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Antioxidant and immune modulatory activities of fruit and vegetable extracts after "cascade fermentation"

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ABSTRACT

Macerates of diverse fruits and vegetables sequentially fermented by various strains of *Lactobacilli*, so-called "Regulates", are in use as health promoting food additives in traditional medicine. They contain a wealth of polyphenolic compounds well known for their antioxidant activities. With the aid of biochemical model reactions simulating pathological processes such as inflammations (activated PMNs, peroxynitrite), induction of atherosclerosis (LDL oxidation) and ischemia-reperfusion (xanthine oxidase, Fenton chemistry) we demonstrate that these regulates 1) exhibit excellent antoxidant properties compared to the critical standard gallic acid, and 2) preactivate human polymorphonuclear leukocytes in whole blood at low concentrations, reversing this effect at higher ones. From our data we conclude that consumption of Regulates may actively support immune reactions of the leukocytes by two mechanisms: pre-stimulation of the innate immune response in the sense of a probiotic tuning and attenuation of oxidative stress accompanying such processes.

KEYWORDS: antioxidants, hypochlorite, peroxynitrite, OH-radical, superoxide radical, fermentation, immune modulation

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ABBREVIATIONS

ACC, 1-aminocyclopropyl-1-carboxylic acid; cfu, colony forming units; KMB, α-Keto-S-methyl butanoic acid; LDL, low-density lipoprotein; LTA, lipoteichoic acid; MPO, myeloperoxidase; PMN, polymorphonuclear leukocytes; ROS, reactive oxygen species; RT, retention time; XOD, xanthine oxidase

INTRODUCTION

The knowledge about the interrelationships and connections of protective systems both in plants and in animals is exponentially increasing leading to novel insights concerning both medical-pharmacological and nutritive aspects. These new insights are based on the common problem during most diseases: "oxidative stress". The biochemistry of oxygen activation and detoxification analyzed in the past has led to the identification of many similar or more or less identical features in both plants and animals. Biochemical model reactions simulating these common situations allow to predict possible functions of plant´s defence molecules and/or systems, to find new fields of application and to exploit up to date unknown resources [1-3].

A diet rich in vegetables and fruits is well known to be associated with health-promoting effects. Secondary plant metabolites such as polyphenols, are correlated with the reduction or attenuation of certain diseases [4]. Studies showed that flavonoids exhibitseveral positive health aspects; they possess anti-carcinogenic, anti-mutagenic, antioxidant,

anti-viral, immune-stimulating and oestrogen-active properties. They also inhibit lipid- and thus LDL-peroxidation, and chelate transition metals [5-7]. Regulates are macerates of different fruits and vegetables subject to so-called cascade fermentation [8].

In this report the potential of Regulat on co-operatively protecting properties against pathological oxidations was tested. The remedy "Rechtsregulat" is commercially available in pharmacies in Germany and Austria and represents a member of food adjuvants containing both selected higher plant and microbial (cell-wall) components thus representing a new profile of concentrated "sour food".

Corresponding biochemical activities, namely both antioxidant and immune stimulatory properties, are documented with the aid of model reactions, both *"in vitro"* and *"ex vivo"* [9, 10].

MATERIALS AND METHODS

Chemicals

1-aminocyclopropane-1-carboxylic acid (ACC), dextran, Na2CO3, Folin & Ciocalteu's Phenol Reagent 2.0 normal, gallic acid, hydroxylamine, hyperchlorite (HOCl), α-keto-S-methylbutanoic acid (KMB), N-(1-naphthyl) ethylenediamine (NED), NADH, NaCl, sulphanilamide, trypan blue, xanthine and zymosan A were purchased from Sigma Munich, Germany. $CuSO_4$*$5H_2O$, EDTA, H_2O_2, KBr, and sulfonic acid were obtained from Merck Darmstadt, Germany. Diaphorase and Xanthine oxidase (XOD) were from Roche Mannheim, Germany. The gases for gas chromatography were purchased from Messer Griesheim Darmstadt, Germany. The carrier gas was N_2 (type 5.0; degree of purity 99.999%), 25 ml/min; the burning gases were H_2 (type 5.0; degree of purity 99.999%), 25 ml/min and synthetic air, 250 ml/min; ethene calibration gas (mixture of ethene and synthetic air): 1ml = 254,55 pmol, 1 bar.

Regulat

The preparation "Rechts-Regulat®" is commercially available in German and Austrian pharmacies. Regulat is derived from a stepwise ("cascade") fermentation of 17 vegetable and fruit species by five different members of Lactobacillae according to the European Patent EP No 1153549 issued 11/17/04.

Instruments

Gas chromatograph: Aerograph 3300 with Integrator; column: 0.125 inches x 60 cm aluminium oxide; column and injector temperature: 80 °C; FID detector temperature: 225 °C, Varian GmbH, Munich, Germany.

Spectrophotometer: Kontron Instruments Uvikon 922, Kontron Instruments, Eching, Germany.

Microscope: Leitz Wetzlar SM-Lux, 25/0.55, Leica Microsysteme Vertrieb GmbH, Bensheim, Germany.

HPLC: Injector: Type 231 from Gilson-Abimed, Den Haag, The Netherlands; Pumps: Type 422 from Kontron Instruments, Eching, Germany and Type 300C for post-column derivatization from Gynkotek, Germering, Germany; Column: Nucleosil 120-3 C18 250 x 4 mm, 3 µm from Macherey-Nagel, Düren, Germany; Detectors: Diode array Type Bio Tek 540+ (DAD) for wavelength band 200 - 600 nm from Kontron Instruments, Eching, Germany; Capillary for post-column derivatization: knitted PTFE capillary, length 10 m, inner diameter 0.5 mm; Software: Geminyx Version 1.91 from Dionex, Germany.

Differential analysis of phenolic groups after concentration of Regulat

Several groups of phenolics have been identified by different runs of HPLC after extraction of a 5-fold concentration of the original Regulat with different solvents (aqueous, ethyl acetate) and after different treatments (acid or enzymatic hydrolysis), as described earlier [11, 12]; different so-called "response factors" have been used asreferences(Table 1).

Quantifications

Quantification of the individual substances separated on HPLC was done on the basis of the peak surface as compared to chromatogrammes of the pure substances ("response factors").

Table 1. Response factors

Substance	RF
Gallic acid	1,1x10-5
4-Hydroxybenzoic acid	2,5x10-5
Protocatechuic acid	2,2x10-5
Ellagic acid	1,9x10-5
p-Coumaric acid	6,4x10-6
Naringenin	1,4x10-5
Apigenin	1,4x10-5

Identifications

The individual peaks were compared to the absorption spectra, maxima and retention times of the pure standards available and subdivided into the different mentioned phenolic group of substances or, if possible, identified individually.

Determination of Folin-values with Folin-Ciocalteu assay

The reducing potential is given as Folin-values using a calibration curve of gallic acid in the range of 50 – 1000 µM (equation for the linear fit of the regression line: $A_{720} = 0.0294$ AU + 0.000945 AU/µM * c with: A_{720} = absorption at 720 nm (AU), c = concentration (µM), r = 0.99755; data not shown).

The assay is as follows: 0.2 ml sample are mixed with 1 ml of a 10% solution of Folin & Ciocalteu's Reagent 2.0 normal. 1 ml of a 7% solution of Na_2CO_3 is added after 2 min, the assay is mixed thoroughly and the absorption is determined after 60 min at λ = 720 nm [13].

Biochemical test systems

The xanthine/xanthine oxidase-system

Hydroxylamine oxidation:

XOD generates superoxide anion radicals during oxidation of xanthine into uric acid. In the XOD reaction, superoxide anion radicals are detected by the conversion of hydroxylamine into nitrite.The hydroxylamine oxidation is a photometric assay to detect specifically the produced $O_2^{\bullet -}$ via oxidation of hydroxylamine to nitrite which is then measurable after azo-coupling with sulphanilamide and NED [14].

An assay contains in a final volume of 1 ml: phosphate buffer pH 7.4 0.1 M, NH_2OH 1 mM, xanthine 5 µM, xanthine oxidase 0.04 U/ml, samples (amounts indicated). After an incubation of 30 min at 37°C, the nitrite determination of 300 µl aliquots of the assay is started with 300 µl sulphanilamide 1% and 300 µl NED 0.02%. The evaluation of the assay is done after 15 min at 540 nm.

KMB fragmentation:

The xanthine–xanthine oxidase reaction produces superoxide, hydrogen peroxide and in the presence of transition metal ions OH–radical. Transition metal ions like Fe^{2+} are always found in traces in enzyme preparations. The methionine derivative KMB is fragmented by the formed ROS (mainly $OH^{\bullet}$) into ethene which is detectable via gas-chromatography. The conduction of the experiments is as follows: the assay is incubated with and without (=control) the test substance in gas-tight sealed reaction tubes with known volume. The formed ethene is measured after incubation by withdrawing 1 ml gas of the headspace with a gas-tight syringe. The ethene amount within this 1 ml is determined gas chromatographically and quantified with the aid of ethene-calibration gas [15].

A final volume of 2 ml contains: phosphate buffer pH 7.4 0.1 M, KMB 1 mM, xanthine 0.5 mM, xanthine oxidase 0.04 U/ml, sample (amounts indicated). The sampling was done after an incubation of 30 min at 37 °C.

The Fenton-system

In this system OH-radicals are generated by hydrogen peroxide in the presence of Fe^{2+}-ions. As earlier KMB is acting as indicator molecule. In the presence of strong oxidants of the $OH^{\bullet}$-type KMB is fragmented and ethene as one product can be detected gas chromatographically [1-3, 9, 10, 15].

A final volume of 2 ml contains: phosphate buffer pH 7.4 0.1 M, KMB 1 mM, $Fe(SO_4)$ 10 µM, H_2O_2 10 µM, sample (amounts indicated). The sampling was done after an incubation of 30 min at 37 °C. For ethene determination see KMB fragmentation.

The NAD(P)H-oxidase/diaphorase-system

NAD(P)H–oxidases/diaphorases are conservative enzymes; in all living cells they are present as soluble enzymes in the cytoplasm, in mitochondria and in all cellular membranes. In leukocytes they play a dominant role during the "respiratory burst". At the expense of NAD(P)H, the flavin- cofactor becomes reduced which in turn is able to reduce a wide range of autoxidable substances. The autoxidation of these substances again leads to ROS which are detectable as ethene formation from KMB. A decrease of ethene may indicate an inhibition of the enzyme or a scavenging activity of the substance investigated [16].

An assay contains in 2 ml final volume: 0.1 M phosphate buffer pH 7.4, 1 mM KMB, 75 µM NADH, 2,2 U diaphorase, sample (amounts indicated). The sampling was done after an incubation of 30 min at 37 °C. For ethene determination see KMB fragmentation.

Peroxynitrite

Peroxynitrite, a strong oxidant, can cause nitration of proteins – especially tyrosine residues and is an indicator for inflammation.

$ONOOH / ONOO^-$ is detected by ethene formation during oxidation of KMB. A decrease of ethene production in the presence of the investigated substance is equivalent to detoxification of peroxynitrite [17, 18] Peroxynitrite was synthesized according to [19]. The alkaline peroxynitrite solution was checked by $E_{302nm} = 1670\ M^{-1}cm^{-1}$ after dilution with 0.1 M NaOH. An assay contains in 2 ml final volume: 0.1 M phosphate buffer pH 7.4, 1 mM KMB, 50 µM ONOOH (20 µl of 5 mM $ONOO^-$ in 0.1 M NaOH) and samples (amounts indicated). After incubation of 30 min at 37°C, 1 ml aliquots of the headspace of the samples were withdrawn with gas tight syringes and analyzed by gas chromatography for their content of ethene.

The hyperchlorite/ACC-system

HOCl is generated by polymorphonuclear leukocytes as a product of myeloperoxidase. Ethene formation from ACC with Hypochlorite is a specific indicator and a decrease in ethene production indicates a protective ability of the test substance [1, 2].

An assay contains in a 2 ml final volume: phosphate buffered saline (PBS) pH 7.2 ; ACC 1 mM; HOCl 25 µM; sample (amounts indicated). After incubation of 30 min at 37°C, 1 ml aliquots of the headspace of the samples were withdrawn with gas tight syringes and analyzed by gas chromatography for their content of ethene.

The copper induced LDL oxidation

Oxidised LDL plays a major role in the process of atherogenesis. Its oxidation can be continuous monitored as formed dienes according to a previously published method [20]. A final volume of a 1 ml assay contains: 0.02 M PBS (pH 7.4), LDL, 0.25 µg protein, 1.67 µM $CuSO_4$, and the test substance in various concentrations. The measurement is started immediately with adding the copper. The formed dienes are measured as change in the extinction at 234 nm every 10 min for 1000 min at 37 °C.

Activated leukocytes in whole blood

In inflammatory processes activated polymorpho-nuclear leukocytes (PMN) are involved, thereby producing different reactive oxygen species.

EDTA blood (1 mg / ml blood) was obtained from healthy donors (male and female, age 20-30 years). Addition of zymosan to whole blood samples activates neutrophil granulocytes, which release the enzyme myeloperoxidase (MPO) into the extracellular environment during the degranulation process. In turn, the oxidative burst product hydrogen peroxide of the zymosan - activated neutrophils catalyses MPO dependent HOCl formation, which is indicated by ethene release from ACC [1, 2].

The assay contains in a total volume of 2 ml: 1 ml blood (freshly withdrawn from healthy donors), 5 mg zymosan, 1 mM ACC, and the indicated amounts of Regulate. All substances were solubilized in PBS-buffer pH 7.2 (containing plasma amounts of Ca^{2+} and Mg^{2+}). After incubation for 45 min at 37°C in gas-tight sealed test tubes in a water bath in the dark, 1 ml gas of the headspace was retained with a gas-tight syringe and analyzed gas chromatographically for its amount of ethene.

Statistics

The results shown are means of three individual experiments (n = 4), except in case of copper-induced LDL oxidation (n = 1). The Standard deviations are given as σ_{n-1}. All experiments were repeated two times to guarantee reproducibility.

RESULTS

Antioxidant contents of "Rechtsregulat"

Total phenolic contents in Regulat were determined with the Folin-reaction and quantified as 3 millimolar. As standard gallic acid, a key substance of the polyphenols with outstanding antioxidant properties was used. More details on these groups of substances will be given below, using a more sophisticated method.

Differential analysis of phenolic groups after concentration of Regulat

Ethyl acetate phase

In Figure 1 a wealth of peaks detectable at 280 nm are shown in the ethyl acetate phase of 5-fold concentrated Regulat. The green coloured peaks have been tried to be evaluated as to their polyphenolic origins by means of their retention times, absorption

spectra and maxima, as compared to authentic reference substances.

Simple phenolic acids

Compounds attributable to simple phenolic acids are listed in the following:

Peak 1 (RT 9.94) was clearly identified as gallic acid, while peak 5 (RT 16.05) was shown to represent protocatechuic acid. Peak 8 (RT 25.37) was identical with the properties of the standard of 4-hydroxy-benzoic acid and peak 11 (RT 38.4) could be identified as vanillic acid. Peak 20 with a RT of 66.99 with an absorption maximum at 316 indicate a derivative of p-coumaric acid. The double maximum at 254/366 nm and a retention time of 149,7 min (Peak 31) revealed to represent ellagic acid.

Flavanones

Due to their absorption-spectra as compared to the standards, eriodictyol, naringenin und hesperetin the peaks No. 12, 22, 23, 25, 27 and 28 were ordered as flavanon-derivatives. In addition enzymatic hydrolysis (with tannase) and co-chromatography of these peaks with corresponding standards support the assumption that these peaks represent derivatives of the aglykons eriodictyol, hesperetin and naringenin in a ration of 1: 0,6: 0,2.

Flavones

Comparing the absorption spectra of the flavon-aglykones, luteolin and apigenin (double peaks around 260 and 340) with the spectra of peaks No.14, 15, 16, 24, 29, 32, 33, 35, 36, 37, 38, 39 and

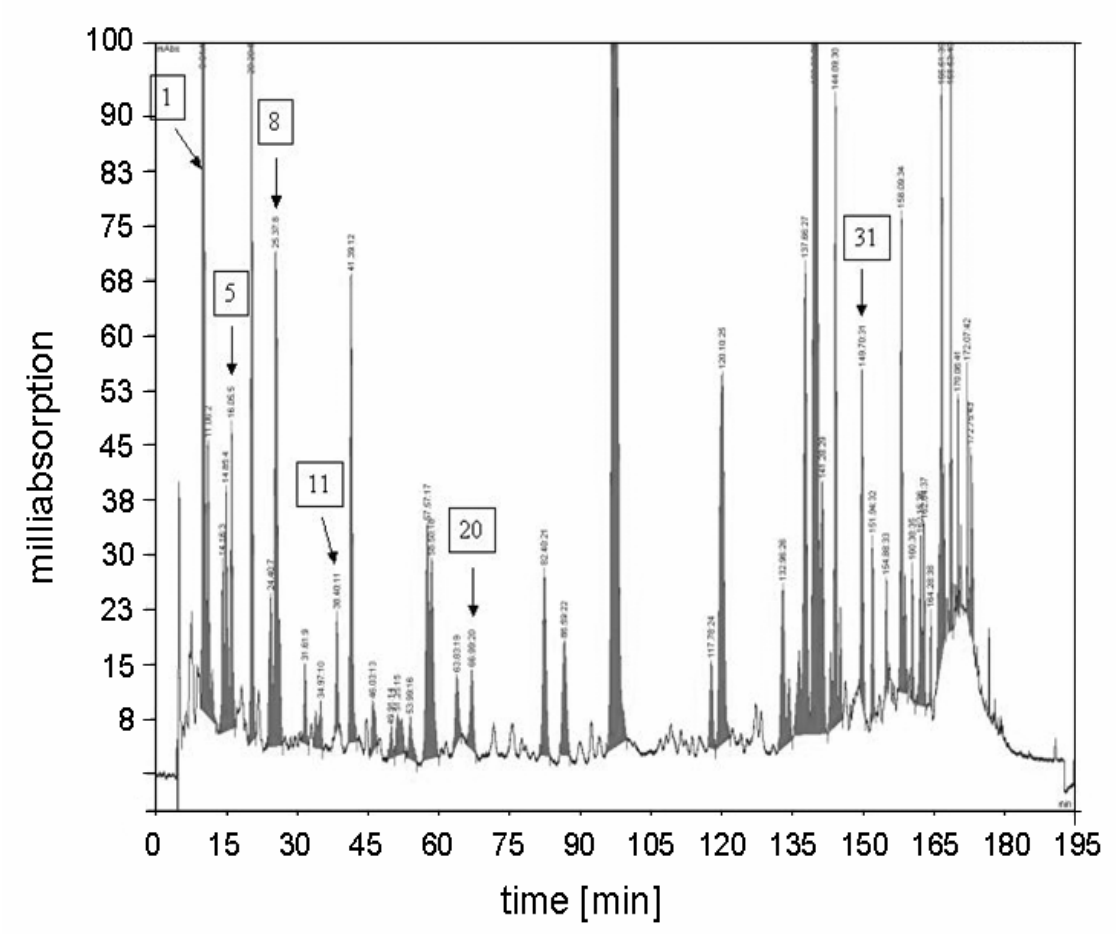

Figure 1. Phenolic ingredients of the ethyl acetate phase at 280 nm.
Peaks identified: 1: gallic acid; 5: protocatechuic acid; 8: 4-hydroxybenzoic acid; 11: vanillic acid; 20: p-coumaric acid; 31: ellagic acid;
Groups identified: peaks 1-11, 13, 17-20, 31: simple phenolic acids; peaks 12, 22, 23, 25-28: flavanone derivatives; peaks 14-16, 24, 29, 32-39, 41: flavones.

41 indicates a close relationship of these compounds with the mentioned flavones.

Unknown phenols

The peaks 4, 6, 7, 9, 10, 13, 17, 18 and 21 do not represent flavanoles despite their absorption maxima at 274-278nm, since these compounds after post-column derivatization exhibit no complex formation detectable at 640 nm.

It may be assumed that they represent structurally simple phenolics. The same holds for peaks 2, 3 and peaks 34-43.

Aqueous phase

After solubilization with ethyl acetate the remaining aqueous phase was analysed by HPLC in order to make sure that all phenolic compound were detected. Figure 2 shows a chromatogramme presenting nine major peaks.

The detected substances (peaks 5, 7 and 6, 8) due to their spectra were identified as flavones and flavanones. Peaks 1-4 and 9 could not be qualified, however.

Quantifications

The above identified substances in the ethyl acetate and the aqueous phase were quantified as to the peak surfaces of the corresponding authentic references ("response factors").

Per ml solution of Regulat a total phenolic content of 164,0 µg was identified.

From these simple acids contribute with 14,97 µg, and hydroxy cinnamic acids with 0,24 µg flavanones with 83,80 µg, flavones with 21,38 µg and unknown phenols with 43,59 µg. The largest part comprise the flavanones with 83,8 µg/ml. The content in flavones with 21,38 µg/ml comprises only ca. 30% of the flavanones where the main part is due to the aqueous phase peaks 5 (8,15 µg/ml) and 7 (4,5 µg/ml).The simple acids mainly comprise gallic acid with 4,8 µg/ml and 4-hydroxybenzoic acid with 6,23 µg/ml beside the yet unknown compounds with 43,59 µg/ml.

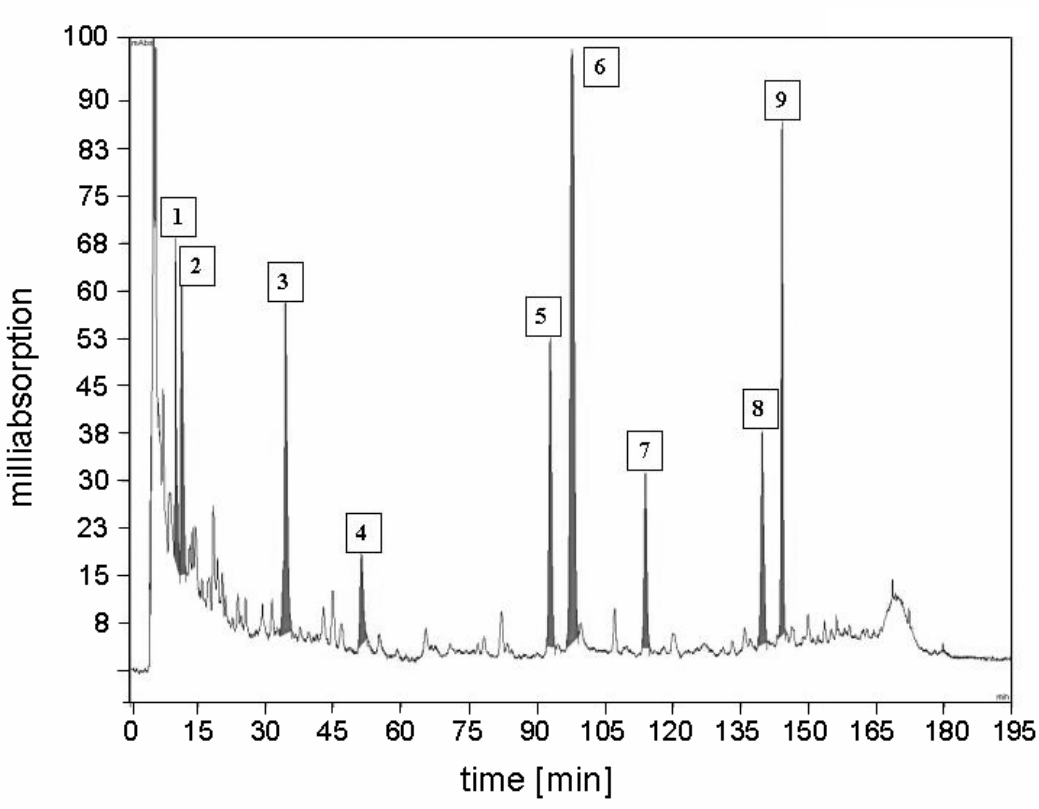

Figure 2. Phenolic compounds of the aqueous phase detected at 280 nm.
Peaks identified: 5: Flavone; 6: Flavanone; 7: Flavone; 8: Flavanone.

Living microbes

In microbiological tests i.e. breeding on different nutrient agars with different inoculi for different time scales gave no indication of living Lactobacillae. Thus Regulat is different to probiotic nutrients such as several yoghurts sold as probiotic, milk-derived food.

The biochemical model systems for determination of antioxidant capacities

In the following biochemical test systems the antioxidant capacity of Regulat is determined and compared with the reactivity of gallic acid as a known polyphenol with high antioxidant potential. In all test systems a concentration dependence of Regulat and gallic acid was investigated and the IC_{50} values (50% inhibition of the basic reaction) specified.

The xanthine oxidase model system

Xanthine oxidase (XOD) generates $O_2^{\bullet -}$, H_2O_2 and uric acid using xanthine or hypoxanthine and molecular oxygen as substrates. In the presence of transition metal ions OH-radicals can be generated via Haber-Weiss-chemistry. XOD activity is characteristic for reperfusion injury after ischemic events. It is detectable as nitrite production from hydroxylamine catalyzed by superoxide radicals, which can be decreased by superoxide scavengers or XOD inhibitors. It is also detectable as release of ethene from KMB due to the reaction with the formed ROS.

Superoxide–driven hydroxylamine oxidation forming nitrite

As shown in Figure 3 gallic acid has a great affinity to superoxide since the concentration yielding a 50%

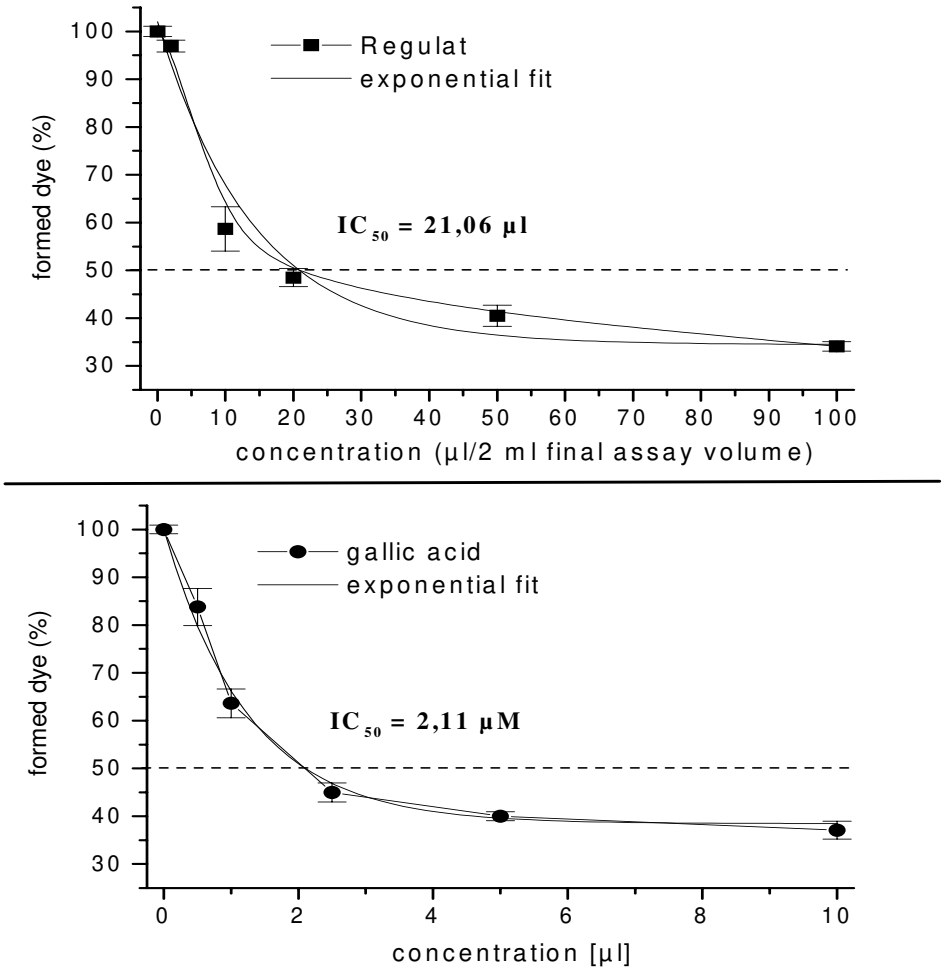

Figure 3. Inhibition of superoxide-dependent hydroxylamine oxidation by Regulat in comparison to gallic acid.

inhibition (IC_{50}) is as low as 2,11 µM or 0,40 µg gallic acid in the reaction mixture. This is one of the reason why gallic acid is of great value as a standard: the three vicinal hydroxyl groups are excellent superoxide scavengers. The IC_{50} value of Regulat lays at 21,06 µl or 3,45 µg total polyphenol content respectively.

KMB fragmentation by xanthine oxidase

The xanthine–xanthine oxidase reaction produces superoxide, hydrogen peroxide and in the presence of transition metal ions OH–radical. Transition metal ions like Fe2+ are always found in traces in enzyme preparations. Regulat and gallic acid "inactivate" these species insofar, as fragmentation of KMB into ethene is inhibited in a concentration dependent manner (data not shown).

For a 50% inhibition of the basic reaction only 10,61 µl Regulat (corresponding to 1,74 µg total polyphenols) are necessary. To achieve this inhibition 7,25 µM or 1,36 µg gallic acid are needed (Figure 4).

The Fenton-reaction

In this system OH-radials (or reactive equivalents) are not produced enzymatically by the xanthine oxidase, but chemically by the reaction of hydrogen peroxide with Fe^{2+} detected again by the KMB reaction.

Comparison of the IC_{50}-Values shows that in this critical test the inhibitory capacity of the used "gold" standard of antioxidant potential, gallic acid, is much weaker as compared to the XOD system since 113 µM (or 21,26 µg) gallic acid are required. In contrast, an amount of only 3,45 µl (or 0,57 µg total polyphenols) of Regulat achieves the 50% inhibition rate, indicating excellent antioxidant properties against the aggressive OH-radical (Figure 4).

The NAD(P)H-oxidase/diaphorase system

NAD(P)H–oxidases/diaphorases are present as soluble enzymes in the cytoplasm, in mitochondria and in all cellular membranes. In leukocytes they play a dominant role during the "respiratory burst". At the expense of NAD(P)H, the flavin-cofactor becomes reduced which in turn is able to reduce a wide range of autoxidable substances. The autoxidation of these substances again leads to ROS which are detectable as ethene formation from KMB.

Regulat was tested with commercial diaphorase and NADH as electron donor. The IC_{50} values for Regulat and gallic acid were almost identical. (4,58 µl corr. to 0,75 µg total polyphenolic content) and pure gallic acid (4,26 µM or 0,80 µg) indicate that in this system primarily superoxide, and in the sequence OH-radicals may be produced (Figure 4).

Peroxynitrite at sites of inflammation

Peroxynitrite is a strong oxidant and nitrating agent, oxidizing preferentially reduced sulfur compounds like thiols or methionine residues. During inflammation, it is formed by the extremely ($k>>10^9$) rapid reaction of superoxide anion radical with nitrogen monoxide, produced from arginine, catalayzed by different NO synthases at inflammatory sites. Like OH-radicals produced by the XOD or Fenton systems, peroxynitrite can be quantified by KMB. Thus we used ethene formation from KMB as a sensitive test for peroxynitrite mediated damage of methionine like residues. Whereas OH-radical induced formation of ethene from KMB is completely blocked by ethanol (>100 mM), peroxynitrite mediated ethene formation is still observable in ethanolic solutions. Thus peroxynitrite is only negligible forming OH-radical via homolytic decomposition into NO_2-radical and OH-radical [15].

Regulat in this reaction with an IC_{50} of 11.67 µl (corr. to 1,91 µg total polyphenolic content) in comparison to gallic acid (IC_{50}: 35,83 µM or 6,74 µg) exhibits much stronger antioxidant potentials toward peroxynitrite damage of methionine structures, although this does not seem to be a free radical mediated reaction (Figure 4).

Antioxidant capacities against hypochlorous acid (HOCl)

Under the conditions in our model (pH 5,7), commercial available MPO catalyses the formation of the strong microbicidal oxidant hypochlorous acid in the presence of hydrogen peroxide and chloride. Ethene formation after chlorination of the detector molecule ACC was detected.

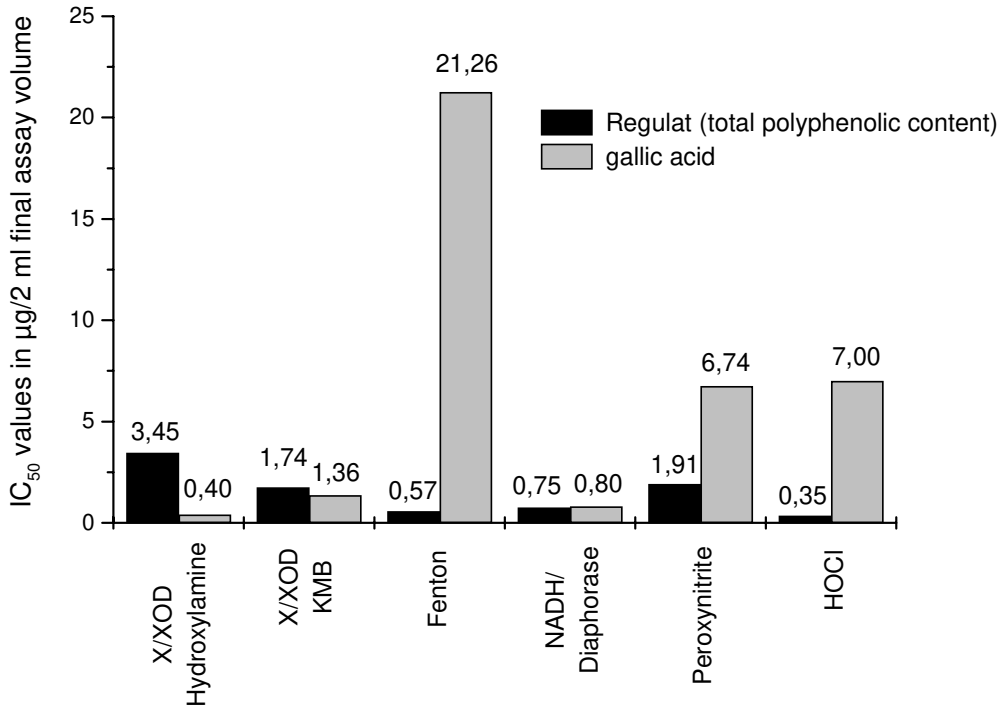

Figure 4. Comparison of the IC_{50}-values of Regulat and gallic acid in different model systems.

In this model system, Regulat exhibits its lowest IC_{50}-value (2,12 µl or 0,35 µg total polyphenols) thus offering the best protection. The IC_{50}-value of gallic acid is close to 37,21 µM (or 7,00 µg).

Comparison of the IC_{50} values from all antioxidant test systems

Figure 4 shows the reactivity of Regulat in comparison to gallic acid in six different systems documented as the amounts in the assays reaching a 50% inhibition (IC_{50} values).

In the applied biochemical model systems Regulat showed antioxidant potentials directly comparable to pure gallic acid. Particularly in the Fenton system but also against peroxynitrite and HOCl Regulat exhibits IC_{50} concentrations well below the values of the standard.

Protection of Low Density Lipoprotein (LDL) oxidation by Regulat

As a human lipoprotein fraction LDL carries major parts of cholesterol in plasma. LDL cholesterol levels are regulated by receptor-mediated clearance via LDL receptor in the liver and to a lesser extent in peripheral tissue. LDL is the main lipoprotein fraction involved in atherogenesis, the major cause of heart disease and stroke. The concept that oxidized lipoproteins are involved in atherosclerotic lesions development was established from the evidence that LDL can injure cells under certain conditions. LDL modified by malondialdehyde as well as by cultured endothelial cells were taken up by monocytes/ macrophages via scavenger receptor (reviewed in [15]). This uptake of lipoprotein-derived lipid by macrophages lacks back regulation of their cholesterol content, thus leading to an unlimited uptake and to an invasion of these macrophages into the endothelium of the blood vessels inducing formation of atheroma.

One process of modification of LDL is its copper induced oxidation, which can be measured as increase in absorption at 234 nm, reflecting lipid peroxidation-associated diene conjugation. The delay of the onset of dienconjugation, i.e. the increase of the extinction at 234 nm reflects protection of LDL by intrinsic antioxidants [21].

If we add increasing concentrations of Regulat to preparations of LDL the time lag of maximal diene conjugation is clearly shifted backward; after addition of 2.5 µl Regulat (to 1 ml LDL– buffer-solution) the start of oxidation is retarded for additional 220 min. The addition of 5 µl causes a

delay for additional 560 min clearly indicating the powerful protection of LDL from copper-mediated oxidation (Figure 5).

Cooperative action of Regulat with ascorbic acid

If 1 µM vitamin C (ascorbic acid) is added to the same LDL-buffer mix a slightly accelerated LDL oxidation is observed: the onset of diene conjugation is shifted for 12 min from 120 min to 108 min. This effect is well known and based on the reduction of CuII+ to CuI+ by ascorbate. The combination of 2.5 µl Regulat/ml LDL-buffer mix with 1 µM ascorbate however extends the time lag from 344 min with Regulat alone to 432 min in combination with ascorbate. Thus the negative effect of ascorbate alone of minus 12 min is converted into plus88 min by the presence of Regulat indicating cooperative effects of this two components (Figure 6).

Inflammatory reactions: activated leukocytes in whole blood

MPO-catalyzed ethene formation after chlorination of the detector ACC was performed with whole blood samples as a natural environment. Upon activation with zymosan, leukocytes (PMNs) present in the whole blood sample release both, MPO and hydrogen peroxide, into plasma (which contains chloride, 100 mM). MPO catalyses the formation of the strong microbicidal oxidant hypochlorous acid (pKs = 7.5) in the presence of hydrogen peroxide and chloride.

Simultaneously cell viability (=cytotoxicity) was determined. 100 µl Regulat in the test system showed no signs of cell toxicity (data not shown).

After activation by zymosan the expected activation of PMNs in whole blood was significantly measurable and set as 100%.

In the presence of 1 µl Regulat the reaction was not visibly influenced while with 10 µl Regulat a statistically significant stimulation was observable. In contrast, 100 µl Regulat caused a clearly significant inhibition of this activity (Figure 7).

If this experiment is performed in whole blood in the absence of zymosan it can be shown that 10 and 100 µl Regulat yield an activation of PMNs in whole blood (Figure 8).

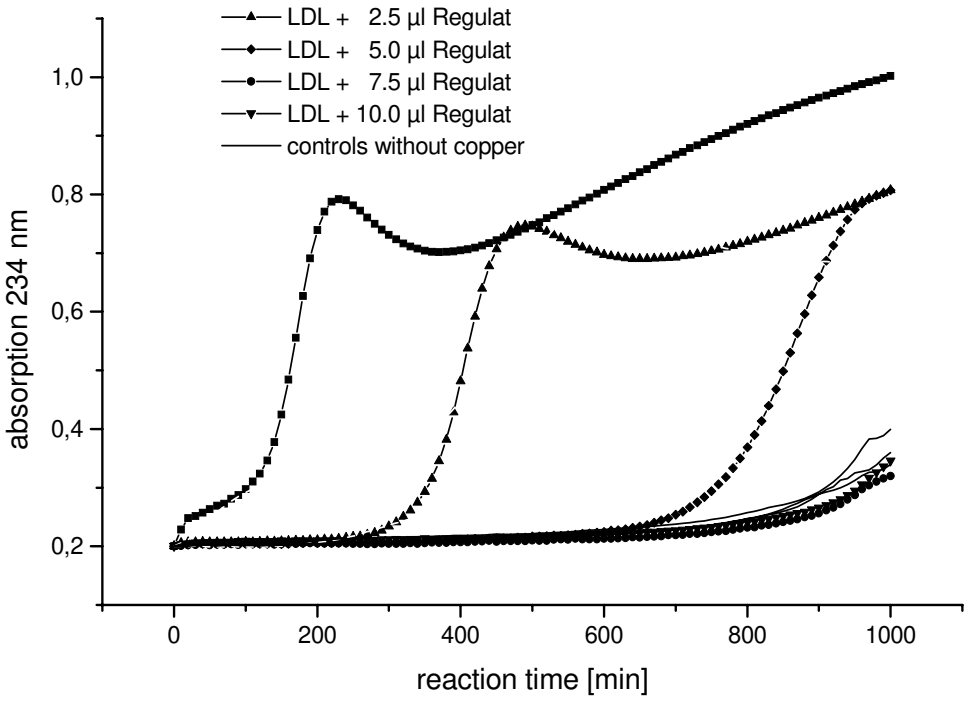

Figure 5. Influence of Regulat on copper induced formation of conjugated dienes in LDL.

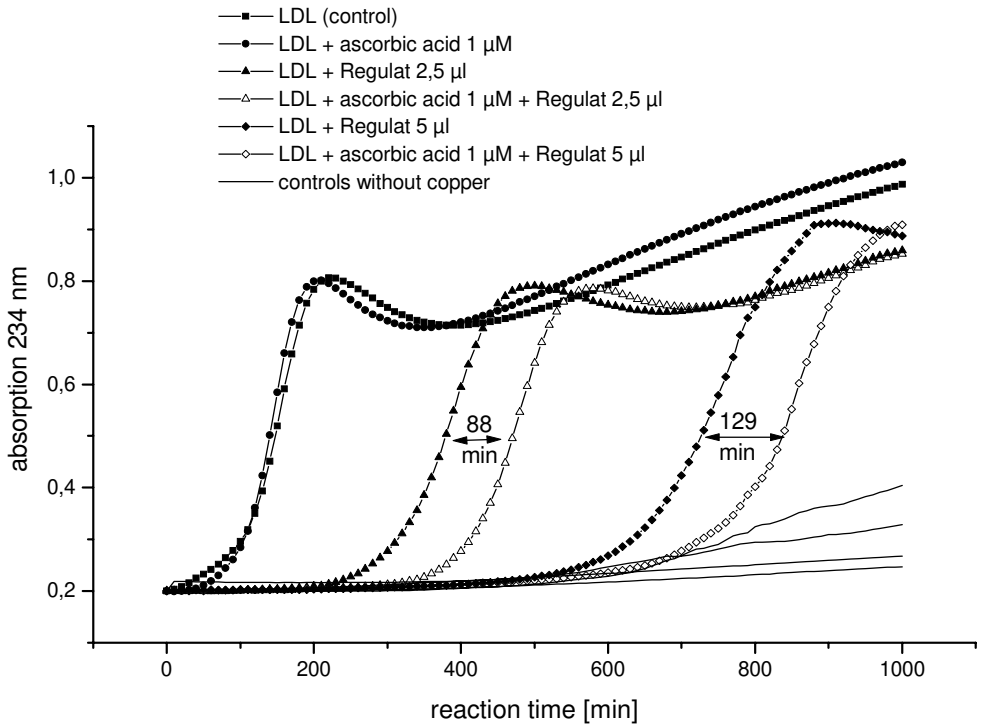

Figure 6. Cooperative effect of Regulat and ascorbic acid on copperinduced formation of conjugated dienes in LDL.

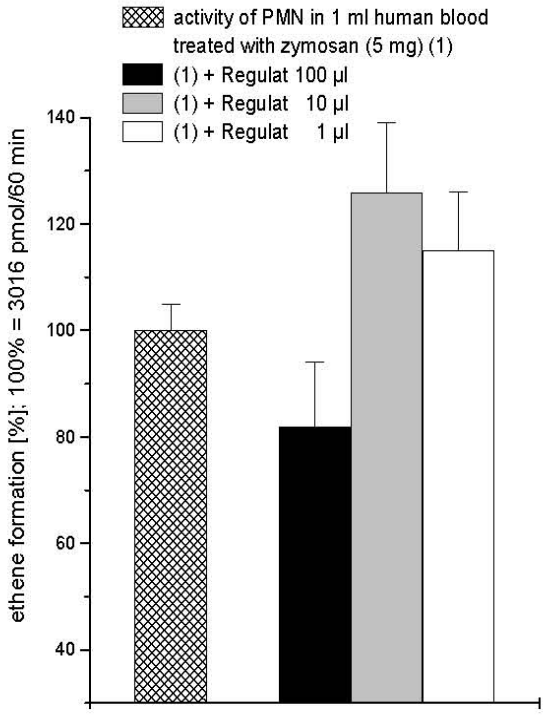

Figure 7. Influence of Regulat on activity of PMNs in whole blood stimulated with zymosan.

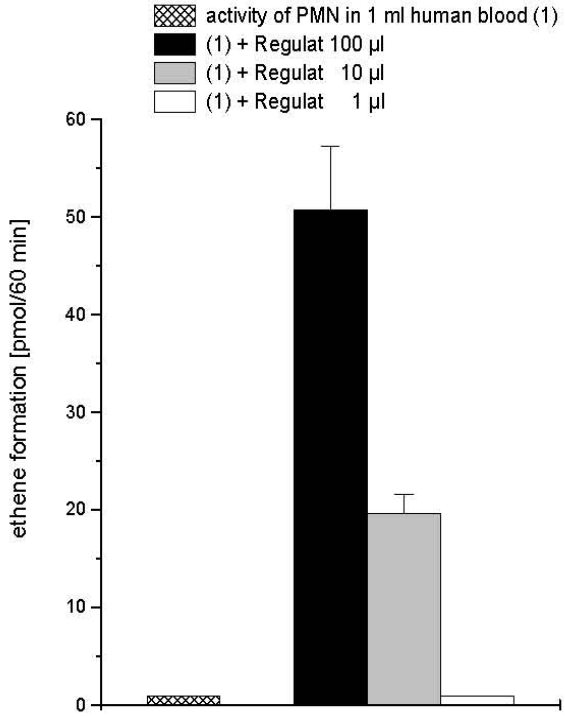

Figure 8. Stimulation of PMN activity in whole blood in the absence of zymosan.

DISCUSSION

Regulat is derived from a stepwise ("cascade") fermentation of 17 vegetable and fruit species by five different members of Lactobacillae.

Regulates thus contain increased contents of "secondary" plant metabolites concentrated by the fermentative degradation of proteins and carbohydrates thus indirectly increasing phenolics, carotenoids and minerals, together with bacterial matter, thus yielding new and until now unknown synergistic effects.

The antioxidant principle

The model reactions shown under results and also used in earlier work [9, 10, 15, 20, 22] represent "key reactions" of certain diseases such as ischemia-reperfusion, gout (xanthine oxidase and "Fenton-chemistry"), inflammations, i.e. unspecific immune reactions (myeloperoxidase of neutrophil granulocytes), blood vessel damage (peroxynitrite) as well as intoxications and side effects of chemotherapy (oxidoreductases coupled to redox-cycling quinones such as adriamycin).

These mentioned reaction systems are mostly initiated by enzymes creating reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hypochlorous acid, peroxynitrite and OH-radicals. Under the influence of these oxidants *"in vivo"* biologically important molecules and structures such as nucleic acids, enzyme proteins, membrane lipids and others are damaged or destroyed finally inducing disease-typical symptoms. In our models "molecular baits" (such as KMB or ACC) are used in order to sensitively detect both oxidative destructions as well as respective protections by the variation of the individual products i.e. ethene formation.

With the aid of the mentioned systems the different components in Regulat can be documented as highly active antioxidants effectively preventing the formation or abolishing the mentioned ROS quantified as diminished product formation from the "molecular baits".

The evaluation of these activities is done on the basis of the IC_{50} -keys: IC_{50} = concentration or amount of Regulat or standard inhibiting the product formation from the "bait molecules" by 50% .

Since Regulat is a polyphenol-rich product containing simple phenolic acids like gallic acid, flavanones and flavones, besides many others, it is conceivable that in the applied biochemical model systems Regulat showed antioxidant potentials directly comparable to pure gallic acid. As extremely though test system the "Fenton" system may be addressed (see Figure 4).

According to the presented results Regulat contains highly potent antioxidants acting both in hydrophilic and in lipophilic environments i.e. in aqueous enzymic systems and as protection of LDL, potentially cooperating with ascorbic acid.

On the other hand Regulat contains stimulator(s) of the unspecific immune response of neutrophils: are these leukocytes inactive, compounds in Regulat cause an upregulation of the production of hypochlorous acid already at low concentrations (10 µl) while higher (100 µl) concentration of Regulat inhibit HOCl- dependent chlorinations of already stimulated neutrophils thus potentially avoiding overshoot reactions of the respiratory burst and subsequent transformation of oxidants. Similar results are obtained with a lipophilic system: LDL oxidation by copper ions is cooperatively retarded by ascorbic acid and Regulat while ascorbic acid alone is prooxidant (Figure 6).

The immune modulatory principle

There is no doubt that certain lactic acid producing bacteria exhibit immune-modulatory activity in humans, animals and in vitro known as "probiotic" effect. Since more than ten years a continuously growing flood of publications on this topic has been published.

E.J. Schiffrin *et al.* [23] reported on the immune modulatory influence after intake of Lactobacillae:

Healthy volunteers received either *Lactobacillus acidophilus* or *Bifidobacterium bifidum* suspensions and were tested as to the phagocytosis of peripheral leukocytes: after 3 weeks of intake this activity was doubled with both preparations; this activity was retained for more 3 weeks after the stop of intake with *L. acidophilus* and decreased slowly in the case of *B. bifidum*. The authors concluded that intake of the mentioned bacteria included in the specific milk products may help to stimulate the immune functions of elderly people and newborns

thus suggesting signal functions from the intestinal tract to the blood system.

O. Vaarla [24] published a review on the same subject discussing several clinical studies:

a) The intestinal flora of Swedish and Estonian children directly correlates with the risk for allergies: the Estonian children had less allergic symptoms and more Lactobacillae were found in the intestinal flora as compared to Swedish children which showed stronger allergic symptoms.

b) Children from Finland which developed atopic sensitivities had more Clostridia and less Bifidobacteria and Lactobacillae; similar results were reported from Japan concerning atopic dermatitis.

From this and other studies one may conclude that Gram-positive bacteria in the intestinal flora reversely correlates with atopic risks thus recommending an intake of Lactobacillae as a prophylactic protection. This correlation was also established for Gram-negative bacteria but due to security reasons these micro organisms cannot be used for clinical studies: Gram-positive Lactobacillae are on the save side.

c) Capsules containing *Lactobacillus GG*, were administered pregnant young females for 2-4 weeks before the birth date. In a prospective study over 2 years the development of atopic eczema were followed: in the "probiotic" group 15 out of 64 children (23%) developed eczema, in the control group, however, 31 out of 68, (46%) were counted. The risk thus decreased by approximately 50% through Lactobacillus–intake.

After 4 years another test was elaborated:

In the Placebo-group 25 out of 54 children had eczema, in the verum-Group, however only 14 out of 53. Another Study of a group from Finland showed that hydrolysates from Gram-positive bacteria were able to positively modify immunological parameters and factors in blood, urine and faeces.

Likewise, a Japanese group investigating nutritional allergies showed that early probiotic treatment via "induction" through the intestinal tract decreases allergies and increases oral tolerances.

In elderly people effects on cellular immunity were investigated. Since the activity of natural killer cells decreases with age and likewise also the sensitivity towards viruses and tumours. The number of killer cells after a 3 week supplementation with probiotics increased significantly in comparison to young people.

f) Rheumatoid arthritis, similarly to atopic or autoimmune diseases, correlates with a reduced formation of interferon (IFN)-gamma, which is significantly increased by probiotics.

Experiments on investigating dosage

Data from the corresponding literature [24, 25] show, that it seems to be clear that lactic acid bacteria induce the "Innate Immunity". Thus the question as to the amount of bacteria necessary and the time course of this effect was raised. Two groups contributed significantly to these questions namely the NESTEC team in Lausanne and the "Milk and Health Research Centre" of the Massey University in New Zealand.

Sustainable bacteria in the intestine

First the question as to the stomach-intestine passage was raised by Schiffrin and colleagues [26]: with 28 volunteers of age between 23 and 64 years both the survival of bacteria in the gut and the attachment onto the intestine epithelia was important for the immune reactivity of the volunteers. This holds for both *Lactobacillus acidophilus* as well as for *Bifidobacterium bifidum*, where 1-7 x 10^{10} cfu ("colony forming units") over a time period of 6 weeks seemed to be sufficient. A measurable success was seen already after 3 weeks: phagocytosis of granulocytes and monocytes was significantly increased by both the applied bacteria strains in milk products.

Effective dosis

Arunachalam *et al.* [27] tested 25 persons over a time period of 6 weeks where 12 persons got twice a day 180 ml milk and 13 persons 180 ml milk plus 1.5 x 10^{11} cfu of *Bifidobacterium lactis,* Strain HN019. Only the persons supplemented with the bacteria exhibited increased Interferon alpha–values, stimulated peripheral monocytes

and stimulated phagocytosis of the neutrophilic granulocytes.

Donnet-Hughes *et al.,* again members of the NESTEC group [28] stated, that via *Lactobacillus johnsonii* (earlier known as *L. acidophilus*) fermented milk (150 ml for 3 weeks) no increased numbers of bacteria of this strain were counted in the gut or the faeces, but already 10^9 bacteria of *L. johnsonii* over a period of 3 weeks stimulated the "respiratory burst", (formation of reactive oxygen species) by the granulocytes as well as the phagocytosis.

In summary the administration of ca. 10^8-10^9 cfu of *L. johnsonii* over 3 weeks causes an induction of immunological functions (i.e. phagocytosis and the respiratory burst).

Apparently the life-passage of the bacteria through the gut does not seem to be a prerequisite for this function.

Mechanism of activation of innate immunity

The mechanisms of induction of immunological changes by Lactobacillae has mainly been studied *in vitro* with cell cultures, isolated blood cells from animals and humans and *in vivo* animal models and seems to proceeds via specific pattern recognition molecules (PRMs) [29]. Two main ways appear to be opened: Type 1 helper T-cells (TH1-cells), or Type 2 helper cells (TH2-cells), seem to be involved in activation, which in turn proceeds either the cell-mediated immunity (TH1) producing Interferon gamma, interleukin 2 (IL 2) and tumour necrosis factor or the humoral immunity pathway (TH2), producing the interleukins 4, 5, 6 and 10. The balance of the pathways is governing the homeostasis between defence and inflammatory disease [29]. In Gram positive cells two main cell wall components seem to be recognized as primary inducers: peptidoglycans [29] and lipoteichoic acids (LTA) [30]. With the Dlt- mutant of *Lactobacillus plantarum* which incorporates less D-alanin (D-Ala) into LTA, it could be shown that less D-Ala in LTA favours the anti-inflammatory way via enhanced production of IL 10 by human monocytes as compared to the wild type, WT. While the WT strain exhibited 41% D-Ala in LTA the Dlt-strain contained only ca. 1%. The IL 10/IL 12 ratio produced in monocytes after exposition of human blood from 11 donors towards either WT or Dlt-cells (10^7 cfu of *L. plantarum*) increased form 3.5% to 122% [31].

From these reports it seems clear that Gram positive bacteria such as lactic acid producers are able to modulate innate immunity in mammals.

In consequence to this report very recently Foligne *et al.* [32] presented a model to predict immunogenic activity of lactic acid bacteria by comparing the cytokine pattern with an animal model for acute inflammation.

We obtained corresponding results with regulate:

As shown under results, the remnants of lactic acid bacteria in the Regulate activate the production of HOCl (via respiratory burst and degranulation of neutrophilic granulocytes in whole blood in a concentration-dependant manner (Figure 8) in the absence of zymosan where higher concentration of regulate are inhibitory in the presence of zymosan (Figure 7). This fact may be due to both the antioxidant functions in Regulat and down regulatory activities of Lactobacillae at higher concentration in respect to the stimulation by zymosan. Further experiments will be necessary to clarify this phenomenon in respect to the corresponding cytokine responses.

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