Components in *Lentinus edodes* mushroom with anti-biofilm activity directed against bacteria involved in caries and gingivitis

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Abstract

The present research investigated the compounds present in the low molecular mass fraction of *Lentinus edodes* mushroom (shiitake) extract and their anti-virulence activity against oral pathogens (reference and clinical *Streptococcus mutans*, *Actinomyces naeslundii*, and *Prevotella intermedia* strains). Oxalic, succinic, and quinic acids, and adenine, inosine, and uridine were identified by HPLC-DAD-ESI-MS/MS. Their anti-biofilm production and preformed biofilm disaggregation activities were studied using commercial standard compounds at different concentrations. As regards *S. mutans*, the highest activity was shown by adenine 5mg mL^{-1} both in the biofilm inhibition (BI 50%) and in the biofilm disaggregation tests (BD 20%). Considering *A. naeslundi*, BI values close to 80% were registered for oxalic acid at 1mg mL^{-1} and 2mg mL^{-1} and BD 50% for quinic acid 3mg mL^{-1}. Weaker activity was found against *P. intermedia*. Furthermore, different mixtures of the commercial standards were tested showing that the activity of a compound can be strongly and sometime negatively affected by the presence of the other compounds.

Keywords: *Lentinus edodes*; anti-caries and anti-gingivitis activities; biofilm, adhesion, organic acids, nucleobases, nutraceuticals

1. Introduction

Lentinus edodes is one of the largest cultivated edible mushroom in the world for its appreciable taste and flavour, and important nutritive properties.[1-4] *L. edodes* has a long history in oriental folklore for treatment of different health problems and thus it is considered as the elixir of life and used also for specific medical conditions.[5-6]

Its use has increased over the last two decades in Western countries and a large number of investigations on its components and biological properties has been carried out. Therapeutic effects against different pathologies such as tumors[7-10], heart diseases[1], obesity[11], diabetes[12], liver ailments[13-14], respiratory diseases[15], inflammation[16-17], viral[18-19] and bacterial infections[20-21], caries and gingivitis[22-24] have been confirmed or recognized.

Several bioactive components have been identified in *L. edodes* including both high and low molecular mass (MM) compounds possessing important pharmacological and functional properties. Such components belong to different chemical classes, including sugars[25], polyalchols[26], polysaccharides[27-30], polyphenols[31-32], glicoproteins[33], proteins[34], nucleotides, nucleobases, peptides, and amino acids.[2]

In recent studies we identified a low MM (LMM) fraction of *L. edodes* possessing the ability to inhibit virulence-related properties (*i.e.*, biofilm formation, adherence to epithelial cells, and hydroxyapatite) of oral pathogenic bacteria involved in tooth decay (*Streptococcus mutans*) and gingivitis (*Actinomyces naeslundii*, *Prevotella intermedia*).[3, 35] We have also recently shown that quinic, succinic, and oxalic acids are the main compounds responsible for the capacity of the LMM fraction of *Cichorium intybus* var. *silvestre* to affect virulence-related traits of bacteria involved in caries and periodontal infections.[36] The present study was undertaken to verify both the presence of these acids in *L. edodes* extract and to identify any other compounds potentially responsible for its biological activities. This study was focused on the analysis of the capability of the identified compounds to inhibit the formation of biofilm by *S. mutans*, *A. naeslundii*, and *P. intermedia*

bacteria, and to induce the disaggregation of preformed biofilms. The potential interactions among the different compounds when present in mixtures were also investigated.

2. Materials and methods

2.1 Chemicals

HPLC-grade and HPLC-MS grade water, formic acid, methanol, ethanol (70%), acetone, oxalic acid (grade purity analytical standard, O), succinic acid (grade purity ≥ 99.0%, S), adenine (grade purity ≥ 99.0%, A), inosine (grade purity ≥ 99.0%, I), uridine (grade purity ≥ 99.0%, U), and crystal violet were purchased from Sigma-Aldrich (Milan, Italy); quinic acid (grade purity ≥ 98.0%, Q) was purchased from Acros Organics (Geel, Belgium).

2.2 Instrumental

A vacuum freeze-drier Modulyo System (5Pascal, Italy) was used for the freeze-drying process. Plate Reader (das srl Rome, Italy) was used to evaluated biofilm formation, inhibition and disaggregation. Anaerobic chamber (Whitley DG 250 Anaerobic Workstation, Don Whitley Scientific, Shipley, UK) was used to create an anaerobioc atmosphere to culture anaerobes. Microtiter plates (96-well flat-bottomed microtiter plates- Sartedt AG& Co., Germany) were used to grown bacterial biofilms. Vivaflow 200 complete system equipped with 5,000 MWCO PES membrane was used for the first fractionation of *L. edodes* extract. Gel filtration chromatography (GFC) analyses were performed using an Agilent 1100 series liquid chromatography system (Agilent, Waldbronn, Germany) equipped with a with a quaternary pump, a vacuum degasser, and a diode array detector. HPLC-DAD-ESI-MS^n analyses were performed using a Thermo Finningan Surveyor Plus HPLC apparatus (Thermo Fischer Scientific, Waltham, MA, USA) equipped with a quaternary pump, a Surveyor UV-Vis photodiode-array detector, a Surveyor Plus autosampler, and a vacuum degasser connected to a LCQ Advantage Max ion trap spectrometer through an ESI source.

2.3 Mushroom extract preparation

Aliquots of shiitake mushroom (500 g) were homogenized for 1 min, centrifuged for 10 min at 6,900 x g. The juice, after separation from solids, was filtered using a paper filter and then submitted to sterile ultrafiltration using a 0.20 μm PES membrane.

2.4 Isolation of active compounds

A first extract fractionation was performed using a 5,000 MWCO PES membrane as reported by Spratt.[35] The active LMM fraction obtained was further fractionated using gel filtration chromatography (GFC). A Merck Superformance Universal glass cartridge system (300 mm x 10 mm) was used for GFC separation and the analyses were performed with a TSK gel Toyopearl HW-40F (exclusion limits 100-10,000 Da) with Millipore grade water as the mobile phase, at a flow rate of 0.5 mL min^{-1}. UV spectra were recorded in the 190-600 nm range, and chromatograms were acquired at 210 nm. The Agilent Chemstation software was used for HPLC system control and data processing. Five fractions (M1-M5) were collected and the corresponding eluates were freeze-dried and then used in a number of assays.

2.5 Chemical characterization of M4 fraction by LC-MS/MS

The active M4 fraction was separated and characterized by liquid chromatography with tandem mass spectrometry. The analyses were performed with a Gemini® C18 analytical column (150 ×2.0 mm, i.d., 5 μm), connected to a Hypersil Gold C18 guard column (10 × 2.1 mm i.d., 5 μm), with a binary mobile phase methanol/water acidified with 0.1% formic acid 5/95 (v/v) at a flow rate of 0.3 mL min^{-1}. Column and autosampler temperatures were held constant at 4 °C. Spectral data were acquired in the range of 200–600 nm for all peaks. The ion trap mass spectrometer was set to operate in data-dependent, zoom scan, full scan, and MS^n mode to obtain fragment ion *m*/*z*. MS operating conditions (negative and positive ion mode) had previously been optimized by flow injection analysis, using oxalic, quinic, and uridine (5 ppm water acidified with 0.1% formic acid–

methanol solution, 50:50, v/v) to 4.5 kV ionization voltage, a capillary temperature of 270 °C, a sheath gas flow rate of 65 arbitrary units, and an auxiliary gas flow rate of 10 arbitrary units; organic acids and uridine were fragmented with 35% collision energy. For full MS analysis, spectra were recorded in the range of 60–1000 *m*/*z*. The width used to isolate precursor ions was set at 3.0 units. MS^n data were acquired in the automatic data-dependent mode. ThermoFisher Scientific Excalibur 2.0 software was used for data acquisition and processing.

2.6 Bacterial strains used and growth conditions

A. naeslundii ATCC 19039, *S. mutans* UA159, *P. intermedia* ATCC 25611, and two additional strains for each species, isolated from clinical specimens, and belonging to the collection of the Department of Diagnostics and Public Health, Section of Microbiology, University of Verona, were used throughout this study. *A. naeslundii* (ANV4 and ANV6) and *P. intermedia* (PIV1 and PIV3) clinical strains were isolated from subgingival plaque from patients with periodontitis; the strains of *S. mutans* (SMV20 and SMV21) were isolated from supragingival plaque of healthy donors.

A. naeslundii strains were cultured in Brain Heart Infusion Broth (BHIB, Difco Laboratories, Detroit, Mich.), *S. mutans* strains were grown in BHIB supplemented with sucrose (0.2%, final concentration), and *P. intermedia* strains were grown in BHIB supplemented of haemin (5 mg mL^{-1}, final concentration) and Vitamin K (1 mg mL^{-1}, final concentration). *S. mutans* strains were incubated at 37 °C in an atmosphere enriched with 5% CO_2[37] while *P. intermedia* and *A. naeslundii* strains were incubated at 37 °C in an anaerobic chamber with an atmosphere composed of 85% nitrogen, 10% hydrogen, and 5% CO_2.[38]

2.7 Antibacterial activity of tested samples

All samples were assayed for their antibacterial activities in a standard Minimum Inhibitory Concentration (MIC) assay determined following the microdilution procedure detailed according to the Clinical and Laboratory Standards Institute (CLSI)[39]. Bacterial cultures were prepared as

mentioned above. After overnight growth, the bacterial culture was diluted in broth to contain 10^5 cfu mL^{-1}. Two-fold dilutions of test samples and fractions in 0.1 mL of BHIB were placed into wells of flat-bottomed microtiter plates. A 10 μL volume of bacterial culture was then added. Following incubation of the plates for 18 h at 37 °C in ambient air or anaerobically, the MICs were determined. The MICs were recorded as the lowest concentration of test sample that completely inhibited visible growth of the bacteria.

2.8 Inhibition of biofilm formation by the target organisms

The capability of the selected fractions/standard compounds at different sub-MIC concentrations (see legends of Figures 2 and 3) to prevent biofilm formation was evaluated by the microtiter plate assay described by Cramton.[40] Briefly, bacterial suspensions were prepared in the appropriate growth medium containing different concentrations of the test material (pH adjusted to 7.0±0.1). The final concentration of bacteria was either 3–5 × 10^5 cfu mL^{-1} (*S. mutans* and *A. naeslundii*) or 5–8 ×10^6 cfu mL^{-1} (*P. intermedia*). Aliquots (200 μL) of the cell suspensions were inoculated into the wells of 96-well polystyrene microtiter plates. The wells were preconditioned (saliva-coated) in processed whole unstimulated pooled human saliva. The collection of saliva and the preconditioning have been previously described [35,41] (all saliva donors were given oral and written information about the purpose of the study and were asked to sign an informed consent. The study was conducted in accordance with the Ethical Principles of the 64th World Medical Association Declaration of Helsinki and consistent with good clinical practices). For each strain, test material untreated controls were included. Plates were then incubated at 37 °C in either 5% CO_2/air (*S. mutans*) or anaerobic conditions (*P. intermedia, A. naeslundii*), with incubation media changed every 24 h or 48 h for aerobic and anaerobic bacteria, respectively. Biofilm formation was quantified after 48 h (*S. mutans*) and 5 day incubation for *A. naeslundii* and *P. intermedia*. To this end, the growth medium was removed by aspiration; wells were gently washed with water and air dried; adherent bacteria were then stained with 0.01% crystal violet (100 μL). After 15 min

incubation at room temperature, wells were gently washed with water, and bound dye was extracted from stained cells by adding 200 μL of ethanol:acetone (8:2, v/v). Biofilm formation was quantified by measuring the absorbance of the solution at 540 nm. Values obtained in the treated sample were compared to the controls (100%) and expressed as % biofilm inhibition (IB). Experiments were run in triplicate and performed twice.[37] Since results obtained with reference and clinical strains were similar, Tables 2 and 4 showed combined values with statistical analysis.

2.9 Disaggregation of pre-existing biofilms of the target organisms

Mature biofilms of each of the test organisms were grown into preconditioned (saliva-coated) saliva wells, on 96-well polystyrene microtiter plates as previously described. The biofilms were grown for 48 h for *S. mutans* (aerobic organism) and 5 days for *A. naeslundii* and *P. intermedia* (anaerobic organisms). The mature biofilms were then incubated with the test compounds overnight. To this end, aliquots of fresh medium, containing different concentrations of GFC fractions or standard compounds were added to the wells (at least in triplicate). After overnight incubation the culture medium was discarded and biofilm formation was quantified as described above (section 2.8). Values obtained in the treated sample were compared to the controls (100%) and expressed as % biofilm disaggregation (BD). Experiments were run in triplicate and performed twice.[37] Since results obtained with reference and clinical strains were similar, Tables 2 and 5 show combined values with statistical analysis.

2.10 Statistical analyses

Data representing the mean ± SD of at least two experiments in triplicate were analyzed by the Mann-148 Whitney U test ($p<0.05$).

3. Results

In order to identify mushroom compounds able to interfere with the growth and virulence-related traits of *S. mutans*, *A. naeslundii*, and *P. intermedia*, the components of LMM fraction isolated from *L. edodes* extract were fractionated by the GFC technique (Fig. 1). Five fractions were collected (M1-M5) according to the nominal molecular mass of their components; each fraction was submitted to sterile ultrafiltration and freeze-dried. The fractions were then tested for their antimicrobial activity (MIC assay, section 2.7) towards clinical and reference *S. mutans*, *P. intermedia*, and *A. naeslundii* strains (Table 1). In addition, their capability to prevent biofilm formation on saliva conditioned microtiter plates and induce pre-existing biofilm disaggregation was also studied (see Material and Methods, sections 2.8 and 2.9) (Table 2).

As shown in Table 1 that reports average values obtained with the different reference and clinical strains, all GFC fractions were able to inhibit A. *naeslundii* and *P. intermedia* growth exhibiting different values, with the exception of M1 fraction toward *A. naeuslundii* and *P. intermedia*, and M2 fraction toward *P. intermedia* only. In contrast, no activity against *S. mutans* was observed with any fraction.

Considering biofilm formation and disaggregation, the results obtained showed that the GFC fractions had different effects on the three bacterial species tested (Table 2 reports the average values obtained with both reference and clinical strains). All the fractions possessed very low BI activity as regards *S. mutans* (BI = 2-6%) while they were more active against the anaerobic bacteria (BI = 42-78%). In the case of biofilm disaggregation (BD) tests, all the fractions were able to disaggregate the biofilm preformed by cariogenic *S. mutans* bacteria (BD = 22-50%) and also by gingivitis bacteria *A. naeslundii* (BD = 30-47%) and *P. intermedia* (BD = 26-74%).

As a whole, M4 fraction showed the highest activity, and therefore it was selected for further investigations to identify the active components.

By comparing their retention time, UV-Vis spectra and MS/MS fragmentation data with those of standard compounds, in the M4 fraction three organic acids, i.e. quinic, oxalic and succinic acids,

two nucleosides, i.e. inosine and uridine, and a nucleobase (adenine) were identified as major components.

Quinic acid, in full negative ion mass spectrum, exhibited a molecular ion at *m/z* 191 and the further fragmentation yielded to the base peak $[M\text{-}H\text{-}CO_2]^-$ at *m*/*z* 147 (corresponding to the decarboxylated acid), and also to secondary peaks at *m*/*z* 111, and *m*/*z* 85. Oxalic acid showed $[M-H]^-$ at *m*/*z* 89; in the mass spectrum, the ion corresponding to its dimeric form $[2M\text{-}H]^-$ at *m*/*z* 179 was also revealed. This was confirmed in the MS^2 spectrum by the presence of the base peak at *m*/*z* 135 corresponding to $[2M\text{-}H\text{-}COO]^-$. Succinic acid (MM 118) fragmented giving the ions at *m*/*z* 73 and at *m*/*z* 99 corresponding to the loss of a carboxylic group and a water molecule, respectively.

Nucleobase and nucleosides were detected in positive ionization mode. Adenine, uridine, and inosine showed in full mass spectrum molecular ions at *m/z* 136, 245, and 269, respectively. Adenine in MS/MS spectrum showed the presence of the typical fragment due to the loss of ammonium residue at *m/z* 119 ($[M+H\text{-}NH_3]^+$; uridine and inosine firstly lost the ribose residue (132 amu) giving the molecular ions at *m/z* 113 and 137, respectively (Table 3).

Quinic, oxalic, and succinic acids were also identified in a *Cichorium intybus* fraction previously studied; these acids showed anti-biofilm activity, at various degree, towards *S. mutans*, *A. naeslundii* and *P. intermedia*.[36] Studies performed by others have also shown that succinic acid inhibits growth of bacterial and fungal strains[42] while quinic acid derivatives are active against different bacterial strains[43,44], and oxalic acid is active against *Bacillus cereus*, *Staphylococcus aureus*, and *Enterococcus faecalis*.[45,46] Regarding basis, it was previously found that uracil influences quorum sensing and biofilm formation in *Pseudomonas aeruginosa*.[47] In addition, nucleosides and/or nucleotides and their analogues showed inhibitory activity towards different viruses (*e.g.*, HIV, HBV)[48,49], and regulate virulence factors, biofilm formation and quorum sensing in *Pseudomonas*. [50]

Our previous investigations showed about 25 fold higher concentration of quinic acid in an edible mushroom[51] (478 μg g^{-1} fresh) than in *C. intybus* red chicory (<20 μg g^{-1} fresh). Unpublished results

from our laboratory showed that the concentration of oxalic acid in mushroom is about two fold higher than in chicory (47.5 ± 2.4 and 26.4 ± 2.8 μg g^{-1} fresh vegetable for mushroom and chicory, respectively), while succinic acid concentration is about three fold lower than in chicory (37.2 ± 5.1 and 108.2 ± 4.7 μg g^{-1} fresh vegetable for mushroom and chicory, respectively). Quinic and oxalic acids higher concentration in mushroom could explain the higher activity of mushroom extract, as compared to chicory extract and related LMM fraction.[36] As the results obtained for mushroom extract[35] and its fractions (Table 2) were not always consistent, thus indicating the possibility of interactions among the different components present in mushroom, it was decided to study the activity of each characterized compounds and the possibility of interactions among them, using the commercial compounds.

These standards were tested on both reference and clinical strains at increasing concentrations starting from the average values reported in literature for plant materials [52-58] (Tables 4-5 report the average values obtained with both reference and clinical strains; for each standard compound the minimum active concentration is also reported).

Quinic and oxalic acids, uridine, adenine, and inosine, when tested alone (Table 4), inhibited weakly biofilm formation by all tested *S. mutans* strains (BI = 21-52% at the highest concentration tested for each substance), while no activity was registered for succinic acid at any tested concentrations. In contrast, the mixtures consisting of the two commercial nucleosides, i.e. uridine 5mg mL^{-1} and inosine 1mg mL^{-1} (U5+I1), or of a nucleoside with adenine, i.e. uridine 5mg mL^{-1} and adenine 2mg mL^{-1} (U5+A2) or adenine 2mg mL^{-1} and inosine 1mg mL^{-1} (A2+I1) (Figure 2a) strongly inhibited biofilm formation (BI = 70%). High values were given also by quinic acid (3.5 mg mL^{-1}, Q3) plus each nucleoside or nucleobase at the lowest tested concentrations (BI = 40-70%). These results highlight additive or even synergistic action between the compounds in such binary mixtures. The presence of oxalic acid did not increase the nucleoside or nucleobase activity values (Figure 2a). When succinic acid was mixed with quinic or oxalic acid, strong negative interactions were showed and activity values lower than 35% were registered (data not shown).

Testing mixtures consisting of more than two standards, contrasting results were obtained (Figure 2b). In fact, ternary and pentenary mixtures showed in some cases higher activity values ($p<0.05$) than those registered for single compounds or binary mixtures (e.g., Q3+I2+A3 or Q3+O2+I2+A5+U5), while in others a decreased activity depending on the tested concentrations was observed (e.g., Q1+O2+I2+A5+U5) (Figure 2b). This comparison points out apparent negative interactions.

Regarding reference and clinical *A. naeslundii* strains, very high activity was shown by oxalic acid (BI = 81-85%), quinic acid at 3.5mg mL^{-1} (BI = 74%), and by inosine (BI > 67%), while lower activity was presented by adenine (BI = 41-52%) and no activity was found for uridine and succinic acid (Table 4). When a couple of basic compounds was considered (Figure 2a), a significant increase in the inhibition value was registered in comparison with the values found for the single active nucleoside or nucleobase ($p<0.05$). If quinic (3.5mg mL^{-1}, Q3) or oxalic acids were mixed with a nucleoside or nucleobase, nearly the same activity of each single acid was registered. Even the mixture of the two active acids gave about the same activity as the single acid and in this case the maximum of percentage inhibition reached corresponded to BI 70-80% (Figure 2a). These results seem to indicate that when an acid is present, nucleoside or nucleobase are not able to act. The more complex mixture of the five active tested standards (succinic acid was not considered because not active) gave low values close to those registered for the single active compounds and this may indicate strong interactions among the compounds present. Considering the mixture containing quinic or oxalic acid and adenine plus inosine, the activity profiles were the same registered for *S. mutans*, even if with higher values (Figure 2b).

Biofilm formation by the different *P. intermedia* strains was weakly inhibited by the tested mushroom compounds. In fact, the highest BI values obtained ranged from 28 to 39% for uridine and inosine alone (Table 4); regarding their mixtures, the highest BI values was 34% (Figure 2a). Any other tested mixture did not increase the inhibition of biofilm production that was lower than 26% (Figure 2b).

The compounds exhibited weak activity either alone or in mixtures in disaggregating biofilm formed by *P. intermedia* and *S. mutans* reference and clinical strains (Table 5) with the exception of quinic acid when mixed with adenine (Q3+A2, BD = 35%) or inosine (Q3 + I3, BD = 37%) (Figure 3a), or with both adenine and inosine (Q3+I2+A3, BD = 42%) (Figure 3b).

A notable activity in the disaggregation of preformed *A. naeslundii* biofilm was found for quinic acid at the highest concentration (Q3, BD about 50%) and inosine at all tested concentrations (BD = 37-38%); adenine at all tested concentration showed a lower activity (BD = 29-31%) (Table 5). Regarding mixtures, a remarkable BD activity was observed for quinic 3.5mg mL^{-1} + oxalic acid 2mg mL^{-1} (Q3 + O2, BD = 36%) (Figure 3a), and quinic acid 3.5mg mL^{-1} + inosine 2mg mL^{-1} + adenine 3mg mL^{-1} (Q3 + I2 + A3) (Figure 3b). The other couples of basic compounds and the mixture quinic acid plus a single basic compound showed low activity values (BD = 30%) indicating negative interactions confirmed by the results given by the pentenary mixture containing the two acids and the three basic compounds that showed activity values closed to 20% (Figure 3a).

Considering *P. intermedia* strains, the activity of uridine 5mg mL^{-1} (U5, BD = 20 - 25%, depending on strain) and uridine mixed with adenine 2mg mL^{-1} (BD = 23 %) was weak (Table 5, Figure 3b).

4. Discussion

The reported results showed that the identified compounds and all their tested mixtures are able to inhibit *S. mutans* biofilm production by at least 21% (with the exception of succinic acid) and *A. naeslundii* by at least 32% (with the exception of uridine and succinic acid). In the case of *S. mutans*, the mixtures quinic acid with inosine or uridine were able to inhibit the biofilm production more than 70% and in the case of *A. naeslundii* the highest detected activity was due to quinic acid plus adenine (Q3 + A2) and quinic acid plus oxalic acid (Q3 + O2), that inhibited 80% of biofilm production. Weaker activity was found for *P. intermedia*. In fact, only inosine, uridine and the mixture of these two standard compounds showed BI values higher than 30%.

Considering biofilm disaggregation, when biofilm was produced by *S. mutans*, only quinic acid in mixture with adenine or inosine gave BD values higher than 35%. Conversely, as regards *A. naeslundii*, BD activity higher than 50% was found for quinic acid at the highest concentration; adenine, inosine and the mixtures of quinic acid with oxalic acid and of adenine with inosine showed 30-40% BD capacity. Considering *P. intermedia*, BD activity higher than 20% was registered only for uridine. As a whole, these compounds are much more active in inhibiting biofilm formation than disaggregation. This can be explained by the fact that planktonic bacteria are more susceptible to the action of compounds present in the external environment than bacteria inside a biofilm. It is well known that cells embedded in biofilm are up to 1000-fold more resistant to antibiotics compared to their free living counterpart.[59] However, the fact that our results pointed out that the disaggregation action occurs with the inhibition of biofilm production by *A. naeslundii* and *S. mutans* could be important in reinforcing capability of these compounds in their potential use as anti-caries and anti-gingivitis agents.

Another important point of this study is the demonstration that the outcome of exposure of bacteria to mixtures is either a summation effect of each compound present in the mixture (although a complete inhibition of biofilm formation was never observed) or an antagonistic effect. The outcome seems to be dose dependent. In general, low doses of the compounds in combination produce additive effects, whereas higher doses produce antagonistic effects probably as a result of chemical-physical negative interactions among the different compounds.

When evaluating the relevance of the obtained results, we must also consider that bacteria were exposed to the tested compounds (alone or in combination) up to 5 days. Therefore, the possibility that the compounds are transformed and modified in their biological activity by growing bacteria should be taken into account. Although elucidation of the molecular mechanisms of the bioactivity of the identified compounds is beyond the scope of this study, it can be hypothesized that they might either inhibit the activity of key enzymes involved in matrix formation or hamper quorum sensing system. It is also possible that the tested components interfere with adhesion/co-aggregation

mechanisms by binding outer membrane proteins of Gram-negative bacteria and disorganizing the phospholipid bilayer.[60] In Gram-positive cells, particularly as regards acid components, it is possible that the active components modify the electrical charge of the cell wall thus altering the electrical potential and/or the hydrophobicity of the surface. This is supported by previous observations showing that exposure to phenolic acids reduces the negative charge of the bacterial surface.[61] These options are currently under investigation in our laboratory.

5. Conclusion

The evolution, selection and spread of bacterial resistance to a wide range of antibiotics makes the development of novel strategies to prevent and treat bacterial infections crucial. An increasing interest in identifying anti-infectious components that exist in dietary sources has been registered since the 1980s.

In this context, the data reported here show that *L. aedodes*, a mushroom to which many beneficial effects on human health have been attributed, contains a number of LMM compounds potentially useful for combating some of the bacteria responsible for certain oral cavity diseases, as they were active in *vitro* against some of the virulence-related traits of *S. mutans*, the main etiological agent of dental caries in humans, and *A. naeslundii* and *P.intermedia,* both of which are involved in gingivitis.

However the results obtained are based on *in vitro* studies only, caution is therefore advised in the interpretation of the *in vivo* significance of our findings. Further investigations are needed to test the safety and efficacy of *L. aedodes* LMM compounds in *vivo* and to define the actual impact on oral health of the consumption of *Lentinus* mushrooms consumption in the diet.

Overall, these studies contribute to both the design of new antibacterial strategies and to reverse the common perception of the negative effects of dietary constituents on the oral microbiota and oral health, in that foods/food components are not necessarily deleterious (*e.g*. sucrose and dental caries) but could possibly exert a beneficial effect.

Conflicts of interest

There are no conflicts of interest to declare.

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Table 1. MICs of mushroom GFC fractions, expressed as mg dry residue mL^{-1}.

Target organism					
	M1	M2	M3	M4	M5
S. mutans UA159	>3.99[a]	>3.68 [a]	>0.48 [a]	>0.51 [a]	>1.13 [a]
S. mutans SMV20	>3.99 [a]	>3.68 [a]	>0.48 [a]	>0.51 [a]	>1.13 [a]
S. mutans SMV21	>3.99 [a]	>3.68 [a]	>0.48 [a]	>0.51[a]	>1.13[a]
A. naeslundii ATCC 19039	>3.99 [a]	1.84	0.48	0.51	1.13
A. naeslundii ANV4	>3.99 [a]	1.84	0.48	0.51	1.13
A. naeslundii ANV6	>3.99 [a]	1.84	0.48	0.51	1.13
P. intermedia ATCC 25611	>3.99 [a]	>3.68 [a]	0.48	0.51	1.13
P. intermedia PIV1	>3.99 [a]	>3.68 [a]	0.48	0.51	1.13
P. intermedia PIV3	>3.99 [a]	>3.68 [a]	0.48	0.51	1.13

[a] this value indicated no activity.

Table 2. Effects of mushroom GFC fractions (at sub-MIC) on biofilm formation by *S. mutans*, *A. naeslundii*, and *P. intermedia* and its disaggregation. Reported data are the mean values obtained from triplicate experiments performed with three strains per species (one reference and two clinicals) (mean ± SD, *n* =9).

Assays	M1 (%)	M2 (%)	M3 (%)	M4 (%)	M5 (%)
Prevention of biofilm formation of					
S. mutans[1]	6.3 ± 0.9	6.1 ± 1.4	1.8 ± 0.8	4.2 ± 1.0	6.4 ± 0.9
A. naeslundii[2]	41.7 ± 3.1*	44.3 ± 2.2*	72.3 ±4.1*	78.2 ± 1.9*	53.0 ± 4.6*
P. intermedia[3]	42.1 ± 2.5*	41.9 ± 2.6	68.6 ± 1.5*	73.7 ± 4.1*	52.2 ± 6.9*
Disaggregation of pre-existing biofilms of					
S. mutans[1]	49.7 ± 2.9*	22.6 ± 2.2*	32.0 ± 5.0*	44.6 ± 2.4*	21.8 ± 3.3*
A. naeslundii[2]	47.3 ± 2.2*	30.4 ± 4.6*	35.0 ± 3.0*	36.9 ± 2.3*	29.7 ± 2.7*
P. intermedia[3]	25.9 ± 3.7*	37.4 ± 2.1*	26.7 ± 4.4*	74.3 ± 4.4*	51.8 ± 3.1*

[1] Sub-MIC concentrations: M1 = 3.99, M2 = 3.68, M3 = 0.48, M4 = 0.51, M5 = 1.13 mg dry residue mL^{-1};
[2] Sub-MIC concentrations: M1 = 3.99, M2 = 0.46, M3 = 0.12, M4 = 0.13, M5 = 0.28 mg dry residue mL^{-1}
[3] Sub-MIC concentrations: M1 = 3.99, M2 = 3.68, M3 = 0.12, M4 = 0.13, M5 = 0.28 mg dry residue mL^{-1}

*differences between treated and control samples were statistically significant ($p<0.05$)

Table 3. MS and MS/MS data of the detected compounds in M4 fraction.

Precursor ion (*m/z*)	HPLC-ESI/MS^2 *m/z* (% of base peak)	Compound identity
191	MS^2 [191]: 147(100), 111(40), 85(55)	quinic acid
89 179[a]	MS^2[179]: 135(100), 89(60)	oxalic acid
117	MS^2 [117]: 99(70), 73(100)	succinic acid
136[b]	MS^2 [136]: 119(100)	adenine
245[b]	MS^2 [245]: 113(100)	uridine
269[b]	MS^2 [269]: 137(100)	inosine

[a] dimeric ion

[b]positive ionization mode

Table 4. Effects of standard compounds on *S. mutans*, *A. naeslundii*, and *P. intermedia* ability to form biofilm. Reported data are the mean values obtained from triplicate experiments performed with three strains per species (one reference and two clinical) (mean ± SD, *n* =9).

Standard #	Concentration (mg mL^{-1})	*S. mutans* BI ± SD (%)	[a]MCIA (mg mL^{-1})	*A. naeslundii* BI ± SD (%)	[a]MCIA (mg mL^{-1})	*P. intermedia* BI ± SD (%)	[a]MCIA (mg mL^{-1})
Q1	1.0	27.6±6.9*	1.0	32.0±3.8*	1.0	17.2±2.8*	1.0
Q2	2.0	28.3±4.6*		43.2±3.6*		22.0±2.5*	
Q3	3.5	31.6±2.5*		73.8±4.1*		31.6±2.9*	
O1	1.0	23.0±3.4*	1.0	84.6±4.3*	1.0	5.0±2.1	[b]n.a.
O2	2.0	20.6±3.3*		89.1±8.2*		4.8±3.0	
U2	2.0	6.9±2.1	5.0	1.0±0.9	[b]n.a.	6.0±2.5	5.0
U5	5.0	32.6±4.4*		2.6±1.3		38.7±5.2*	
A2	2.0	43.3±4.6*	2.0	40.9±2.7*	2.0	16.2±3.2*	2.0
A3	3.0	40.4±4.4*		42.9±3.4*		19.7±3.5*	
A5	5.0	52.1±4.1*		51.8±3.6*		14.1±2.6*	
I1	1.0	46.6±6.0*	1.0	66.8±4.0*	1.0	35.4±5.0*	1.0
I2	2.0	33.8±3.8*		68.7±5.3*		29.0±4.2*	
I3	3.0	48.4±7.6*		69.1±7.4*		27.7±5.8*	
S1	1.0	6.2±2.3	[b]n.a.	1.0±1.1	[b]n.a.	2.4±1.9	[b]n.a.
S2	2.0	6.8±1.6		2.0±1.0		3.9±2.0	

Q, quinic acid; O, oxalic acid; U, uridine; A, adenine; I, inosine; S, succinic acid
[*] differences between treated and control samples were statistically significant ($p<0.05$)
[a]MCIA, Minimum Concentration with Inhibitory Activity
[b]n.a., no inhibitory activity was observed at any tested concentration.

Table 5. Effects of standard compounds on disaggregation of biofilm formed by *S. mutans*, *A. naeslundii*, and *P. intermedia*. Reported data are the mean values obtained from triplicate experiments performed with three strains per species (one reference and two clinical) (mean ± SD, *n* =9).

Standard #	Concentration (mg mL^{-1})	*S. mutans* BD ± SD (%)	[a]MCDA (mg mL^{-1})	*A. naeslundii* BD ± SD (%)	[a]MCDA (mg mL^{-1})	*P. intermedia* BD ± SD (%)	[a]MCDA (mg mL^{-1})
Q1	1.0	2.0 ± 0.3*	[b]n.a.	14.9 ± 1.7	1.0	1.0 ± 0.3*	[b]n.a.
Q2	2.0	8.2 ± 0.7*		19.6 ± 1.7		3.8 ± 2.0*	
Q3	3.5	10.8 ± 2.7		51.6 ± 3.4		4.0 ± 1.2*	
O1	1.0	1.3 ± 0.4*	[b]n.a.	15.2 ± 2.2	1.0	15.7 ± 1.9	1.0
O2	2.0	3.3 ± 0.4*		15.1 ± 2.1		16.1 ± 2.0	
U2	2.0	6.4 ± 2.0*	[b]n.a.	1.3 ± 0.4*	[b]n.a.	7.6 ± 2.8*	5.0
U5	5.0	9.3 ± 2.2*		2.1 ± 0.5*		23.3 ± 4.5	
A2	2.0	15.3 ± 1.8	2.0	30.0 ± 3.6	2.0	15.1 ± 3.4	2.0
A3	3.0	15.1 ± 2.4		29.1 ± 2.9		14.7 ± 3.0	
A5	5.0	18.4 ± 1.4		30.8 ± 4.1		19.4 ± 1.5	
I1	1.0	10.4 ± 1.1	1.0	38.2 ± 7.3	1.0	1.0 ± 0.6*	[b]n.a.
I2	2.0	11.1 ± 1.1		37.0 ± 4.9		4.7 ± 1.7*	
I3	3.0	13.5 ± 1.4		38.0 ± 4.1		5.4 ± 1.1*	
S1	1.0	5.7 ± 2.2*	[b]n.a.	3.9 ± 0.6*	[b]n.a.	3.6 ± 1.0*	[b]n.a.
S2	2.0	8.2 ± 0.7*		5.6 ± 1.8*		6.1 ± 1.8*	

Q, quinic acid; O, oxalic acid; U, uridine; A, adenine; I, inosine; S, succinic acid
[*] differences between treated and control samples were statistically significant ($p<0.05$)
[a]MCDA, Minimum Concentration with Disaggregation Activity
[b]n.a., no disaggregation activity was observed at any tested concentration.

Figure captions

Figure 1. GFC chromatogram of LMM mushroom extract registered at 210 nm.

Figure 2. Biofilm Inhibition (BI) percentage of binary mixtures (a) and ternary and pentenary mixtures (b). White bars referred to *A. naeslundii*, grey bars to *S. mutans*, and black bars to *P. intermedia*. Reported data are the mean values obtained from triplicate experiments performed with three strains per species (one reference and two clinical) (mean ± SD, *n* =9).

Q1= Quinic acid 1 mg mL^{-1}; Q2= Quinic acid 2 mg mL^{-1}; Q3= Quinic acid 3.5mg mL^{-1}

O1= Ossalic acid 1 mg mL^{-1}; O2= Ossalic acid 2 mg m L^{-1}

I1= Inosine 1 mg mL^{-1}; I2= Inosine 2 mg mL^{-1}; I3= Inosine 3mg mL^{-1}

A2= Adenine 2 mg mL^{-1}; A3= Adenine 3 mg mL^{-1}; A5= Adenine 5 mg mL^{-1}

S1= Succinic acid 1 mg mL^{-1}; S2= Succinic acid 2 mg mL^{-1}

U5 = Uridine 5mg mL^{-1}

Figure 3. Biofilm Disaggregation (BD) percentage the pre-formed biofilm of binary mixtures (a) and ternary and pentenary mixtures (b). White bars referred to *A. naeslundii*, grey bars to *S. mutans*, and black bars to *P. intermedia*. Reported data are the mean values obtained from triplicate experiments performed with three strains per species (one reference and two clinical) (mean ± SD, *n* =9).

Q1= Quinic acid 1 mg mL^{-1}; Q2= Quinic acid 2 mg mL^{-1}; Q3= Quinic acid 3.5mg mL^{-1}

O1= Ossalic acid 1 mg mL^{-1}; O2= Ossalic acid 2 mg m L^{-1}

I1= Inosine 1 mg mL^{-1}; I2= Inosine 2 mg mL^{-1}; I3= Inosine 3mg mL^{-1}

A2= Adenine 2 mg mL^{-1}; A3= Adenine 3 mg mL^{-1}; A5= Adenine 5 mg mL^{-1}

S1= Succinic acid 1 mg mL^{-1}; S2= Succinic acid 2 mg mL^{-1}

U5 = Uridine 5mg mL^{-1}