (Fr.: Fr.) Fr. or Fleecy Milk-cap is a mushroom belonging to the Russulaceae family which is distributed across Europe and North America. The main characteristic of the genus *Lactarius* is the milky cellular juice that issues from the laticifers of the injured fruiting bodies, and which rapidly becomes pungent (Daniewski and Vidari 1999). Sterner et al. (1985) have shown that, in injured *Lactarius vellereus* fruiting body, non-polar precursor compounds (fatty acid esters of velutinal) are rapidly changed into substances that are toxic and mutagenic (vellelral and isovelleral), but then degraded into substances which are not mutagenic or toxic to bacteria. It has been suggested that these mechanisms constitute a chemical defence against parasites and predators (Sterner 1995).

Isovelleral has been shown to be highly mutagenic to *S. typhimurium* TA98 (Johanssohn et al. 1997). The methanol extract Lv of *L. vellereus* was prepared from injured mushrooms in which isovelleral would be formed and extracted. The Lv extract did not induce mutations in *S. typhimurium* but, when tested without metabolic activation at concentrations above 500 µg/plate, it inhibited bacterial growth slightly. The extract also did not induce DNA damage in HepG2 cells, as determined by the comet assay, while viability was reduced only at the highest applied concentration (500 µg/ml). This indicates that the extract contained no mutagenic compounds (i.e. isovellelral) but did contain a compound or compounds with antibacterial and cytotoxic activity. Previous studies showed that *L. vellereus* contains sesquiterpenes with antibacterial and cytotoxic activity (Abraham 2001).

In our previous study we showed that a methanol/water extract of *L. vellereus* suppressed the UV induced SOS response in *S. typhimurium* TA1535/pSK1002 and UV induced mutations in *Escherichia coli* WP2 (Filipič et al. 2002). In this study we used as a direct acting mutagen 4-NQO, but the antimutagenic potential of the extract against this mutagen could not be evaluated, since exposure to the combination strongly inhibited bacterial growth.

The methanol extract of *L. vellereus* strongly inhibited mutagenesis by the heterocyclic amine, IQ in *S. typhimurium* TA98 without inhibiting bacterial growth. IQ is an indirect mutagen that is activated by cytochrome P450 1A2 to an N-hydroxylated product, 2-hydroxyamino-3-methylimidazo(4,5-*f*)quinoline, which then spontaneously, or by the subsequent action of sulphotransferase or acetyltransferase, generates an electrophilic nitrenium ion that causes DNA damage and induces mutations (Sniderwine et al. 1988). The mechanisms by which the extract Lv inhibited IQ induced mutagenesis in bacteria may include inactivation of heterocyclic amines and their metabolites by direct binding, inhibition of enzymes involved in metabolic activation and/or interaction with DNA repair processes.

Bacterial antimutagenicity assays are, due to their cost- and time-effectiveness, very useful for screening purposes and for identifying antimutagens. However using bacterial antimutagenicity assays against indirect mutagens, where exogenous metabolic activation is used, it is not possible to predict whether compounds that inhibit activating enzymes or that inactivate metabolites extracellularly act inside the cell as well. It is also not possible to detect those antimutagens that induce detoxifying enzymes. And finally it is also not known if antimutagens which act at the level of DNA repair in bacteria are also effective in mammalian cells. We therefore explored whether the protective effect of *Lactarius vellereus* extract against IQ-induced genotoxic effects is also expressed in mammalian cells. We used human hepatoma HepG2 cells because they retain the activities of various phase I and phase II enzymes which play a crucial role in the activation/detoxification of genotoxic procarcinogens. They also better reflect the metabolism of such compounds *in vivo* than experimental models with metabolically incompetent cells and exogenous activation mixtures (Knasmüller et al. 1998). HepG2 cells have been used in several antimutagenicity studies, and it has been shown that they can identify mechanisms not detected in conventional *in vitro* systems, such as the induction of detoxifying enzymes, inactivation of endogenously formed DNA-reactive metabolites, and intracellular inhibition of activating enzymes (Knasmüller et al. 2002).

Exposure of HepG2 cells to 1 mM IQ for 24 h induced significant DNA damage, in agreement with previous reports (Uhl et al. 2000). In the presence of the extract Lv, the IQ-induced DNA damage was suppressed in a dose dependent manner. In the presence of non-cytotoxic concentrations of the extract the cells were also protected against the cytotoxicity of IQ. This result demonstrates that the protective effect of the extract observed in the bacterial test system is expressed in mammalian cells. Moreover, the protective effect was even more pronounced in HepG2 cells, since a 5 times lower concentration of the extract was required to reduce the genotoxic effect of IQ by 50% than in bacteria.

Heterocyclic amines are mutagenic carcinogens generated by cooking meat and fish (Skog et al. 1998), therefore exposure to these substances is scarcely avoidable. Some heterocyclic amines, including IQ, are implicated as aetiological factors for cancer in humans and have been classified by IARC as probable human carcinogens (IARC 1993). Therefore the ability of the extract Lv to attenuate IQ induced genotoxicity in human cells is of particular interest, as it could have pharmacological application in cancer prevention. *Lactarius vellereus* can be considered as a natural source of antimutagenic and anticarcinogenic substances.

4. Experimental

4.1. Test fungal material

Fruiting bodies of *Lactarius vellereus* (Fr.: Fr.) Fr. were obtained in autumn 1999 in the north-west of Slovenia, identified by Mr Andrej Piltaver from the Mycological Society of Slovenia and stored at −20 °C. Voucher specimens are deposited at the Department for Pharmaceutical Biology, Faculty of Pharmacy, Ljubljana, Slovenia (designation Lv 99/1).

4.2. The preparation of methanolic extracts

Frozen fruiting bodies were ground in an electric grinder, weighed and freeze-dried (Beta 1–8, Christ, Germany). Dry fruiting bodies were pulverized, weighed and extracted with methanol as follows: maceration at room temperature for 30 min, extraction in an ultrasonic bath for 15 min, maceration at room temperature for 12 h and repetition of the procedure in the ultrasonic bath. Extracts were filtered and the solvent removed by evaporating to dryness under reduced pressure, at temperatures not exceeding 35 °C. Dry extracts were dissolved in sterile DMSO to obtain a final concentration of 50 mg/ml. Samples were stored at −20 °C.

4.3. The Salmonella typhimurium mutagenicity/antimutagenicity assay

Mutagenicity and antimutagenicity of methanolic extracts of *L. vellereus* (Lv) were tested with *Salmonella typhimurium* TA98 using the standard plate incorporation procedure (Maron and Ames 1983). 4-Nitroquinoline-*N*-oxide (4-NQO, 1 μg/plate) was used as a directly-acting mutagen and 2-amino-3-methylimidazo(4,5-*f*)quinoline (IQ, 5 nM/plate) as an indirectly acting mutagen. The assay was conducted by adding 0.1 ml of the overnight bacterial culture, 0.1 ml of diluted extract Lv alone or in combination with 4-NQO or IQ, and 0.5 ml of PBS or S9 mix (containing 10% S9 – Arachlor induced rat liver microsomal fraction (Moltox, Boone, NC, USA)) to 2 ml of top agar. After mixing and plating on minimal bottom agar plates, His^+ revertants were counted after 48 h incubation at 37 °C. Three plates per experimental point were used and at least two independent experiments were performed. Plate backgrounds were inspected visually (with the aid of an inverted light microscope) to ensure that the reduced number of His^+ revertant colonies was not due to non-specific toxicity of the test substance.

The % reduction of the mutagen induced His^+ revertants by the extract was calculated from the number of His^+ revertants in mutagen treated bacteria (PC), the number of revertants in combined anti-mutagenicity treatment (AC) and the number of spontaneous revertants (NC), according to the equation:

$$\% \text{ of reduction} = 100 \times (PC - AC)/(PC\text{-}NC).$$

4.4. Human HepG2 cells and culture conditions

The HepG2 cells were a gift from Dr Firouz Darroudi (Department of Radiation Genetics and Chemical Mutagenesis, University of Leiden, Netherlands). They were grown in William's medium E (Sigma, St. Louis, USA) containing 10% foetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin at 37 °C in 5% CO_2. The cells were used at passages between 5 and 12.

4.5. Treatment of HepG2 cells

Single cell suspensions of HepG2 cells (400 000 cells/ml) were prepared and aliquots of 900 μl distributed to the wells of ultra low attachment cluster plates (Corning Costar Corporation, New York, USA). 100 μl of diluted extract Lv was added to the cell suspension to final concentrations of 125, 250 and 500 μg/ml. The negative control consisted of PBS containing 1% DMSO (the same amount of the vehicle as at the highest concentration of the samples). The suspensions were incubated for 24 h at 37 °C in 5% CO_2.

For the combined treatment with IQ and the methanol extract Lv, 100 μl of IQ (final concentration 1 mM) and 100 μl of appropriately diluted Lv extract, or PBS containing 1% DMSO, were added to 800 μl of cell suspension and incubated for 24 h at 37 °C in 5% CO_2.

4.6. Comet assay

After the treatment described above an aliquot (30 μl) was removed and processed for the comet assay as described by Singh et al. (1988). 30 μl of cell suspension was mixed with 70 μl of 1% LMP agarose and added to fully frosted slides that had been covered with a layer of 1% NMP agarose. The slides were then immersed in 2.5 M NaOH, pH 10, 0.1 M EDTA, 0.01 M Tris and 1% Triton X-100 for 1 h at 4 °C to lyse the cells, rinsed with distilled water, placed in the electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min to allow DNA unwinding, and electrophoresed for 20 min at 25 V and 300 mA. After neutralising with 0.4 M Tris buffer (pH 7.5), the slides were stained with ethidium bromide (5 μg/ml). Slides were scored using an image analysis system (VisCOMET, TillPhotonics, Germany) attached to a fluorescence microscope (Nikon, Eclipse 800) equipped with appropriate filters. The final magnification was x400. The extent of DNA damage was quantified as % tail DNA. Images of 100 randomly selected cells were analysed for each experimental point. In each experiment two parallel cultures were treated and two separate experiments were performed. The data are expressed as the mean ± S.E of median values of four cultures for each treatment group.

4.7. Cytotoxicity assay

Cell survival after exposure to Lv extract, IQ and the combination of the extract with IQ, was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), according to Mosmann (1983) with minor modifications. HepG2 cells were seeded at a density of 2×10^4 cells/well into 96-well microtiter plates in six replicates and incubated at 37 °C for 24 h to attach. The medium was then replaced with fresh medium containing the indicated amounts of extract Lv, IQ or combination of Lv with IQ, and the cells incubated for 24 h. The cells were then washed with PBS, fresh growth medium containing 0.5 μg/ml of MTT was added to each well, and the cells further incubated for 3 h at 37 °C. The medium was removed and the formazan crystals were dissolved in DMSO. The absorbance (A_{570}) was measured (reference filter 690 nm) using an ELISA microplate reader. Survival was determined by comparing the $A_{570/690}$ of the wells containing treated cells to those of non-treated cells. Survival is expressed as a % of the untreated controls, using the equation: ($A_{570/690}$ of treated sample/$A_{570/690}$ of untreated sample) × 100.

4.8. Statistical analysis

Student's t-test was used to compare the mean values of the number of revertants per plate in the bacterial test and for comparison of the mean values of $A_{570/690}$ values in the MTT test. Since the distribution of % tail DNA departed significantly from normality (Kolmogorov-Smirnov test), differences between cells exposed to IQ alone and those exposed to IQ in combination with the extract Lv were evaluated by applying the non-parametric Mann–Whitney U-test. All analyses were conducted using the Analyse-it for Microsoft Excel 1.68 statistical package.

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