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ORIGINAL PAPER

The synergism of natural compounds in the pursuit of safe and healthier food

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Abstract Food producers apply modern processing techniques and use a variety of preservative additives to guarantee safe food and a longer shelflife. Regrettably many of these impact the sensory characteristics of the foodstuffs, such as colour, texture, and flavour, which can result in low consumer acceptance. Additionally, strategies used to reduce growth of spoilage and pathogenic bacteria are not selective enough and may inactivate also desired microbiota. Food is usually overdosed with antimicrobials that are supplemented 'just in case.' Consequently, food producers are searching for natural preservation methods that are not harmful to humans. Nature offers a wide spectrum of biologically active (phyto) chemicals that can be used as potential natural preservatives. Compounds with bacterial growth-limiting properties are detected in all parts of plants, including their leaves, flowers, fruits, roots, etc. These are mostly acids, alcohols, medium and long-chain organic acids, terpenic compounds, and their derivatives. This study focused on the effectiveness of plant extracts, i.e., synergism between terpenoids and medium chain fatty acids in cured cooked meat. Bacterial strains that were tested include typical members of the spoilage microflora in vacuum (*Lactobacillus curvatus*) and MA-packed meats (*Brochothrix thermosphacta*). These were isolated and identified in a separate study. *L. curvatus* was observed to be very resistant against either terpenoids or fatty acids when used separately, whereas its growth was strongly inhibited when both chemicals were combined. Growth of *B. thermosphacta* was significantly inhibited when antimicrobial compounds were solely applied, whereas a blend of terpenoids and fatty acids showed an almost bactericidal effect.

Keywords Food preservation · Functional food · Synergism · Plant extracts

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Introduction

Antibacterial properties of plant extracts have been studied for years. The scientific literature focuses especially on terpenoid compounds since they display the widest spectrum of antibacterial activity [1–5]. Other effective antibacterials such as medium and long chained fatty acids are less researched. Amongst terpenoids, the strongest antibacterial compounds are found in cloves, allspice, thyme, sage, oregano, and garlic, with the most active molecules

being thymol, carvacrol, carveol, cymene, geraniol, and eugenol [2]. Two mutual enantiomers, thymol and carvacrol, are of special interest to scientists because of their dissimilar activity. According to Lambert [6] and Bagambula [7], thymol is a stronger inhibitor, whereas Yamazaki [8] displays the opposite. Their antibacterial activity strongly depends on the environmental conditions. It is however certain that the combined application of both compounds results in a stronger antibacterial effect than if applied separately [2, 9, 10]. Similarly, cymene (a precursor of carvacrol) shows poor antibacterial activity when applied alone, but used in combination with other (terpenoid) compounds, the blend results in an enhanced growth-limiting activity [7]. This suggests that there is a synergetic interaction between compounds and that the antimicrobial activity can be strengthened by mixing antimicrobial agents. Furthermore, by mixing their concentration can be significantly reduced, which possibly eliminates their undesired sensorial impact on preserved food [11–13]. The antibacterial properties of terpenic compounds are due to the presence of delocalised electrons in the molecule, which according to Ultee [14] allows for easier deprotonation, playing an important role in their antibacterial activity. Additionally, it is reported that terpenoid compounds have a greater inhibiting effect on gram-positive rather than gram-negative bacteria [15]. Some researchers even report poor or no growth-limiting activity against gram negatives [2]. The most extensively studied fatty acids are C4–C16 compounds. Huhtanen [16] determined that the number of carbons in a chain of aliphatic alcohols determines the antibacterial effectiveness. The longer the chain, the stronger the bacteriostatic signal is, up to a certain limit. This is most likely determined by their solubility in a hydrophilic environment, which consequently influences the penetration of bacterial cells and their subsequent inhibition. Huhtanen found that for alcohol C4 the value of MIC (minimal inhibitory concentration) was 10 mg/kg and subsequently decreased to 0.16 mg/kg for C16, whereas with two additional carbons in the chain (C18), the MIC rapidly increased to 25 mg/kg. Also Delaquis [9] found a relationship between the chain length of organic alcohols and their antibacterial activity. Nakai and Siebert [17] studied bacteriostatic activity against spoilage bacteria of 16 different organic acids and observed the strongest inhibition in cases of medium-chained fatty acids (C8–C14). Immerseel [18] reports strong antimicrobial activity of fatty acids against the human pathogen *Salmonella enteritica*.

Physical treatment of food purports to rapidly inactivate undesired bacteria, with the use of different types of packaging to safeguard against contamination. Alternatively, preservative supplements are used to safeguard food products by suppressing the uncontrolled growth of pathogenic and spoilage bacteria. Long time exposure of bacteria to small quantities of growth-limiting agents leads to the activation of defence mechanisms in bacteria and thus their adaptation [10]. Microorganisms can survive harsh environments if they are exposed earlier to a low dose of antimicrobials or adverse conditions [12, 13, 19, 20]. One explanation is that genes responsible for the development of resistance mechanisms such as the production of enzymes, changes in bacterial membrane, etc., are activated when cells are exposed to unfavourable environmental conditions. An example is the gene *horA*, coding production of ATP-dependent carriers that can expel undissociated antimicrobials outside of the cell [21]. Microorganisms also have specific membrane proteins that act as sensor detectors inducing a defence mechanism after detecting a menacing signal. Other mechanisms involve active transport of molecules passively entering the cell. In that case molecules can be encapsulated in proteins and be actively expelled to the external environment [21]. Also, bacterial membranes of cells living in beverages rich in alcohol contain a large number of long chain fatty acids that increase their resistance to alcohol.

Materials and methods

Broth medium

A brain heart infusion broth (VWR International, Belgium) was used as a base in a laboratory medium in order to imitate nutritional conditions of cured cooked meat. The broth was supplemented with nitrite curing salt (2.00%), an antioxidant (sodium ascorbate, 0.05%), sugar (glucose syrup, 0.50%), and yeast extract (0.30%). Values of pH and water activity were adapted, using a phosphate buffer and NaCl, to meet conditions in reference cooked cured meat (5.85 and 0.975, respectively) purchased in the supermarket (average of $n = 150$ samples). After autoclaving, sterile test tubes were separately inoculated with *Lactobacillus curvatus* and *Brochothrix thermosphacta* at an initial concentration of 2 log CFU/ml and thoroughly mixed on vortex. These inoculums were incubated for 2 weeks at +12°C. In order to select the most representative temperature, conditions were inspected ($n = 25$) in open display refrigerators in supermarkets in Flanders. The highest recorded temperature was +12°C, and the average temperature was +7°C. So for the current study the average and highest temperatures were selected for incubation.

Preparation of antibacterial products

For the current study three terpenoids (thymol, carvacrol, and cymene) and two medium chain fatty acids (capric and caprylic) were selected. Values of MIC (minimal inhibitory

concentration) were estimated in a separate study using an MIC method similar to that described by Delaquis [9]. The following MIC concentrations were used (mg/kg) respectively for terpenoids and fatty acids: 1,000, 750, 2,500, 1,000 and 1,500. In order to examine the synergistic activity, chemicals were applied at concentrations that met the conditions necessary for synergism where $FIC_{AC,\ AR} < 1$. In case all products were applied together (SYN), the concentrations were additionally decreased by 50% so that $FIC_{SYN} < 1$.

In the test tube analysis, terpenoid compounds[1] and fatty acids were initially dissolved in alcohol supplemented with Tween 20 (0.10%) and filter-sterilised using a 0.20-µm pore size cellulose filter applied with a syringe (Schleicher & Schuell, Belgium). The final ethanol concentration in the target test tubes was 0.10%.

For experimentation using the meat model, all antimicrobial compounds were initially separately dissolved in alcohol, filter-sterilised and subsequently suspended in a sterile sodium caseinate solution (2.50%) in saline water and left in a sonic bath for 3 × 2 min in order to decrease the concentration of alcohol by 100-fold (comparing to broth assays) and sustain organic compounds in the water phase.

Cured meat model

Porcine muscles *Longissimus dorsi* of freshly slaughtered animals (24–48 h) were used in the meat model preparation. Muscles were initially manually cleaned of skin, fat, connective tissue, and veins, and minced (8 mm size pores) with a meat mincer (OMEGA, type M32s, Milan, Italy). The next step involved the addition of cured brine containing phosphate 0.50%, glucose syrup 0.50%, nitrite curing salts 2.00%, and Na-ascorbate 0.10%. The application of antimicrobial compounds in appropriate concentrations preceded the canning (round-shaped cans 50 mm high, 70 mm diameter) and the cooking process ($T_{ambient} = 72°C$, $T_{internal} = 70°C$). Products were cooked until a thermodynamic value F of 45 min was achieved. Afterwards cans were rapidly cooled in an ice water bath to +7°C and stored for 24 h in a refrigerator at +7°C.

Microbiological tests

Prior to opening the cans, all surfaces were disinfected with a 75% ethanol solution. After slicing, the meat (1.00 mm thick) was inoculated with a 2 log CFU/g bacterial suspension, vacuum packed, and incubated 4 weeks at +7°C. The chosen temperature was the average from ($n = 25$) supermarket display refrigerators. At regular time intervals (48 h), samples were blended for 60 s in a stomacher together with adequate volumes of 0.1% sterile peptone water. For each condition three samples were analyzed.

Subsequently samples were serially diluted 10-fold in sterile peptone water. Appropriate dilutions were plated on selective agars, i.e., de Man Rogosa & Sharpe (MRS) agar (ref. 1.0660.0500; VWR International, Belgium) and Streptomycin-thallous acetate-actidione (STAA) agar (ref. CM0881B) complemented with a selective supplement (ref. SR0151E; Oxoid, Belgium). Bacteria were incubated 48 h at 35 and 22°C, respectively.

Data analysis

The following data were processed: F-cooking value, bacterial growth parameters, bacterial inhibition rate, and synergism rate of compounds.

During thermal processing, temperature and time were recorded. The cooking process was terminated when the thermodynamic parameter F had reached 45 min. For the purpose of calculation, the standard equation was used (1), where t and T are measured parameters of time and temperature, respectively: $T_{ref} = 70°C$, $z = 10$

$$F = \sum_{i=1}^{n} 10^{\frac{T - T_{ref}}{Z}} \times \Delta t \tag{1}$$

The parameters of bacterial growth (μ_{max}, λ, d) were determined using standard equation (2) proposed by Monod $\frac{dN(t)}{dt} = \mu N(t)$ from which maximal specific growth rate (μ_{max}) was calculated as follows:

$$\mu \int_{t_1}^{t_2} dt = \int_{x_1}^{x_2} \frac{dx}{x} \Rightarrow \mu_{max} = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \tag{2}$$

The growth-limiting capacity of the applied antimicrobials was calculated by using the original formula (2) and expressed in percentage of inhibition:

$$\Delta\mu_{max}[\%] = \frac{\mu^{ref} - \mu}{\mu^{ref}} \times 100;\ \Delta\lambda_{max}[\%] = \frac{\lambda - \lambda^{ref}}{\lambda} \times 100$$

Synergism of products was expressed by FIC index (Fractional Inhibitory Concentration), equation (3), where FIC <1; A, B, … are particular products applied in a certain concentration, *An*, *Bn*, … fractional concentration of the particular agent within the mixture of compounds; *MIC* stands for the minimum inhibitory concentration of the agent against a particular bacterial strain:

$$FIC = \frac{\sum_{i=1}^{n} MIC_{An}}{MIC_A} + \frac{\sum_{i=1}^{n} MIC_{Bn}}{MIC_B} + \cdots; \tag{3}$$

Collected data were then analysed using one-way ANOVA to determine whether the treatment had a

[1] Chemicals (terpenoid compounds and fatty acids) were purchased in Aldrich, Belgium.

significant impact on the monitored growth parameters. Each of the antimicrobial combinations were tested in triplicate. For statistical analysis, the SPSS programme was used. For the collected response variables, ANOVA analyses were always carried out on the mean of triplicate measurements of samples in order to analyse independent observations. When factor effects were detected, a multiple comparison technique was used to locate differences among the factor levels.

Results and discussion

Natural products and particularly plant-derived compounds may have wide application in the preservation of different foodstuffs. However, the challenge is selectively targeting undesired bacteria and omitting any side effects on the sensory characteristics of foods such as original flavour or colour. Natural products demonstrate different modes of action [22–26], and in order to achieve the desired inhibition effect, products belonging to different groups should be combined as shown in the current study.

Table 1 summarises the values of bacterial growth kinetics (maximal specific growth rate-μ_{max} [d-1]; lag phase-λ [d]; generation time at exponential growth phase-g_{max} [h] and achieved maximal bacterial count-logN_{max}) and inhibition rate of both bacteria ($\Delta\mu_{inhib.}$ [%] and $\Delta\lambda_{inhib.}$ [%]) in the meat model. As expected, both types of agents studied demonstrated different growth-limiting activity on selected bacteria. Fatty acids showed stronger inhibition than terpenoids. Also, it was observed that *Lactobacillus curvatus* was the most resistant to the compounds tested. Dorman and Deans [27] also investigated the ability of natural products to inhibit bacterial growth and found that *L. curvatus* was the most resistant among the tested gram-positive bacteria. Experiments in broth (data not shown) showed significant inhibition of *Brochothrix thermosphacta* either by aromatic compounds (AR) or a mix of fatty acids (AC), $P < 0.02$. Bacterial count of *Lactobacillus curvatus* in samples containing terpene compounds (AR) reached a similar level as in the reference compound, while in the presence of fatty acids (AC), the growth was inhibited ($P > 0.05$). Even despite the presence of thymol and carvacrol, the growth inhibition was poor. Bagambula [7] demonstrated that thymol had the strongest inhibitory impact on selected bacteria; however, the concentration of the agent was much higher than that used in the current study. Further, combining fatty acids and terpenoides (SYN) resulted in a stronger inhibition in comparision to using fatty acids (AC) ($P < 0.05$) alone. In the case of *Brochothrix thermosphacta*, the synergism between terpenes and fatty acids (SYN) showed an almost bactericidal effect; the bacterial count remained at the level of the inoculation after 2-week incubation.

Table 1 Growth kinetics of *Lactobacillus curvatus* (L) and *Brochothrix thermosphacta* (B) in the cooked cured meat model

Products	μ_{max} (d-1)	λ (d)	g_{max} (h)	log (N_{max})	$\Delta\mu_{inhib.}$ (%)	$\Delta\lambda_{inhib.}$ (%)
Ref						
L	0.66	–	1.04	7.77	–	–
B	0.46	0.56	1.50	6.77	–	–
AR						
L	0.59	–	1.17	7.22	10.9	–
B	0.34	0.74	2.02	5.52	25.7	23.9
AC						
L	0.50	0.25	1.39	6.75	25.0	–
B	0.32	1.65	2.16	4.95	30.3	65.8
SYN						
L	0.24	1.95	2.84	4.77	63.3	–
B	0.14	3.20	5.07	3.30	70.3	82.3

ref reference product, *AR* terpeniods, *AC* fatty acids, *SYN* terpenoids and fatty acids

Assays carried out in the meat model showed clear differences in the antibacterial efficacy of the agents used, demonstrating a strong potential of synergism between terpenes and fatty acids. A blend of terpene compounds (AR) decreased the specific growth rate of *B. thermosphacta* from 0.46 (ref) to 0.34, giving a growth reduction of 26%, whereas a blend of fatty acids (AC) 0.32 [d-1] reduced the growth rate by 30%. Additionally, it was observed that terpenic compounds particularly influenced the specific growth rate of *B. thermosphacta*, whereas AC only extended the lag phase. Strong antibacterial activity of fatty acids was also observed by Mbandi et al. [24]. Researchers report that among ten studied fatty acids, decanoid acid was the third strongest in bacterial growth suppression. Synergism between terpenes and fatty acids (SYN) restrained bacterial growth, keeping the count at the inoculation level during the 4-week incubation period. In the case of *Lactobacillus curvatus*, separate application of terpenes in the meat model (AR) resulted in a minor growth inhibition, decreasing the maximum growth rate by 10.9%, whereas the blend of fatty acids (AC) had a stronger effect, reducing the value of μ_{max} by 25.0%. Synergism between terpenes and fatty acids showed a significant growth reduction. The maximum bacterial count after a 4-week incubation period was lower by 4 log units in comparison to the reference compound. The specific growth rate was decreased from 0.66 (ref) to 0.24 (SYN) [d-1], resulting in a 63.3% reduction of μ_{max}. Moreover, during the growth of *L. curvatus* almost no lag phase was observed in the reference compound and AR, whereas samples containing fatty acids (AC and SYN) showed a growth delay and a clear λ [d]. This demonstrates a significant contribution of

fatty acids to bacterial inhibition. Studies prove that the combination of multiple compounds can result in a stronger antibacterial effect than a single compound application. Antibacterial activity is enhanced because of synergetic interaction even at low concentrations of the ingredients (SYN). This is in accordance with the observations of Lambert [6] and Lin [12]. Marounek [28] studied the antibacterial activity of fatty acids (C2–C18) and a found limited dose-dependent relation. In some cases products applied above a certain limit can turn from growth inhibitors into growth enhancers. Very strong inhibiting effects were observed when fatty acids were combined with terpene compounds (SYN), thus suggesting their accumulation in bacterial cells. This could lead to an enhanced diffusion and thus a stronger effect. It is reported that lipophylic compounds such as cymene or carvacrol can accumulate in the bacterial membrane, leading to its deformation and the development of trans-membrane channels mainly in the space between parallel-situated phospholipid chains through which other molecules can passively flow into the cell core and cause an efflux of ions from the cell, 'leaking cells' [6, 29, 30]. Such changes also impair 'van der Waals' forces between components building the membrane, disturbing its integrity. The aggregation of lipophylic compounds in the membrane changes its fluidity, allowing bacteria to resist the channel formation [10], but it also enables their survival in unfavourable temperature conditions. This phenomenon is commonly observed in bacteria incubated at different temperatures [31]. In this case other modifications have also been reported: a change in the length of the fatty acid chain, in the ratio of saturated and non-saturated carbon bonds, or in the content of the fatty acid's building membrane from C18:0 into C14:0 and iso-C13:0 [32, 33].

Conclusions

This study demontrated that the growth of selected spoilage bacteria was best restricted by the combination of natural compounds. The sole application of fatty acids (AC) showed stronger growth-limiting activity than terpenoid compounds (AR) with both tested bacterial strains. *Lactobacillus curvatus* was observed to be very resistant to the single application of either terpenoids (AR) or fatty acids (AC). Growth of *Brochothrix thermosphacta* was significantly inhibited when antimicrobial compounds were separately applied. Strong antibicrobial activity was observed despite a radical decrease in the concentration of all antimicrobials (SYN), resulting in a significant growth inhibition of resistant *Lactobacillus curvatus* and an almost bactericidal effect on *Brochothrix thermosphacta*.

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