Tryptophol acetate and tyrosol acetate, metabolites secreted by a probiotic yeast, halt cytokine storm

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Abstract

Probiotic fermented foods are perceived as contributing to human health and capable 1
of protecting against inflammation, however solid mechanistic evidence for the 2
presumptive therapeutic benefits is lacking. Here we report that tryptophol acetate and 3
tyrosol acetate, small molecule metabolites secreted by the probiotic milk-fermented 4
yeast *Kluyveromyces marxianus* exhibit remarkable anti-inflammatory properties. 5
Comprehensive *in vivo*, *ex vivo* and *in vitro* experiments, employing LPS-induced 6
‘cytokine storm’ models, reveal dramatic effects of the two molecules, added in tandem, 7
on mice morbidity, laboratory parameters and mortality. In parallel, significant 8
attenuation of pro-inflammatory cytokines including IL-6, IL-1 α , IL-1 β and TNF- α , 9
and reduction of reactive oxygen species were recorded. Importantly, tryptophol acetate 10
and tyrosol acetate did not completely suppress cytokine generation, but rather brought 11
their concentrations back to baseline levels, further maintaining core immune functions, 12
including phagocytosis. The anti-inflammatory effects of tryptophol acetate and tyrosol 13
acetate were mediated through downregulation of TLR4, IL-1R, and TNFR signaling 14
pathways and increased A20 expression, attenuating NF- κ B level. In addition, the two 15
molecules had a significant impact on mice microbiome, increasing the abundance of 16
the genus *Bactericoides*, known to exhibit anti-inflammatory properties. Overall, this 17
work illuminates pronounced and broad-based immune modulation properties of 18
probiotic yeast-secreted metabolites, uncovering their mechanism of action and 19
underscoring potential new therapeutic avenues for severe inflammation. 20

Introduction

Sepsis is a life-threatening condition manifested by severe inflammation leading to multiple organ dysfunction. The inflammatory host response associated with both innate and adaptive immunity mechanisms play an important role in the development of the clinical and pathological manifestations of sepsis¹. Recently, it has been found that the prognosis of the disease is dependent not only on the virulence of the microorganisms, but, mainly, on the host response affected by the pathogen-associated molecular patterns (PAMPs)^{2,3}. Recruitment of immune cells and secretion of soluble mediators by the cells can exacerbate the severity of the disease⁴. Specifically, the release of pro-inflammatory molecules, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α) and interleukin-1 α and 1 β (IL-1 α and IL-1 β), trigger "cytokine storms", systemic inflammation often leading to multi-organ failure and adverse clinical outcomes with high mortality rates⁵. Cytokine storms occur in various disease conditions, including sepsis and septic shock⁶ and acute stages in chronic diseases⁷. Severe inflammation and the occurrence of cytokine storms were also shown to be major causes of mortality from COVID-19^{8,9}.

Despite the significant progress in inflammation treatment following the discovery of antibiotics, high mortality from sepsis still exists. Thus, new approaches to improve conventional therapies are highly sought. Varied food products have been touted to endow anti-inflammatory properties and as such attract significant interest¹⁰. Food-extracted substances have been particularly explored as anti-inflammatory agents, including probiotics^{11,12}, curcumin¹³, resveratrol¹⁴, plant extracts¹⁵, and phenolic compounds from natural sources¹⁶. However, the therapeutic benefits of most such substances against severe inflammation have been limited¹⁷, and, moreover, detailed

mechanistic understanding of their perceived anti-inflammatory properties are 44
generally lacking. 45

Probiotics, particularly milk-fermented microorganism mixtures (yogurt, kefir), have 46
been known to bolster the innate immune system and host-defense mechanisms against 47
pathogens^{18,19}. In a recent study, we reported on a yet-unrecognized mechanism for 48
cross-kingdom inhibition of pathogenic bacterial communication and virulence by a 49
small molecule - tryptophol acetate – secreted by the probiotic yeast *Kluyveromyces* 50
marxianus in a milk-fermented probiotic microorganism mixture²⁰. Specifically, 51
tryptophol acetate was found to disrupt biofilm formation and reduce virulence of 52
several human pathogenic bacteria, underscoring a novel mechanism for combating 53
bacterial colonization and pathogenicity. 54

Here, we report that tryptophol acetate and tyrosol acetate, another *K. marxianus* – 55
secreted metabolite, exhibit remarkable anti-inflammation activities, in *in vitro*, *ex vivo* 56
and *in vivo* models. Through application of LPS-induced cytokine storm, we observed 57
that the two molecules had synergistic anti-oxidation, anti-inflammatory, clinical, 58
histological and hematological systemic protective effects against severe inflammation. 59
Importantly, tryptophol acetate and tyrosol acetate did not give rise to immune system 60
shutdown, rather reduced pro-inflammatory cytokine production to baseline levels 61
while retaining core immune processes including phagocytosis and generation of anti- 62
inflammatory cytokines. Detailed molecular analysis indicates that the anti- 63
inflammatory activities of tryptophol acetate and tyrosol acetate are mediated through 64
downregulation of *TLR4*, *IL-1R*, and *TNFR* signaling pathways and suppression of NF- 65
 κ B activity. In particular, we discovered that the molecules enhanced expression of 66
A20, a key modulator of NF- κ B signaling pathways²¹. Overall, this study demonstrates 67
remarkable anti-inflammatory properties of probiotic yeast-secreted metabolites and 68

furnishes a detailed mechanistic description of the effects, underscoring their
significant therapeutic potential.

Results and Discussion

Anti-oxidation properties of tryptophol acetate and tyrosol acetate

Scheme 1 depicts the chemical structures of tryptophol acetate (**1**) and tyrosol acetate (**2**), recently identified as metabolites secreted by the probiotic fungus *Kluyveromyces marxianus* in milk-fermented microorganism mixture (“kefir”), and shown to exhibit intriguing antibacterial properties through blocking quorum sensing²². Importantly, no inflammation-modulatory activities have been previously reported for neither tryptophol acetate nor tyrosol acetate.

Scheme 1. Chemical structures of tryptophol acetate (1) and tyrosol acetate (2).

We first analyzed the anti-oxidation properties of the molecules (Figure 1) as oxidation processes and generation of reactive oxidative species (ROS) are recognized as contributing to toxic effects in diverse diseases and pathological conditions²³. Figure 1 depicts application of a spectrophotometric assay monitoring the visible absorbance of

the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, which is widely employed for evaluating the anti-oxidation activity of biomolecules²⁴. In this assay, the DPPH free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to a colorless ethanol solution. The bar diagram in Figure 1A shows the degree of quenching of DPPH radical absorbance (and concomitant anti-oxidative properties) by tryptophol acetate and tyrosol acetate, when added individually as well as together. Figure 1A indicates a dramatic synergistic anti-oxidation effect for the mixture of the two molecules. Specifically, while **1** individually gave rise to 10% quenching of DPPH absorbance and **2** alone reduced the absorbance by ~3%, when the **1** and **2** were added together (at the same concentrations) to the DPPH solution, 30% absorbance attenuation was recorded (Figure 1A).

Figure 1. Anti-oxidation effect of tryptophol acetate and tyrosol acetate. Anti-oxidative capacity of the molecules as measured by DPPH (A) and ORAC (B) assays. A. The results are presented as a percentage of absorbance inhibition (517 nm, the absorbance maximum of DPPH) in the presence of tryptophol acetate (**1**) alone, tyrosol acetate alone (**2**) alone, and **1+2** together. B. Fluorescence decay of fluorescein induced by AAPH in the absence or presence of **1** and **2**. Reaction mixtures containing fluorescein (60 nM) and AAPH (18.75 mM) in 200 μ l of phosphate buffer (75 mM, pH 7.4) were incubated at 37°C for 800 min. Changes in fluorescence intensity emitted by fluorescein were monitored. Results are presented as the net area under the curve (AUC). Each value is the mean $\pm$ SD of triplicate experiments. *** p <0.0001

The results of the oxygen radical absorbance capacity (ORAC) assay in Figure 1B further demonstrate substantial synergistic anti-oxidation activity by tryptophol acetate and tyrosol acetate when added together. The ORAC experiment measures the quenching of fluorescein emission by peroxy radicals, and inhibition of the oxidation reaction by molecular species added to the solution²⁵. As shown in Figure 1B, in the control sample, the fluorescence emission recorded for fluorescein decreased instantaneously because of the significant quenching induced by the peroxy radicals. However, upon co-addition of **1** or **2**, the fluorescence decays were significantly longer, up to around 400 min (in the case of **1**, purple curve) and 800 min (for **2**, beige curve), likely accounting for scavenging of the peroxy radical by the molecules. Strikingly, significant inhibition of peroxy-induced fluorescence quenching occurred when tryptophol acetate and tyrosol acetate were added together to the solution (green curve in Figure 1B), underscoring their synergistic anti-oxidation effect.

Oral uptake of tryptophol acetate and tyrosol acetate mixture provides protection against LPS-induced cytokine storm in mice

As tryptophol acetate and tyrosol acetate exhibit significant synergistic anti-oxidation activity, we next investigated the anti-inflammatory properties of the **1+2** mixture *in vivo* using a model of lipopolysaccharide (LPS)-induced inflammation, which triggers a massive pro-inflammatory cytokine release identified as a "cytokine storm"^{26,27}. In the experiments, C57BL/6 mice were injected with LPS (30 mg/kg), and the effects of the **1 + 2** mixture administered orally by gavage (each molecule at 150 µg/Kg per mouse) were monitored up to 156 hours after LPS injection (scheme in Figure 2A).

Figure 2. Tryptophol acetate and tyrosol acetate mixture shield mice from LPS-induced severe inflammation. (A) Experimental scheme. After intraperitoneal (IP) injection of LPS (30 mg/kg), mice were randomly divided into two groups, PBS-treated and treated with 1+2 immediately after LPS injection (B, C). A third group treated with the molecules 28 hours after LPS injection (D, E). Survival (B, D) and weight loss (C, E) of the mice were monitored for 156 hours. A 2-way ANOVA statistical analysis was performed, and P-values were calculated for the weight loss experiments (P-values < 0.001; n=12).

Initially, mice were injected with LPS and at the same time interval were treated with **1+2** mixture. The percentage survival and weight loss in the LPS-administered mice, with and without treatment, show that the molecules mixture significantly improved the disease outcome. Remarkably, while all LPS-treated mice died within 72 hours due to the severe inflammation (i.e. the “cytokine storm”), 100% of the mice orally given the mixture of **1 + 2** survived (Figure 2B). Furthermore, the LPS-administered mice experienced substantial weight loss prior to mortality (Figure 2C, black line). In contrast, mice which were orally treated with the molecular mixture, initially lost weight (up to 72 hours), but reverted to their initial values within 156 hours (Figure 2C, green line).

To prove the therapeutic effects of the molecules, we carried out another experiments, in which **1+2** mixture was given to mice that were already impacted by the onset of LPS-induced inflammation (Figure 2D-E). In these experiments, the molecular mixture was administered to the mice by gavage 28 hours after LPS injection. At this time-interval, severe signs of the disease were already evident, including decreased motor activities, ruffled fur, diarrhea, substantial eye discharge and respiratory distress. Notably, the results in Figure 2D demonstrate that while all untreated mice died within 48 hours after LPS injection, 100% of the mice treated with the **1 + 2** mixture survived. The weight lost patterns in mice treated with the molecules 28 hours after LPS administration when severe inflammation was already developed (Figure 2E) were similar to the case of mice that were orally given the two molecules simultaneously with LPS (e.g., Figure 2C). Overall, the results presented in Figure 2 demonstrate an exceptional therapeutic effect of the tryptophol acetate and tyrosol acetate mixture.

Oral uptake of tryptophol acetate and tyrosol acetate decreased tissue damage 166
induced by LPS 167

Cytokine storms generally account for multisystem failure manifested in considerable 168
damage to the lungs, liver, and the gastrointestinal (GI) tract ²⁸. To investigate whether 169
the healing effects of tryptophol acetate and tyrosol acetate (e.g., Figure 2) also lead to 170
reduced organ damage, we carried out histology analyses of different tissues obtained 171
from LPS-injected mice treated or non-treated with the **1+2** mixture (Figure 3). The 172
representative hematoxylin and eosin (H&E) staining image of lung tissue from a 173
healthy mouse displays normal alveolar walls and no inflammatory cell infiltration 174
(Figure 3A, top row). In LPS-injected mice, signs of severe lung injury characterized 175
by lung edema, hemorrhage, intensive cellular infiltrate, and accumulation of fibrin 176
were observed already during the first 6 hours (Figure 3A, middle panel). 177

Figure 3. Tryptophol acetate and tyrosol acetate prevent inflammation-associated tissue damage. A(i), B and C Representative pictures of H&E-stained sections of

obtained organs from healthy and LPS-injected mice with or without administration of molecule mixture **(A(i))** lung, **(B)** liver and **(C)** small intestine samples. Original magnification x20 for lung and small intestine, x10 for liver. Quantification of tissue damage was performed, as discussed in Materials and Methods (M&M) and is presented as a bar diagram on top of each panel. **(A (ii))** Lung tissues obtained 6 and 24 h after LPS administration were stained with anti-myeloperoxidase (MPO) antibodies for neutrophils detection. Original magnification x10. of the amount of neutrophils was counts as discribed in M&M and presented in the graph. **(A(iii))** Monocytes were detected by anti-Ly6C antibodies and macrophages by anti-F4/80 antibodies. Quantification of the positive cells was assessed, as described. Insert in panel B represents an amount of hepatocytes with preserved cell structure. Insert in panel C represents quantification of intestinal crypt damage. Representative pictures are presented in magnification x20. *p < 0.05; **p < 0.01; ***p < 0.0001 ****p<0.00001

Strikingly, lung tissue damage was largely eliminated in mice to which the **1+2** mixture was co-administered with LPS [Figure 3A(i), bottom row]. Indeed, the pulmonary architecture was mostly preserved in those mice, with only small local zones of inflammation. In addition, the alveolar walls were almost not altered, and minimal accumulation of inflammatory cells was observed at the indicated time intervals. Notably, after 72 hours (in which only mice treated with the molecules survived), the lung structure was similar to the structure observed in healthy mice (Figure 3A(i)). Lung tissue damage was assessed by quantification of pathological score [bar diagram in Figure 3A(i), inset]. Indeed, the increase in mean pathological score after LPS administration, was significantly reduced after treatment with **1 + 2** mixture (at 24 and 72 hours). Those findings demonstrate that tryptophol acetate and tyrosol acetate mixture effectively blocked inflammation-induced damage in lungs.

To further examine the protective effects of the molecules on lung damage after LPS-induced inflammation, we assessed the recruitment of myeloid cells into the lungs (Figure 3A,ii-iii). In the experiments, we performed immunohistochemical (IHC) staining with anti-MPO antibodies for neutrophils, anti-Ly6C antibodies for monocytes

(Ly6C^{positive}) and anti-F4/80 antibodies for macrophages (F4/80^{pos}), considered mature 212
macrophages ²⁹. Figure 3A(ii), top row, shows minimal neutrophil infiltration in lungs 213
from control mice. However, lungs of LPS-treated mice displayed significant 214
recruitment of neutrophils already 6 hours after LPS injection (Figure 3A(ii), middle 215
row). Remarkably, in mice which received the **1+2** mixture, the recruitment of 216
neutrophils was significantly decreased and was comparable to healthy (PBS treated) 217
mice (bar diagram in Figure 3A(ii), inset). 218

Figure 3A(iii) illuminates recruitment of monocytes and macrophages into the lungs. 219
Both types of cells are strongly associated with inflammation-induced lung 220
pathogenesis ^{30,31}. Lung-recruited Ly6C^{pos} monocytes account for microvascular 221
endothelial cell activation and vascular injury in LPS-induced early endotoxemia, 222
leading to enhanced pulmonary vascular leakage ^{32,33}. Figure 3A,iii shows that while 223
the abundance of lung-recruited Ly6C^{pos} monocytes significantly increased following 224
LPS administration both after 6 and 24 hours, treatment with tryptophol acetate and 225
tyrosol acetate mixture effectively reduced Ly6C^{pos} monocytes levels back to baseline 226
(i.e. healthy mice). In contrast, treating mice with **1+2** mixture increased F4/80^{pos} 227
myeloid cells (Figure 3A,iii, bottom row), as these macrophages are associated with 228
suppression of inflammation in lung tissues ^{29,34}. 229

Figure 3B indicates that **1+2** mixture also prevented tissue damage in the liver. The 230
H&E staining image of healthy liver is presented in Figure 3B, top row. In contrast, the 231
structure of liver tissue was significantly damaged 6 and 24 hours after LPS 232
administration, in which acute inflammatory response was apparent, characterized by 233
decreased cell density, enlarged cell gaps and neutrophil infiltration. Morphological 234
changes in the liver tissue, however, were markedly decreased in mice treated with **1+2** 235
mixture (Figure 3B, bottom panels). Indeed, administration of **1** and **2** featured higher 236

number of normal nuclear cells in comparison to LPS-administered mice that were 237
untreated with the molecules (bar diagram in Figure 3B, inset). 238

Similar prevention of tissue damage by tryptophol acetate and tyrosol acetate was 239
apparent in the small intestine (Figure 3C). Specifically, H&E staining of intestinal 240
tissues extracted from LPS-injected mice displayed an extensive damage of both 241
mucosa and sub-mucosa, loss of intestinal mucosal crypt morphology and recruitment 242
of inflammatory cells (Figure 3C, middle row). In comparison, mice treated with **1 + 2** 243
mixture exhibited markedly lower tissue damage (Figure 3C, bottom row). 244
Quantification of crypt injury (Figure 3C, inset) attests to the significantly lower tissue 245
damage, in all time points, in LPS-injected mice treated with the two molecules. 246

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Effects of oral uptake of tryptophol acetate and tyrosol acetate on systemic 248 inflammation. 249

We further assessed the effects of the tryptophol acetate and tyrosol acetate mixture 250
oral uptake on systemic inflammation (Figure 4). In the experiments depicted in Figure 251
4, the peripheral cell blood count (CBC) was evaluated at different time intervals. The 252
results show that in LPS-injected mice that were not treated with **1+2**, significant 253
reductions in red blood cell (RBC) counts, hemoglobin (HGB) and hematocrits (HCT) 254
occurred. Notably, however, addition of **1 + 2** mixture significantly increased these 255
parameters (Figure 4A top row, green lines vs black lines). Treating mice with **1 + 2** 256
mixture also increased thrombocyte counts (PLT), which were reduced after LPS 257
injection (Figure 4A). The CBC data in Figure 4A indicate that tryptophol acetate and 258
tyrosol acetate prevented both inflammation-induced anemia and thrombocytopenia 259
that are usually associated with severe inflammation, especially accompanied by 260

Figure 4. Effects of tryptophol acetate and tyrosol acetate mixture on systemic inflammation. Samples of the peripheral blood were obtained from the tail vein at different time intervals and assessed for CBC. Serums were collected and studied by ELISAs. **A.** The results of RBC, HGB, HCT, PLT, WBC, GRA at different time intervals. LPS-administered mice with PBS treatment (black lines) and treated with **1+2 mixture** (green), * $p < 0.05$. **B.** Cytokine levels in serum, assessed by commercial ELISA kits. Data are presented as mean $\pm$ SD in each group ($n=3$). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$. **(C)** Lungs were obtained from LPS treated mice at indicated time intervals and mRNA was extracted and proceed to RTPCR. Expression of IL-6, IL-1 α and IL-1 β is shown. Data are shown as mean $\pm$ SD in each group ($n=4$). * $p < 0.05$, ** $p < 0.005$.

The clinical hallmark of cytokine storms is the increased secretion of pro-inflammatory cytokines. Accordingly, we determined the effects of tryptophol acetate and tyrosol acetate mixture given orally to the LPS-injected mice on the levels of pro-inflammatory cytokines, including IL-6, IL-1 β and TNF- α in serums (Figure 4B) and lungs (Figure 4C). Figure 4B demonstrates that while LPS injection increased the levels of cytokines in serum, treatment with **1+2** mixture significantly attenuated cytokine concentrations within 6-24 hours (Figure 4B). Specifically, blood concentrations of IL-6 in LPS-administered mice treated with the molecules were lower compared to the untreated LPS-injected mice in all time intervals (note that after 48 hours, LPS-administered mice treated with PBS did not survive, Figure 4B). However, after 48 hours, the IL-6 level of treated mice was almost identical to the healthy mice. Statistically significant reductions of IL-1 β and TNF- α levels in LPS-administered mice that received the **1+2** mixture was observed 12 hours after LPS injection (Figure 4B).

Tryptophol acetate and tyrosol acetate mixture also inhibited the expression of pro-inflammatory cytokines in the lungs (Figure 4C). Specifically, while LPS administration significantly elevated the mRNA levels of IL-6, IL-1 α , and IL-1 β in lung tissues, oral treatment with **1 + 2** mixture reduced the expression of these cytokines at the examined time intervals (Figure 4C). Thus, oral administration of tryptophol acetate and tyrosol acetate mixture decreased cytokine secretion in serum (Figure 4B) and expression (Figure 4C) thereby inhibiting severe systemic inflammation after LPS injection.

Tryptophol acetate and tyrosol acetate reduced the production of ROS and pro-inflammatory cytokines in LPS-activated murine peritoneal macrophages, without reducing phagocytosis function 309
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To investigate the mechanistic basis of the clinical and biological immunomodulation 312
effects of tryptophol acetate and tyrosol acetate (e.g., Figures 1-4), we performed *ex-* 313
vivo experiments using murine peritoneal macrophages. Figure 5A depicts the effects 314
of tryptophol acetate and tyrosol acetate on reactive oxygen species (ROS) generation 315
using the CellRox Deep-Red dye assay. In general, elevated inflammation levels go 316
together with enhanced production of ROS ³⁶. Oxidative stress conditions were attained 317
in the experiments by stimulating the murine peritoneal macrophages with LPS (100 318
ng/ml) with or without co-addition of the **1+2** mixture. The flow cytometry analysis 319
revealed a significant decrease in intracellular ROS after 16 hours incubation of LPS- 320
stimulated macrophages with **1 + 2** mixture, in comparison with the untreated LPS- 321
stimulated macrophages (Figure 5A). 322

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Figure 5. Anti-inflammatory properties of tryptophol acetate and tyrosol acetate *ex vivo*. **A.** Intracellular reactive oxygen species (ROS) after LPS stimulation of murine peritoneal macrophages (three replicates flow cytometry analysis), the results are the Geometric Mean Fluorescent Intensity (GMFI) of the CellRox Deep-Red dye. (Unpaired t-test, $p < 0.05$; $n = 14$). **B.** Representative scatter plots and gating conditions for green-fluorescent beads engulfment analysis, depicting bead uptake after 1 hour incubation of the macrophages, with or without co-addition of 1+2 mixture. Percentages of bead positive cells are indicated in the bottom right quadrants. **C.** Analysis of phagocytosis function of murine peritoneal macrophages after LPS stimulation incubated for 1 or 3 hours with or without molecules in concentration of 100 μ M each by using green-fluorescent beads (Unpaired t-test, two-tailed; $p < 0.05$). **D.** Cytokine levels determined by ELISA from supernatants of *ex-vivo* isolated peritoneal murine macrophages. Macrophages were incubated for 16-hour with and without LPS

and **1+2** mixture. Results presented as mean $\pm$ SD; n=3 from two independent experiments. *p<0.05 ***p<0.0001.

Since phagocytosis is a key function of macrophages ³⁷, we examined macrophage phagocytic action in LPS-stimulated murine peritoneal macrophages with and without co-incubation with the **1 + 2** mixture (Figure 5B,C). Phagocytosis was assessed in the experiments by flow cytometry measuring the uptake of green-fluorescent beads by the cells. The representative gating analysis by flow cytometry (1 hour phagocytosis assay, Figure 5B) together with the bar diagram (Figure 5C) demonstrate no difference in the phagocytic activity of LPS-stimulated macrophages in the absence or presence of the **1+2** mixture (1 and 3 hours of phagocytosis assays). These results indicate that the tryptophol acetate and tyrosol acetate mixture did not adversely affect the phagocytic ability of macrophages.

To further confirm the immunomodulatory effects of tryptophol acetate and tyrosol acetate *ex vivo*, we quantified production of pro-inflammatory cytokines by the LPS-stimulated murine peritoneal macrophages with and without co-incubation with the molecule mixture (Figure 5D). Indeed, while the production of IL-1 α , IL-1 β , IL-6, and TNF- α by macrophages increased within 16 hours after LPS stimulation, addition of the **1+2** mixture significantly reduced the concentrations of all these pro-inflammatory cytokines, in most cases back to the non-stimulated macrophage levels. In contrast, tryptophol acetate and tyrosol acetate did not affect secretion of the anti-inflammatory cytokines TGF- β and INF- β (Figure 5D). These results again demonstrate that the addition of the molecule mixture did not disrupt core anti-inflammatory mechanisms of the host. Together, the results presented in Figure 5 demonstrate that tryptophol acetate and tyrosol acetate decreased intracellular ROS and inhibited generation of pro-

inflammatory cytokines, while not adversely affecting important immune processes, 364
including macrophage phagocytic function and anti-inflammatory cytokine production. 365

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Effect of tryptophol acetate and tyrosol acetate on NF- κ B pathways in LPS-activated 367
macrophages ex vivo and in vitro 368

To obtain further insight into the molecular contours underlining the immune 369
modulating activities of the tryptophol acetate and tyrosol acetate mixture, we 370
investigated intracellular pathways associated with NF- κ B, a key protein in major 371
inflammation gene cascades ³⁸ (Figure 6). Indeed, the representative western blot (WB) 372
image in Figure 6A (i) demonstrates that addition of **1 + 2** mixture to the LPS-stimulated 373
macrophages markedly inhibited generation of the phosphorylated form of NF- κ B 374
(p65) in comparison to stimulation with LPS (relative band intensities are depicted in 375
the bar diagram in Figure 6A (ii)). 376

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Figure 6. Molecular mechanisms underlining the anti-inflammatory activity of the tryptophol acetate and tyrosol acetate mixture. Intraperitoneal macrophages were obtained 72 hours after thioglycolate injection and cultured with LPS with or without 1+2 mixture. (A) At different time intervals, cell lysates were assessed by Western blot (WB). (i) Representative WB of phosphorylated NF- κ B (p65) protein expression, with the effects of the mixture (100 μ M each). (ii) Relative band intensity histograms. The

graph represents an average of three independent experiments. Bars indicate standard errors of the means; ** $p < 0.001$, *** $p < 0.0001$. Beta-actin was used to assess equal protein loading. **(B)** RAW 264.7 cell line was used for definition of the NF- κ B pathway related receptors. Cells were incubated with LPS with and without molecules at the indicated time intervals. RNA was extracted and cDNA was analyzed by RT-PCR. **(C)** Relative protein and mRNA expression of A20 after stimulation with 1 μ g/ml of LPS in the absence and presence of **1+2** mixture (100 μ M), detected by RT-qPCR. Error bars indicate standard deviations of three independent cultures. * $P < 0.05$, **** $P < 0.0001$ ANOVA followed by Tukey's post hoc analysis. **(D)** **(i)** HEK-293T cells were transfected with pCAGGA vector (*HA-A20*) (1.5 μ g) and incubated with and without **1+2** mixture (1 or 2 h). Protein expression of A20 in cell lysates is presented in WB. HEK-293T cells were used as a negative control. **(ii)** Relative band intensity histograms. The graph represents an average of three independent experiments. Bars indicate standard error of the means; statistical analysis calculated by Unpaired t-test, $p < 0.0001$. Beta-actin was used to ascertain equal protein loadings.

To shed further light on the parameters responsible for NF- κ B attenuation incurred by tryptophol acetate and tyrosol acetate in LPS-stimulated macrophages, we evaluated expression of prominent upstream receptors associated with NF- κ B cascades, specifically TLR4, IL-1R and TNFR³⁹ (Figure 6B). The bar diagrams in Figure 6B depicts the levels of the receptors' mRNA recorded in RAW 264.7 macrophage cells, stimulated by LPS in the presence or absence of **1 + 2** mixture. Figure 6B demonstrates that addition of the molecules to the LPS-stimulated cells significantly attenuated expression of the genes of all three receptors. Specifically, LPS stimulation induced expression of *TLR4* during the first hour (black bar, Figure 6B, left), while co-addition of **1+2** mixture together with LPS resulted in suppression of *TLR4* expression, reverting to the baseline (pre-inflammation) level. Similarly, tryptophol acetate and tyrosol acetate significantly attenuated expression of both *IL-1R* (grey bars) and *TNFR* (white bars) 4 hours after LPS stimulation, giving rise to baseline expression levels in both receptors.

Accounting for the reduction of the upstream receptors in the NF- κ B pathways by the
tryptophol acetate and tyrosol acetate (e.g., Figure 6B), we further tested the effects of
the molecules upon expression of the A20 protein, known to suppress signaling
cascades associated with the TLR receptors⁴⁰ (Figure 6C-D). The bar diagram in Figure
6C presents experimental data corresponding to RAW 264.7 cells treated with **1+2**
mixture and the A20 m-RNA levels measured after 30 min and 60 min. As shown in
Figure 6C, both LPS, and the **1+2** mixture co-added with LPS, induced significant
expression of A20 gene within 60 minutes after addition to the cells. However, when
1+2 were co-added to the cells together with LPS, significantly higher expression of
A20 was found.

To assess the protein level of A20, we further carried out WB analysis (Figure 6D),
employing HEK-293T cells transfected with the pCAGGA mammalian expression
vector (HA-A20), allowing transient transfection of A20^{41,42}. Specifically, after
transfection, the tryptophol acetate and tyrosol acetate mixture was incubated with the
cells for different time intervals (HEK-293T cells were used as a negative control,
Figure 6D,i). The WB data in Figure 6D demonstrate that **1+2** mixture induced
significant expression of A20, both after 1 and 2 h of incubation compared to the control
HEK-293T cells comprising the vector. Together, the RT-PCR and WB results in
Figure 6C-D suggest that modulation of A20 levels by tryptophol acetate and tyrosol
acetate is likely a key factor in attenuation NF- κ B expression and concomitant anti-
inflammatory effects of the molecules.

Tryptophol acetate and tyrosol acetate affect mouse gut microbiome 437

In tandem with elucidating the dramatic clinical effects and molecular facets of the 438
inflammation modulatory activities of tryptophol acetate and tyrosol acetate, we also 439
examined the effect of the molecules on gut microbiome of the mice (Figure 7). The 440
relationship between gut microbiota and host immune properties has recently emerged 441
as a prominent factor in systemic response to inflammation. Previous seminal studies, 442
for example, linked inflammatory cytokine production and individual variations in 443
cytokine response to the composition and function of the microbiota ⁴³. In the 444
experiments depicted in Figure 7, mice microbiome analysis was carried out through 445
collecting the stool samples from all participating mice, the day before and at intervals 446
of 6 h and 24 h after LPS injection. The microbial taxonomic 16S rRNA gene sequences 447
were subsequently determined. 448

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Figure 7. Effect of tryptophol acetate and tyrosol acetate mixture treatment on mice microbiome. (A, B) Alpha diversity analysis using the Shannon index after (A) 6 hours (p=0.46) and (B) 24 hours of treatment (p=0.29); quantitative variables were compared between groups with the Wilcoxon U-test, for non-normally distributed data. (C, D) Principal coordinate analysis plot representing beta-diversity based on weighted UniFrac distances after (C) 6 hours (permutational multivariate analysis of variance, P=0.001) and (D) 24 hours of treatment (permutational multivariate analysis of variance, P=0.038). (E) Bacterial taxa *Bacteroides ovatus* relative abundance (%) boxplots for untreated and molecules treated groups. The taxa more abundant in treated group 24 h from LPS injection. (Treated and untreated, n=8)

When comparing the microbial richness (i.e., alpha diversity; Figure 7A, B) before and after treatment with tryptophol acetate and tyrosol acetate (at 6 hours and 24 hours,

respectively), no significant differences were observed (Wilcoxon test, $P=0.46$ and $P=0.29$). However, we found differences in beta diversity (i.e., between sample diversities; Figure 7C, D) between treated and untreated mice 6 and 24 hours after LPS injection (PERMANOVA, $P=0.001$ and $P=0.038$, respectively). This important observation indicates that although the species richness was not significantly altered between the treated and untreated mice, dysbiosis (shift in the microbiome) at the community level was already observed 6 hours after LPS administration. Dysbiosis 6 hours after LPS treatment was characterized by a profound reduction in the relative abundance of commensals such as *Blautia*, *Bacteroides*, *Clostridium*, *Sutterella* and *Parabacteroides* and by an increase in the relative abundance of *Ruminococcus* (Table S1, Supporting information). These changes occurred in both the treated and untreated groups. Twenty-four hours after LPS administration we observed a decrease in the relative abundance of *Blautia*, *Bacteroides*, *Clostridium* and *Oscillospira* and increase in the relative abundance of *Ruminococcus*.

Figure 7E underscores the effect of treating the LPS-administered mice with tryptophol acetate and tyrosol acetate. Importantly, the abundance of *Bacteroides* increased in the mice that were treated with 1+2 compared to the mice that were not treated (24 hours after LPS injection; Figure 7E). This taxon was also abundant in the microbiome of healthy mice (ANCOM test significance $W=68$ and $W=72$ respectively (Table S1)). This result is important as *Bacteroides* has been linked to varied immune-protective and anti-pathogenic activities associated with microbiome modifications ⁴⁴.

Discussion

Current therapy of sepsis is mainly based on anti-bacterial and symptomatic treatments. Important strategies for overcoming sepsis syndromes have been based on blocking cytokines and other pro-inflammatory molecules. While mortality from sepsis significantly decreased in the past century, in many cases of septic shock or cytokine storms, for example associated with the recent SARS-Covid-19 pandemic, mortality is high ^{2,9}. In most patients experiencing such sepsis syndromes, various inflammatory factors are responsible for the development of multiorgan failure and new therapeutic approaches which target upstream pro-inflammatory molecules are highly sought.

In this work, we identified two metabolites secreted by the probiotic fungus *Kluyveromyces marxianus* in a milk-fermented microorganism mixture (kefir) – tryptophol acetate and tyrosol acetate - which exhibit remarkable anti-inflammatory activities. Comprehensive *in vivo*, *ex vivo* and *in vitro* experiments demonstrate an effective blocking of the “cytokine storm” by an equimolar mixture of the two molecules. Specifically, we observed that mice inflicted with LPS-induced sepsis and orally treated with tryptophol acetate and tyrosol acetate, had significantly milder patterns of the disease in comparison with LPS-administrated control mice that received only buffer. Importantly, no mice mortality was observed both upon co-administration of the molecules with LPS (i.e., sepsis preventive effect), as well as upon providing the molecules to mice already displaying signs of severe inflammation (Figure 2).

LPS administration in mice yields clinical signs of human sepsis. Thus, we assessed the effects of tryptophol acetate and tyrosol acetate mixture on systemic inflammation and on various vital organs in LPS-injected mice (Figures 3-5). In sepsis, multiorgan destruction due to severe inflammation leads to hepatic and renal failure,

respiratory distress, dysfunction of gastrointestinal system and hematological changes, 512
such as coagulation disorders ⁴⁵. The reported experimental data demonstrate that orally 513
administered tryptophol acetate and tyrosol acetate mixture prevented tissue damage 514
and attenuated hematological changes. Specifically, the molecules' mixture attenuated 515
alveolar wall degradation, and in parallel decreased lung congestion (Figure 3A). 516
Tryptophol acetate and tyrosol acetate also significantly affected recruitment of 517
myeloid cells, which are intimately involved in exacerbation of lung inflammation ⁴⁶. 518
Indeed, we observed that mixture of tryptophol acetate and tyrosol acetate reduced 519
recruitment of neutrophils and Ly6C-positive monocytes into lungs (Figure 3A, iii). 520
Moreover, histology analyses reveal that, after treatment with the molecules, more 521
abundant mature F4/80-positive macrophages was detected, likely accounting for tissue 522
repair ^{29,34} (Figure 3A, iii). We also recorded substantial healing effects in the case of 523
LPS-induced liver tissue damage, in which the molecules stemmed the loss of hepatic 524
cells (Figure 3B), and reduced crypt damage in intestinal tissue (Figure 3C). 525

The systemic therapeutic effects of tryptophol acetate and tyrosol acetate were 526
further analyzed *in vivo* and *ex vivo* (Figure 4). We found that LPS-administered mice 527
treated with the molecules' mixture displayed lower anemia and thrombocytopenia that 528
usually accompany severe septic syndromes and cytokine storms (Figure 4A). 529
Furthermore, significant reduction in the expression of pro-inflammatory cytokines in 530
lung tissue and in serum were recorded after treating LPS-injected mice with the 531
tryptophol acetate and tyrosol acetate mixture (Figure 4B and 4C). These results are 532
significant, since release of pro-inflammatory mediators, including cytokines, 533
chemokines and ROS is the hallmark of cytokine storms ⁴⁷. 534

Particularly significant in this context were the *in vivo* and *in vitro* data recorded 535
for IL-6, specifically the attenuation of this pro-inflammatory cytokine by orders of 536

magnitude to the baseline level after treatment with tryptophol acetate and tyrosol acetate (Figure 4B, 4C; Figure 5B). Cytokine storm syndromes in COVID-19 patients, for example, have been associated with dramatic increase in blood IL-6 levels, and IL-6 concentrations have been shown as markers for fatality outcomes ⁴⁸. As such, the considerable and rapid reduction of IL-6 levels induced by the tryptophol acetate and tyrosol acetate point to potential therapeutic avenue for severe inflammation syndromes, such as affected by COVID-19. Additional *ex vivo* experiments in Figure 5D assessing pro-inflammatory cytokine secretion echoed the *in vivo* results, demonstrating attenuated secretion of pro-inflammatory cytokines by LPS-stimulated macrophages in the presence of the tryptophol acetate and tyrosol acetate mixture. Importantly, different from numerous anti-inflammatory strategies reported in the literature and clinically employed, tryptophol acetate and tyrosol acetate did not induce complete immunosuppression, rather the molecules reverted the pro-inflammatory cytokine levels to baseline (pre-inflammation) values.

A major question underlying the anti-inflammatory effects of tryptophol acetate and tyrosol acetate concerns their mode of action. *Ex vivo* experiments utilizing LPS-stimulated murine peritoneal macrophages reveal important mechanistic aspects (Figures 5-6). Specifically, in parallel with inhibition of pro-inflammatory cytokines, we recorded a significant reduction in ROS levels. ROS include short-lived bioactive molecules produced by immune competent cells serving as messenger molecules for various physiologic and pathologic processes ⁴⁹. ROS particularly activate multiple inflammatory signaling pathways ⁵⁰. Notably, we recorded a significant reduction in ROS levels after tryptophol acetate and tyrosol acetate were added to LPS-activated murine peritoneal macrophages (Figure 5A). It should be noted that while tryptophol acetate and tyrosol acetate significantly attenuated the pathophysiological

consequences of severe inflammation, the molecules did not adversely affect core
immune processes, particularly phagocytosis activity (Figure 5B-C). These results are
consistent with previous studies, reporting anti-inflammatory and anti-oxidant effects
without impairing macrophage bactericidal activities ^{51,52}.

To account for the broad-based immune modulation effect of tryptophol acetate
and tyrosol acetate, particularly the significant reduction in pro-inflammatory cytokine
levels, we investigated the impact of the molecules' mixture upon the immune signaling
pathway involving NF- κ B (Figure 6). NF- κ B is central to transcription pathways of
many genes encoding pro-inflammatory mediators and plays a crucial role in varied
inflammatory disease conditions ³⁷. Indeed, addition of tryptophol acetate and tyrosol
acetate reduced NF- κ B level in macrophages stimulated with LPS (Figure 6A).
Furthermore, the RT-PCR data in Figure 6 attest to pronounced downregulation of the
main upstream receptors involved in NF- κ B activation - TLR4, IL-1R and TNFR.
These observations are mechanistically significant as it is known that production and
secretion of pro-inflammatory cytokines are governed by the TLR/NF- κ B signaling
pathway, perceived as a major "gateway" cascade in innate immunity, and are
associated with the pathogenesis of severe inflammation that promotes lung, liver, and
intestinal injuries⁴⁴. For example, binding of LPS to TLR-4 was shown to induce lung
parenchymal damage, neutrophil accumulation in the interstitial and alveolar
compartments, elevated vascular permeability and pulmonary edema ^{53,54}.

Further molecular insight upon the underlying immunomodulatory mechanism
of tryptophol acetate and tyrosol acetate was furnished by analysis of A20 protein levels
(Figure 6). A20 is a prominent negative feedback regulator of NF- κ B signaling. It has
been reported that mice genetically deficient in A20 develop severe inflammation,
underscoring the central role of A20 in suppression of NF- κ B-dependent inflammation

and tissue homeostasis⁵⁵. Importantly, we found that the tryptophol acetate and tyrosol acetate mixtures significantly induced, in both gene and protein levels, the expression of A20 (Figure 6 C,D). Thus, our data suggest that the protection furnished against severe inflammation by tryptophol acetate and tyrosol acetate may be mediated by triggering A20 expression and concomitant inhibition of the TLR4/IL-1R/TNFR-NF- κ B pathway.

Due to our observations that administration of tryptophol acetate and tyrosol acetate orally gave rise to the pronounced anti-inflammatory effects, we assessed the effects of the molecules on the gut microbiota (Figure 7). The relationship between severe inflammation and microbial dysbiosis has been extensively studied in recent years^{56,57}. The gut microbiota have been shown to enhance host immunity to pathogens, and dysbiosis has been linked to increased susceptibility of severe inflammation⁵⁸. Accordingly, we characterized the gut microbial compositions of mice prior and after LPS injection, and with / without treatment with the mixture of tryptophol acetate and tyrosol acetate. Indeed, oral administration of the molecules had a significant impact on the community composition, manifested by an increase in the abundance of bacteria with anti-inflammatory properties⁵⁹. Notably, the predominant change recorded was the increase in the genus *Bacteroides* in mice that were treated with the molecules 24 hours after LPS administration (Figure 7E).

Recent studies have reported on the anti-inflammatory properties of *Bacteroides*⁶⁰. The mechanisms of action proposed for these anti-inflammatory activities include inhibition of pathogen colonization⁴⁴ and increased mucosal barrier by modifying goblet cells and mucin glycosylation⁶¹. The higher abundance of *Bacteroides* upon treating the LPS-injected mice with tryptophol acetate and tyrosol acetate may thus account to “cross-talk” between the molecules and host microbiota, promoting intestinal homeostasis. Moreover, the significance of these results are further

underlined by a recent study stressing the need for substances that protect gut microbiota such as *Bacteroides* from the collateral damage of widely used antibiotics such as tetracyclines and macrolides⁶². Thereby, consumption of tryptophol acetate and tyrosol acetate in combination with antibiotics may present a new approach for reducing the harmful side effects of antibiotics on host gut microbiome.

Overall, varied food-extracted substances have been touted as exhibiting powerful antioxidant and anti-inflammatory properties⁶³. However, reports presenting comprehensive clinical, physiological, and mechanistic analyses of the anti-inflammatory activities of probiotic yeast secreted metabolites, such as presented here for tryptophol acetate and tyrosol acetate, have been rare. In particular, to the best of our knowledge, no previous studies have shown an exceptional clinical outcome (i.e. survival and morbidity results, Figure 2), combined with a comprehensive mechanistic analysis. Indeed, our findings uncover, in detail, the immune pathways underlying anti-inflammatory effects of probiotic-sourced molecules.

In conclusion, we discovered that metabolites secreted by a probiotic yeast abundant in a milk-fermented microorganism mixture – tryptophol acetate and tyrosol acetate – significantly inhibit systemic severe inflammation effects. Comprehensive *in vivo*, *ex vivo*, and *in vitro* data demonstrate the healing and protective effects of the two molecules, including prevention of mortality in mice experiencing LPS-induced severe inflammation, blocking organ damage, reduction of ROS production, and maintaining healthy peripheral blood cell profile. Particularly important, the experiments demonstrate that, unlike many anti-inflammatory treatments, tryptophol acetate and tyrosol acetate did not induce immunosuppression, but rather effectively restored baseline levels of pro-inflammatory cytokines and inhibited activation of NF-κB through elevating A20 expression. Overall, the remarkable systemic anti-inflammatory

effects of tryptophol acetate and tyrosol acetate and their presence in a food source that
has been consumed by humankind for millennia may open new avenues for anti-
inflammatory therapeutics.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute
(RPMI 1640), heat-inactivated fetal bovine serum (FBS) were purchased from
Biological Industries (Beit Haemek, Israel). Penicillin, streptomycin, ECL western
blotting substrate, opti-MEM, and Bradford reagent were purchased from Thermo-
fisher. Dihydrochloride (AAPH), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,20 –azobis
(2-amidinopropane, fluorescein, hydrogen peroxide, sodium citrate, acetonitrile, formic
acid, sulfuric acid, ethanol, methanol, sodium carbonate, lipopolysaccharide (from E.
coli O55:B5, L2880), propidium iodide (PI) (Solution P4864), NaCl, Triton X-100,
BSA, NaF, Na₃VO₄, AEBSF, polyethylenimine (PEI), Leupeptin, and Aprotinin were
purchased from Sigma-Aldrich. Xylene was purchased from Epreidia (lot 6601). Tris
buffer was purchased from Bio-lab (Jerusalem, Israel). Thioglycolate was purchased
from life technologies, 2085262 (ultra-pure grade). All reagents and solvents were of
analytical grade and were used as received.

DPPH scavenging activity

The effect of tryptophol and tyrosol acetates on DPPH• radical were estimated
according to recommendations of Marinova and Batchvarov ²⁴ with some
modifications. All of the solutions were prepared in ethanol. The stock solution was
prepared by dissolving 13.8 mg DPPH with 20 mL ethanol and stored until needed. The

control (100%) solution was obtained by mixing 225 μ L ethanol with 25 μ L stock
solution to obtain an absorbance of 1.0 ± 0.1 units at 490 nm. 200 μ L of tryptophol
acetate and tyrosol acetate dissolved in ethanol in a concentration of 200 μ M was
allowed to react with 25 μ L of the DPPH solution for 20 min in the dark at 400 rpm at
25°C. Ethanol (250 μ L) was used for the blank control (100%), and 225 μ L was used
as a blank. The absorbance decrease was recorded at 490 nm. For all evaluated assays,
absorbance measurements were performed in triplicate using a Microtiter Plate Reader
(Varioskan Flash, Thermo) to calculate radical scavenging activity (% of inhibition)
with the formula.

$$\text{Inhibition (\%)} = 1 - \frac{\text{Abs (sample)} - \text{Abs (blank)}}{\text{Abs (control)} - \text{Abs (blank)}} \times 100$$

where Abs (sample) was the absorbance of the reaction in the presence of the sample
(sample dilution + DPPH solution), Abs (blank) was the absorbance of the blank for
each sample dilution (sample dilution + DPPH solvent), and Abs (control) was the
absorbance of the control reaction (sample solvent + DPPH solution).

Oxygen radical absorbance capacity (ORAC assay)

The ORAC method is based on the oxidative degradation of the fluorescent molecules
after mixing with a free radical generator, for example, azo compounds. This method
determines the ability of the sample to neutralize short-lived free radicals. The assay
was carried out according to Ou et al.²⁵, with some minor modifications. Prior to the
measurements, tryptophol acetate and tyrosol acetate were dissolved in acidified
methanol to a concentration of 200 μ M. 30 μ L of each molecule or 30 μ L mixture of
both, as well as blank (methanol) were mixed with 180 μ L of 112 nM fluorescein
solution in a 96-well plate and incubated at 37°C for 15 min. Subsequently, 100 μ L of

100 mM 2,2' -azobis(2-amidinopropane) dihydrochloride (AAPH) solution was added, 685
and the fluorescence was measured every 70 s for 90 min using a Microtiter Plate 686
Reader (Varioskan Flash, Thermo). The excitation wavelength was 485 nm and the 687
emission recorded at 520 nm. All stock solutions and dilutions of samples were 688
prepared fresh daily in phosphate-buffered saline (PBS, pH 7.4). The experiments were 689
done in six repetitions. Results are presented according to the following calculations : 690

Area under the curve (AUC) was calculated for each sample using the equation: 691

$$\text{AUC} = 1 + \text{FU}_1/\text{FU}_0 + \text{FU}_2/\text{FU}_0 + \text{FU}_3/\text{FU}_0 + \dots + 692$$

FU_0 = fluorescence at time zero. 693

FU_x = fluorescence at specific timepoints (e.g., FU_3 is the fluorescence value at three 694
minutes). Net AUC was calculated by subtracting the Blank AUC from the AUC of 695
each sample using the equation: 696

$$\text{Net AUC} = \text{AUC (Antioxidant)} - \text{AUC (blank)} 697$$

Animal studies 698

C57BL/6 mice 699

All experiments were performed in accordance with the Institutional Animal Care and 700
Use Committee at Ben-Gurion University (IL-43-07-2020). Eight-week-old C57BL/6 701
mice (Harlen, Rehovot, Israel) weighing approximately 20 gr were held in specific 702
pathogen-free conditions at the University Central Research Facility for at least one 703
week prior to commencing studies. The mice were fed (*ad libitum*) with V1154-703 704
ssniffTM (Soest, Germany) and were allowed water continuously. Environmental 705
enrichment was provided to all animals based on the requirements of the particular 706
mouse strain. Temperature was maintained at 21°C, and animals were exposed to a 12 707

h light, 12 h dark cycle with a 15 min ramp-up and ramp-down to simulate dusk and dawn. The mice were divided into two study groups: untreated (mice injected with LPS and administered a placebo), Treated (mice injected with LPS and treated by oral administration of mixture of tryptophol acetate and tyrosol acetate). Mice were housed in groups of six per cage. LPS dose was 30 mg/kg to each mouse (~20 g), which was injected intraperitoneally.

Treatment with tryptophol acetate and tyrosol acetate

Tryptophol acetate and tyrosol acetate were synthesized as previously described²² and dissolved in DDW to desired concentrations (final concentration of 150 µg/kg). The molecular mixture was administrated through oral gavage in a volume of 200 µL twice per day, in the morning (08:30–09:30) and evening (19:30–20:30). DDW was used as a placebo and was administrated orally to the control group at the same times.

In one set of experiments, the mixture of molecules was administrated concomitantly with LPS injection and treatment was continued two times per day until the end time of the experiment (156 h). In another set of experiments, the first treatment with the molecules started 28 h after LPS injection and was continued two times per day until the end time of the experiment (156 h).

Clinical evaluation of inflammation

Clinical signs of inflammation were recorded twice daily. Measurement of body weight, signs of diarrhea and appearance of blood traces in stool, as well as signs of dyspnea were assessed. Mean percentage of weight loss was calculated daily as the ratio of measured body weight and weight measured before injection of LPS. Every 6 h after LPS injection till endpoint of the experiments (till 72 h), mice were anesthetized with 5% isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) and blood

samples were got from tail vien. Lungs, livers, colons, and intestinals were obtained at 732
the same time intervals and were cleaned from contents by flushing with 10 ml of sterile 733
PBS. The tissue were taken for extraction of RNA and part of them were embedded in 734
paraffin following a standard protocol. 5- μ m sections using a rotary microtome (MRC- 735
Lab, HIS-202A). Paraffin embedded tissues were cut into 5- μ m sections using a rotary 736
microtome (MRC-Lab, HIS-202A), rehydrated and stained with hematoxylin. 737

Immunohistochemistry Staining 738

Tissue sections were de-paraffinized in xylene and re-hydrated with decreasing 739
concentrations of alcohol. Subsequently, endogenous peroxide was blocked with 740
hydrogen peroxide, and antigen retrieval was achieved by treating sections with 0.01M 741
sodium citrate, pH 6.0 for 1 min in a pressure cooker. After blocking with universal 742
blocking solution (ZYMED Laboratories, San Francisco, CA), tissue sections were 743
stained with the designated primary antibodies. A Vectastain Elite ABC Peroxidase kit 744
or Universal ImmPRESS kit (Vector Laboratories, Burlingame, CA) were used for 745
secondary antibodies, and visualization was performed using 3-amino-9- 746
ethylcarbazole (AEC) as a substrate (ZYMED Laboratories, San Francisco, CA). 747
Sections were then stained with hematoxylin for counterstaining and mounted using 748
VectaMount AQ Aqueous Mounting Medium (Cat-No. H-5501; Vector Laboratories). 749
Myeloperoxidase (MPO)-positive (Abcam), F4/80-positive (Santa Cruz, 377009), and 750
LY6C-positive cells (Abcam, ab15627) were counted in stained sections in six 751
randomly chosen fields ($\times 200$), and bars are indicated standard errors of the means. 752

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Blood count 756

Blood samples were collected from mice tails with EDTA micropipette capillaries 757
(Exigo) and analyzed directly after collection by a veterinary hematology analyzer 758
(Exigo H400, Boule Medical AB, Spånga, Sweden). 759

Cytokine measurements by ELISA 760

To measure the extent of the inflammatory response in mice, serum obtained from the 761
blood of LPS-treated and untreated mice, we customized a highly sensitive milliplex® 762
MAP Kit (Cat # MPXMCYTO-70K; Millipore) with color-coded beads and fluorescent 763
dyes according to the manufacturer's recommendations. 764

In vitro macrophage experiments 765

264.7 cell line 766

The RAW 264.7 cell lines (third passage) were purchased from American Type Culture 767
Collection (ATCC1 TIB-71™). Cells were cultured in DMEM with 10% FBS and 1% 768
penicillin/streptomycin, in an atmosphere of 5% CO₂ and 95% humidity at 37°C. Cells 769
were regularly tested for mycoplasma contamination. 770

Peritoneal macrophages 771

Mouse peritoneal macrophages were obtained from C57BL/6J mice (6–8weeks old) 772
hours after intra-peritoneum injection of Thioglycolate and cultured in complete RPMI 773
medium with and without LPS (100ng/ml) (O55:B5, L2880, Sigma) and in the presence 774
or absence of the tryptophol acetate and tyrosol acetate (1+2 mixture). 775

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Cytokine production in peritoneal macrophages 778

Cells (3×10^5) were seeded in 24-well plates and after 24 h, supernatants were collected 779
and assessed for cytokine secretion. To measure the extent of the inflammatory 780
response in the LPS stimulated peritoneal macrophages, we used murine ELISA kits 781
according to the manufacturers' recommendations for IL-1 β (PeproTech., 900-K47), 782
IL-6 (Biotest, DY406), TNF- α (Biotest, DY410), TGF- β (Biotest, DY1679), IL-1 α 783
(Biotest, MAB400-500; AB-monoclonal BAF400, AB-polyclonal 400-ML-005), and 784
INF- β (BioLegend B292426). 785

ROS analysis 786

Peritoneal macrophage cells were seeded (1×10^5 cells) into 96-well plates for ROS and 787
phagocytosis analysis. 788

After 2 h of incubation in complete medium at 37°C, cells were stimulated with LPS 789
(100ng/ml) and incubated overnight in the absence or presence of tryptophol acetate 790
and tyrosol acetate (100 μ M each). After 24 hours, the cells were washed two times 791
with PBS and incubated for 1 h with CellROX Deep Red Reagent (Invitrogen, Carlsbad, 792
CA) prior to analysis by flow cytometry. Dead cells were gated out using PI. The 793
presence of ROS was analyzed by the fluorescence geometric mean (GM) of the 794
CellROX Deep Red fluorescence. 795

Phagocytosis analysis 796

Peritoneal macrophage cells were seeded in 96-well plates with RPMI medium. After 797
2 adherences at 37°C, all the cells were stimulated with LPS (100ng/ml) and incubated 798
overnight in the absence or presence of the tryptophol acetate and cytosol acetate 799
mixture (100 μ M each). After 24 hours the cells were washed two times with PBS and 800

incubated for 1 h or 3 h at 37°C in 200 µl RPMI medium, alone (control) or with 801
Fluoresbrite® Yellow-Green carboxylate microspheres (1 µm) (Polysciences Inc., 802
15702) in a ratio of 3:1 beads-to-cells. In the flow cytometry analysis, dead cells were 803
gated out using PI. Free beads were analyzed alone and gated out from the analysis. 804
The fraction of live cells positive for beads was used to estimate phagocytosis 805
functionality. 806

Western blot analysis for NF-κB expression 807

Protein expression was monitored using Western blot analysis. 10x10⁶ murine 808
peritoneal macrophages were seeded on petri dishes. After overnight incubation at 37°C 809
the cells were stimulated with LPS (100ng/ml) in the absence or presence of the mixture 810
of tryptophol acetate and tyrosol acetate (100 µM each molecule) and incubated for 30 811
minutes and harvested in lysis buffer (50 mL 3M NaCl, 25 mL 1M Tris pH 7.5, 10 mL 812
Triton X-100, and 10 mL 0.5M EDTA were mixed and added to 905 mL distilled water) 813
in the presence of protease inhibitor cocktail (1:50 complete, Sigma-Aldrich, Israel). 814
Lysates were placed on ice for 30 min and then centrifuged for 30 min (12,000 rpm) at 815
4°C. Supernatants (cytosolic fractions) were collected, and protein concentrations were 816
determined using a Bio-Rad protein assay kit (lot 5000202, Israel). SDS sample buffer 817
was added, and samples were boiled for 5 min and then frozen at -20°C until use. NF- 818
κB (after 30 minutes) was assayed using cell lysates (70µg), separated on SDS-PAGE 819
and analyzed using the following: antibody raised against the anti-NF-κB (p-65) 820
(1:1000; abcam, ab32536), β-actin (1:1000; MP Biomedicals, Santa Ana, CA). 821

Real-time PCR 822

Total mRNA from obtained tissues and RAW 264.7 macrophages was extracted using 823
a RNA extraction kit (ISOLET II RNA Mini Kit, Bioline). cDNA was synthesized from 824

1 µg of RNA using a PrimeScript™ RT reagent kit (Lifegene, Bio-52073). Subsequent
real-time PCR was performed with an iCycler iQ™ Real-Time PCR Detection System
(Bio-RAD). PCR results were analyzed with SDS 2.02 software (Applied Biosystems,
Thermo). The level of target gene expression was calculated following normalization
of the GAPDH gene level in each sample and presented as relative units. Quantitative
PCR was performed with Taqman Master Mix (Rhenium, Israel) for: TLR4 (Cat
4453320, Assay ID Mm00445273_m1), Tnfrsf1b (Cat 4453320, Assay ID
Mm00441889_m1), GAPDH (Cat 4453320, Assay ID Mm99999915_g1), and IL-1R
(Cat 4453320, Assay ID Mm00434237_m1) or with Mix SYBR Green Master Mix
(Applied Biosystems).

Gene	Forward sequences (5'–3')	Reverse sequences (5'–3')
<i>IL-1α</i>	GTTACAGTGAAACGAAGACTAC	TGCAAGTCTCATGAAGTGAGC
<i>IL-6</i>	TTCCTCTCTGCAAGAGACTTC	GTTGGGAGTGGTATCCTCTGT
<i>IL-1β</i>	TGCCACCTTTTGACAGTGATG	TGTGCTGCTGCGAGATTGA
<i>HPRT</i>	AGTCCCAGCGTCGTGATTAG	TGGCCTCCCATCTCCTTCAT

Table 1. Primers used for RT-qPCR of target gene

A20 expression in HEK-293 T cells

Cells: HEK-293 T cells were grown and maintained in Dulbecco's Modified Eagle's
Medium (DMEM) at 37 °C and 5% CO₂, supplemented with 10% fetal calf serum and
antibiotics (50 units/ml penicillin, 50 µg/ml streptomycin).

Antibodies and plasmids 843

Mouse monoclonal antibodies (mAbs) specific for A20 (Santa Cruz Biotechnology, 844
Inc., Santa Cruz, CA). Mouse anti- β -actin mAbs (Merck Millipore, Darmstadt, 845
Germany). Horseradish peroxidase (HRP)-conjugated goat anti-mouse Abs (Abcam 846
Biotechnology). The full-length human or mouse A20 cDNA with a human influenza 847
hemagglutinin (HA) tag cloned in a pCAGGA mammalian expression vector (HA-A20) 848
was kindly provided by Dr. Shai Cohen (Cancer and Vascular Biology Research Center, 849
The Rappaport Faculty of Medicine and Research Institute, Technion, Israel), 850
previously described ⁴² . 851

Transient transfection in HEK-293 T cells 852

HEK-293 T cells were chosen for transient plasmid transfection. Briefly, 5×10^5 HEK- 853
293 T cells were plated and left to grow for 24 hours in the incubator (37 °C and 5% 854
CO₂). The following day, old DMEM medium was replaced with serum free-fresh 855
DMEM and the cells were left in the incubator for 30 minutes. DNA (plasmids) samples 856
were prepared for transfection; 1.5 μ g of purified HA-A20 plasmid DNA was added to 857
250 μ L of Opti-MEM medium, then 5 μ L of (final concentration of 5 μ g/mL) of 858
polyethylenimine (PEI) was added to the DNA, vortexed and incubated at RT for 20 859
minutes. The PEI-DNA mix was, added to the cells steadily drop by drop and the cells 860
were left in the incubator (37 °C and 5% CO₂) for 4 hours. After the transfection, the 861
cells were incubated with the Molecule mix (200 μ M each) for time range of 1 and 2 h. 862
Cells were harvested at indicated time points and lysed in lysis buffer containing 25mM 863
Tris HCl, pH 7.5, 150mM NaCl, 5mM EDTA, 1mM Na₃VO₄, 50mM NaF, 10 μ g/ml 864
each of leupeptin and aprotinin, 2mM AEBSF and 1% Triton X-100) followed by 30 865
min incubation on ice. Lysates were centrifuged at 13,000 rpm for 30 min at 4°C and 866

the nuclear-free supernatants were used further analysis. 20 µg of protein was resolved 867
on 10% SDS-PAGE and blotted on to nitrocellulose membrane. Membranes were 868
immunoblotted with mouse anti-A20 (1:2000) and mouse anti-β-actin mAbs (1:500) as 869
indicated and visualized following ECL exposure. 870

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Protein concentration 873

Protein concentration was measured by the method of Bradford using bovine serum 874
albumin as standard. 875

Electrophoresis and immunoblotting 876

Whole cell lysates were resolved by electrophoresis on 10 % polyacrylamide gels using 877
Bio-Rad Mini-PROTEAN II cells. Gel proteins were electroblotted onto nitrocellulose 878
membranes (Schleicher and Schuell) at 100 V for 1 h, using BioRad Mini Trans-Blot 879
transfer cells. After 1 h of membrane blocking with 3% BSA in TBS at 37 °C, the 880
membranes were incubated with the indicated primary Abs followed by extensive 881
washings in TBST and incubation with HRP-conjugated goat anti-mouse. 882
Immunoreactive protein bands were visualized using the enhanced chemiluminescent. 883

Data analysis 884

All results are expressed as the mean ± SD or mean ±SE, as indicated. Data for all in- 885
vivo and in-vitro experiments were analyzed by a one/two-way ANOVA followed by 886
Tukey post-hoc test using GraphPad Prism (GraphPad Software, San Diego, Ca). A p- 887
value of ≤ 0.05 was considered statistically significant. 888

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Fecal microbiome analysis

Total DNA was extracted from fecal samples using a PureLink microbiome DNA extraction kit (Invitrogen, Carlsbad, CA) followed by a 2-min bead-beating step, as previously described by Shouval, R. et al.⁶⁴. Purified DNA was PCR amplified using PrimeSTAR Max (Takara-Clontech, Shiga, Japan) for the variable V4 region (using 515F-806R barcoded primers) of the 16S rRNA gene. Amplicons were purified using Agencourt AMPure XP magnetic beads (Beckman-Coulter, Brea, CA), and subsequently quantified using a Quant-It Picogreen dsDNA quantitation kit (Invitrogen, Carlsbad, CA). Equimolar DNA amounts from individual samples were pooled and sequenced using the Illumina MiSeq platform at the Genomic Center, Azrieli Faculty of Medicine, Bar-Ilan University, Israel. Sequencing data were processed using QIIME2 version 2019.10.⁶⁵. Single end sequences with a similarity $\geq 99\%$ were assigned as the same feature. Taxonomy was assigned using the GreenGenes database⁶⁶. Chimeric sequences were removed with DADA2 (--p-trunc-len 160)⁶⁷. Samples with <1000 features and features with total frequency <2 were filtered out. Rarefaction was done using 5,000 sequences per sample. Alpha and beta diversity were calculated based on rarefied datasets.

Statistical analysis of microbiome results

The distribution pattern of quantitative variables was examined using a Shapiro-Wilk test. Quantitative variables were compared between groups with a Wilcoxon U-test, for non-normally distributed data, respectively. All tests were 2-tailed, and in all, $p \leq 0.05$ was considered significant. Bacterial alpha diversity was assessed by the Shannon index. Statistical significance of microbial alpha diversity differences was confirmed using a Kruskal–Wallis test, followed by paired Mann-Whitney tests, with Benjamini-

Hochberg correction for the false discovery rate. Beta diversity was calculated using
weighted UniFrac distances. Statistical significance was confirmed using a
permutational multivariate analysis of variance (PERMANOVA). Differences in
relative abundances of bacterial taxa between groups were identified using the ANCOM
method⁶⁵.

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Author contributions

Conceived and designed the experiments O.M., R.M, E.V. and R.J.; experiments and
data analysis O.M., R.M., M.B., M.K., J.S., E.T., E.S., and B.R.; writing—original draft
O.M., R.M., E.V. and R.J.; writing - reviewed and edited the manuscript B.R., O.K.,
and E.V.; All authors read and approved the final manuscript.

Competing interests

A company - Biotic Therapeutics - has been co-founded by O.M. and R.J. based on this
research.

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Supporting information

Tryptophol acetate and tyrosol acetate, metabolites secreted by a
probiotic yeast, display remarkable anti-inflammatory effects

ANCOM results after 6 hours			
ASVs	Healthy	Untreated	Treated
<i>Blautia</i>	High	Low	Low
<i>Bacteroides</i>	High	Low	Low
<i>Clostridium</i>	High	Low	Low
<i>Sutterella</i>	High	Low	Low
<i>Ruminococcus</i>	Low	High	High
<i>Parabacteroides</i>	High	Low	Low

ANCOM results after 24 hours			
ASVs	Healthy	Untreated	Treated
<i>Blautia</i>	High	Low	Low
<i>Bacteroides</i>	High	Low	High
<i>Clostridium</i>	High	Low	Low
<i>Oscillospira</i>	Low	High	High
<i>Ruminococcus</i>	Low	High	High

Table 1 ANCOM results of mice treated and untreated with molecules. Main
bacterial taxa abundance after 6 and 24 hours from LPS administration.