

Preliminary study on stimulatory and inhibitory effects of aldehydes from the green leaf volatiles family on beneficial and pathogenic bacteria from the intestine¹⁾

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Summary

The balance between beneficial and pathogenic bacteria in the intestine has a great importance in terms of gut physiology and immunity. The aim of the study was to investigate the stimulatory and inhibitory effects of aldehydes from the green leaf volatiles family (*trans*-2-hexenal, *cis*-3-hexenal, *trans*-2-nonenal, and *trans*-2-decenal) on beneficial (*Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *infantis*, *Lactobacillus acidophilus*, and *Lactobacillus casei*) and pathogenic bacteria (*Fusobacterium nucleatum* subsp. *nucleatum*, *Clostridium perfringens*, *Staphylococcus aureus* subsp. *aureus*, *Escherichia coli*, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium) from the intestine. The growth of *B. bifidum* was stimulated by *trans*-2-hexenal and *trans*-2-decenal at 3.9-250 µg/mL, by *cis*-3-hexenal at 15.6 and 31.3 µg/mL, and by *trans*-2-nonenal at a dose of 3.9-500 µg/mL ($p < 0.05$). *Trans*-2-decenal also moderately stimulated *L. acidophilus* at concentrations of 31.3 and 62.5 µg/mL ($p < 0.05$). *Trans*-2-hexenal, *cis*-3-hexenal, and *trans*-2-nonenal did not inhibit beneficial intestinal bacteria, with the exception of *B. infantis*. *Trans*-2-decenal was the most effective aldehyde on pathogens, with growth-inhibitory effect on *C. perfringens*, *F. nucleatum*, and *S. aureus* at the concentration of 500 µg/mL. *Trans*-2-decenal also protected beneficial bacteria at the dose at which it inhibited pathogenic ones. All the used aldehydes at a concentration of 500 µg/mL inhibited the growth of *F. nucleatum* as one of the agents of colorectal cancer. Among the pathogens, *E. coli* and *S. Typhimurium* were resistant to all aldehydes while *S. aureus* was inhibited only by *trans*-2-decenal. In conclusion, the use of aldehydes from the green leaf volatiles family might have beneficial effects on gut health by regulating beneficial bacteria and inhibiting pathogenic bacteria.

Keywords: antibacterial, green leaf volatiles, intestinal bacteria, MIC

Intestinal microflora, which live in a symbiotic relationship with the host, have important metabolic, trophic, and protective functions. Commensal or beneficial bacteria in the gastrointestinal tract of mammals form a natural defensive barrier to the invasion of the intestinal surfaces by exogenous pathogenic microbes. They compete with pathogens for available nutrients and adhesion sites (9). Commensal microflora also promote the modulation and proper maturation of the immune system (25). In particular, lactic acid-

producing Gram-positive bacteria in the intestine, such as *Bifidobacterium* spp. and *Lactobacillus* spp., have beneficial effects in the host; as a matter of fact, many probiotics were originally isolated from the gastrointestinal tract (33).

Disruptions of normal intestinal microbiota in humans and animals is a cause of enteric infections by gut pathogens (35). Ulcerative colitis, irritable bowel syndrome, and colon cancer are other intestinal disorders that are closely related to an unbalanced gastrointestinal microbiota (16). Because conventional antibiotics indiscriminately kill both pathogenic and beneficial

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microbes (3) they have a considerable impact on the microbiota. Furthermore, the extensive use of antibiotics causes the development of bacterial resistance to antibiotics, which leads to the increasing concern about the undesirable effects of antibiotics on public health (8). In recent years, intense interest has arisen in plant extracts and secondary plant metabolites as safer alternatives for antibiotics (14, 29). For example, certain essential oils have been reported to show greater toxicity to gut pathogens than to commensals (35). Similarly, extracts of green tea (*Camellia sinensis*) and *Eleutherine americana* did not show activity against all commensal microbiota, but inhibited Gram-positive pathogenic bacteria in the intestine (31). On the other hand, plant extracts are composite mixtures of various metabolites, and identification of active compounds responsible for antimicrobial activity poses difficulties for antimicrobial assay studies (6). Therefore, it is recommended to evaluate the antimicrobial activity of single pure plant compounds instead of plant extracts (12).

Green leaf volatiles are composed of C6 compounds, including aldehydes, alcohols, and esters derived from unsaturated fatty acids, linolenic acids, and linoleic acids, via the lipoxygenase pathway (26). The amount of green leaf volatiles is low in healthy plant tissues. They are produced when leaves are injured, infected, or attacked by herbivores and insects (34). Their name originates from the distinctive scent that is emitted when leaves are crushed. Hexenal, *trans*-2-hexenal, *trans*-3-hexenal, and *cis*-3-hexenal are the most common C6 aldehydes from the green leaf volatiles family (26). *Trans*-2-nonenal (C9) and *trans*-2-decenal (C10) are longer-chain aldehydes that are produced from hemiacetals by plants and have similar properties to all other C6 aldehydes (17, 24). The aldehydes from the green leaf volatiles family, such as nonanal, decanal, dodecanal, octanal, and undecanal, have been granted a “generally recognized as safe” status (15). *Trans*-2-hexenal also tested negatively for mutagenicity (1).

Green leaf volatiles show a broad-spectrum antimicrobial activity against several fungal and bacterial microorganisms (20, 27). However, bacteriostatic activity of aldehyde compounds was greater than that of the alcohol ones (27). In a previous study, we observed that *cis*-3-hexenal increased the abundance of some Gram-positive anaerobic fermenters, such as *Butyrivibrio fibrisolvens* and *Streptococcus bovis*, in a simulated rumen (13). Although there are a few reports about the effects of some green-leaf-derived compounds on some agents of gastrointestinal tract infections (4, 21, 27), the effects of green leaf volatiles on beneficial gut bacteria have not been evaluated previously.

Therefore, the aim of the present study was to investigate the stimulatory and inhibitory effects of aldehydes from the green leaf volatiles family on both beneficial and pathogenic bacteria from the intestine.

Material and methods

Commercial aldehydes. Commercial aldehydes from green leaf volatiles family, *cis*-3-hexenal (W256102), *trans*-2-nonenal (W321303), and *trans*-2-decenal (W236608) were obtained from Sigma-Aldrich. *Trans*-2-hexenal (A11513) was purchased from Alfa Aesar.

Intestinal bacterial strains and culture conditions. Beneficial bacterial species used in antimicrobial tests were *Bifidobacterium bifidum* ATCC 29521, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus casei* ATCC 393. Pathogenic bacterial species tested were *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586, *Clostridium perfringens* ATCC 13124, *Staphylococcus aureus* subsp. *aureus* ATCC 12600, *Escherichia coli* ATCC 11775, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 29630.

The media used to culture different intestinal strains were as follows: *B. bifidum*, Mann Rogosa Sharpe (MRS) broth with 0.05% cysteine (MRS-C); *L. acidophilus*, MRS broth; *E. coli* and *S. Typhimurium*, Luria-Bertani (LB) medium; *S. aureus*, tryptic soy broth (TSB); and *B. infantis*, *L. casei*, *C. perfringens*, and *F. nucleatum*, liquid form of medium 2 (18). Medium 2 was prepared under CO₂, as previously described (18) with only slight modification. Trypticase peptone was used instead of caseitone in medium 2 (Tab. 1). *E. coli*, *S. Typhimurium*, and *S. aureus* were grown aerobically at 37°C for 24 h. All others were grown at 37°C for 24-72 h under an atmosphere of 80% N₂, 10% CO₂, and 10% H₂ in an anaerobic chamber (Whitley DG250, Don Whitley, West Yorkshire, UK).

Tab. 1. Composition of medium 2 (for 100 mL)

Component	
Trypticase peptone (BD 211921 Bacto™)	1.0 g
Yeast extract (Sigma Y1625)	0.25 g
Mineral solution 1	15 mL
Mineral solution 2	15 mL
Clarified rumen fluid	20 mL
Resazurin (Sigma R7017)	0.0001 g
Sodium lactate (70% w/v)	1.0 g
Glucose	0.2 g
Maltose	0.2 g
Cellobiose (Sigma 22150)	0.2 g
Cysteine HCl (Sigma C7880)	0.05 g
NaHCO ₃ (Sigma S5761)	0.4 g
Deionized water	to 100 mL

Explanations: mineral solution 1 – 3 g/L K₂HPO₄ (Sigma P3786); mineral solution 2 – 3 g/L KH₂PO₄ (Sigma P9791), 6 g/L (NH₄)₂SO₄ (Sigma A4915), 6 g/L NaCl (Sigma S7653), 0.6 g/L MgSO₄·7H₂O (Sigma 230391), and 0.6 g/L CaCl₂ (Sigma C1016); clarified rumen fluid – ruminal fluid brought from the slaughterhouse was mixed and filtered through three layers of cheesecloth to partition into liquid and solid (digesta) fractions. The liquid fraction was centrifuged at 15000 rpm, and the clear supernatant was used as a component of the anaerobic media.

Determination of minimal inhibitor concentrations (MIC). The broth dilution method was conducted in the anaerobic chamber. Tests for *E. coli*, *S. Typhimurium*, and *S. aureus* were carried out in a laminar flow cabinet. Aldehydes were dissolved in 50% ethanol. MICs of green leaf aldehydes were evaluated at doses ranging from 0.015 to 1000 $\mu\text{g/mL}$ in the literature (4, 21, 27). In the present study, green leaf volatiles were tested at 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, and 3.9 $\mu\text{g/mL}$ concentrations. Dilutions of aldehydes were made in the bacterial strain specific growth media. Two hundred microliters of each dilution were distributed over a 96-well plate (Corning 3599, Flat bottom). Next, 20 μL of overnight bacterial cultures at a density of 4×10^{10} cell/mL were inoculated into each well (10, 13). Three wells were used for each concentration. At the same time, negative control wells without aldehydes and media control wells without bacteria were maintained for each set. Plates were incubated at 37°C for 24 h in the anaerobic chamber and in an incubator for *E. coli*, *S. Typhimurium*, and *S. aureus*. Bacterial growth was determined at 600 nm using a microplate reader (Epoch, BioTek,

USA). The MIC was recorded as the lowest aldehyde concentration at an OD600 value of ≤ 0.1 .

Statistical analyses. Statistical analysis was carried out by the use of one-way ANOVA followed by Dunnett's test. Each well of a 96-well plate was an experimental unit. A value of $p < 0.05$ was taken to indicate a significant difference.

Tab. 2. Minimum inhibitory concentration (MIC) values of aldehydes from the green leaf volatiles family on beneficial and pathogenic bacteria from the intestine

Bacteria	MIC values ($\mu\text{g/mL}$)			
	<i>trans</i> -2-hexenal	<i>cis</i> -3-hexenal	<i>trans</i> -2-nonenal	<i>trans</i> -2-decenal
Beneficials				
<i>B. bifidum</i>	-	-	-	1000
<i>B. infantis</i>	1000	1000	1000	1000
<i>L. acidophilus</i>	-	-	-	1000
<i>L. casei</i>	-	-	-	1000
Pathogens				
<i>F. nucleatum</i>	500	500	500	500
<i>C. perfringens</i>	-	1000	1000	500
<i>S. aureus</i>	-	-	-	500
<i>E. coli</i>	-	-	-	-
<i>S. Typhimurium</i>	-	-	-	-

Explanations: (-) MIC values are higher than 1000 $\mu\text{g/mL}$

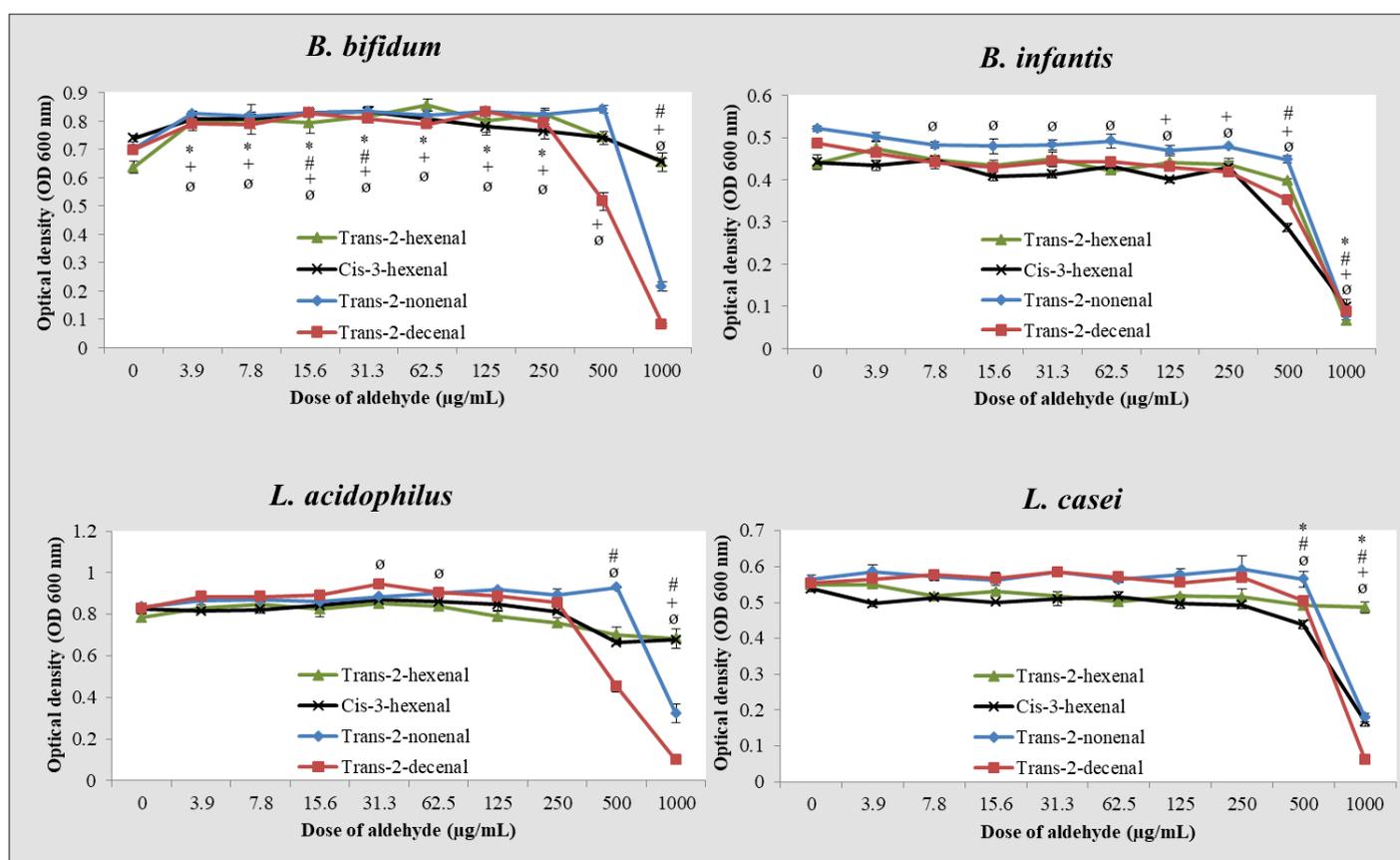


Fig. 1. Effects of aldehydes from the green leaf volatiles family on beneficial intestinal bacteria. The results represent the mean \pm standard error. * $p < 0.05$, difference of the *trans*-2-hexenal-treated culture compared with the control; # $p < 0.05$, difference of the *cis*-3-hexenal-treated culture compared with the control; + $p < 0.05$, difference of the *trans*-2-nonenal-treated culture compared with the control; and o $p < 0.05$, difference of the *trans*-2-decenal-treated culture compared with the control. Control level was 0 $\mu\text{g/mL}$ for each aldehyde.

Results and discussion

Effects of aldehydes from the green leaf volatiles family on intestinal bacteria are presented in Figure 1 and Figure 2, and the MIC values are summarized in Table 2. The growth of *B. bifidum* was stimulated by *trans*-2-hexenal and *trans*-2-decenal at 3.9-250 µg/mL, by *cis*-3-hexenal at 15.6 and 31.3 µg/mL, and by *trans*-2-nonenal at 3.9-500 µg/mL dosage ($p < 0.05$). *Trans*-2-decenal also moderately stimulated *L. acidophilus* at concentrations of 31.3 and 62.5 µg/mL ($p < 0.05$). Green leaf volatiles failed to promote the growth of other beneficial gut bacteria. Nonetheless, *trans*-2-hexenal, *cis*-3-hexenal, and *trans*-2-nonenal did not show growth-inhibitory activity toward beneficial bacteria,

except *B. infantis*, which was inhibited by the use of 1000 µg/mL concentrations of these aldehydes. *Trans*-2-decenal, on the other hand, exhibited antibacterial activity on beneficial bacteria when used at 1000 µg/mL. *Trans*-2-decenal was also the most effective aldehyde on pathogens, with a growth-inhibitory effect on *C. perfringens*, *F. nucleatum*, and *S. aureus* at the concentration of 500 µg/mL. Both *cis*-3-hexenal and *trans*-2-nonenal inhibited *C. perfringens* and *F. nucleatum* at doses of 1000 and 500 µg/mL, respectively. *Trans*-2-hexenal was less toxic to all pathogenic bacteria; it inhibited only *F. nucleatum* at 500 µg/mL. *E. coli* and *S. Typhimurium* from pathogens were resistant to all aldehydes while *S. aureus* was inhibited only by *trans*-2-decenal.

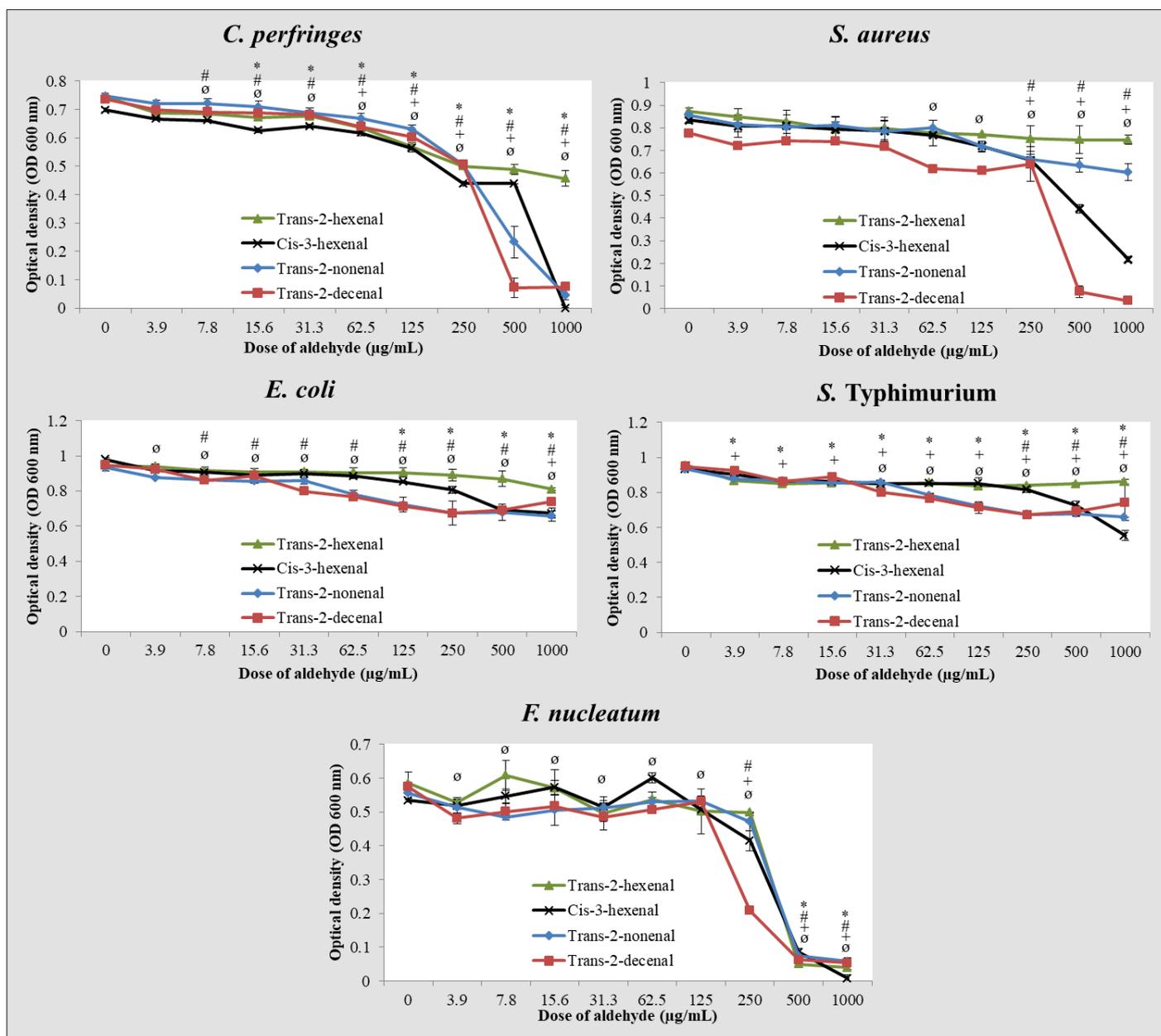


Fig. 2. Effects of aldehydes from the green leaf volatiles family on pathogenic intestinal bacteria. The results represent the mean ± standard error. * $p < 0.05$, difference of the *trans*-2-hexenal-treated culture compared with the control; # $p < 0.05$, difference of the *cis*-3-hexenal-treated culture compared with the control; + $p < 0.05$, difference of the *trans*-2-nonenal-treated culture compared with the control; and o $p < 0.05$, difference of the *trans*-2-decenal-treated culture compared with the control. Control level was 0 µg/mL for each aldehyde.

Beneficial microbes in the gut have collective physiological functions almost equal to an organ within the host (28). The major physiologic function of these symbiotic microbes is the fermentation of nondigestible carbohydrates and generation of short-chain fatty acids, mainly acetate, propionate, and butyrate, which are the primary energy sources of the intestinal epithelium (9). Differently from the others, butyrate inhibits the proliferation and stimulates the differentiation of epithelial cells; it can therefore suppress the viability and growth of colorectal cancer cell lines (7). According to a review of 31 original articles on the role of colon microbiota in colorectal cancer observed in humans and animals, beneficial bacteria such as *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, and *Faecalibacterium* spp. were consistently diminished while some bacteria, including Fusobacteria and Staphylococcaceae, were consistently augmented (5). In particular, *Fusobacterium nucleatum* is linked with colorectal cancer because it induces DNA damage, stimulates inflammation, and can shield tumors from immune attack (7). In the present study, all aldehydes from the green leaf volatiles family inhibited the growth of *F. nucleatum* at a concentration of 500 µg/mL while they protected the beneficial microbes at the same dose. There is no literature on the effects of green leaf volatiles on this bacterium. However, extracts of a series of medical plants did not inhibit *F. nucleatum* isolated from periodontal pockets of periodontic patients up to 16384 mg/L, while the MIC value of spiramycin, a commercially used antibiotic, on *F. nucleatum* was 64 mg/L in the same study (19).

C. perfringens and *S. aureus* are Gram-positive food-borne pathogens that can cause enterotoxaemia and severe gastro-intestinal illness (32). *Trans*-2-decenal, at a concentration of 500 µg/mL, showed antimicrobial activity on both of these bacteria in this study. Bisignano et al. (4) also reported that *trans*-2-decenal was effective on *S. aureus* (ATCC 6538) at a concentration of 250 µg/mL and methicillin-resistant *S. aureus* at concentrations of 125-500 µg/mL, which were similar to our findings. *Trans*-2-decenal was the unique aldehyde that had an antimicrobial effect on *S. aureus* in the present study. Additionally, the MIC value of *trans*-2-decenal was lower than *trans*-2-nonenal and *cis*-3-hexenal on *C. perfringens*. Kubo et al. (21) linked the degree of antimicrobial activity of α/β unsaturated alcohols and aldehydes with the hydrophobic alkyl (tail) chain length and the microorganism being tested. The researchers suggested that aldehydes with a long chain length exhibit more potent activity against Gram-positive bacteria and fungi. They submitted that electronegativity of the oxygen atom in the aldehydes may be influenced by differences in carbon tail length. Greater chain length could cause greater electronegativity, which can cause greater affinity for hydrogen bond formation with nucleophilic groups of the membrane, creating significant disruption in

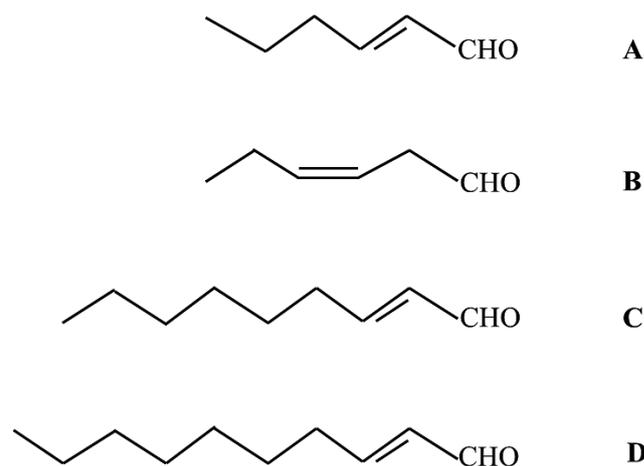


Fig. 3. Chemical structure of (A) *trans*-2-hexenal, (B) *cis*-3-hexenal, (C) *trans*-2-nonenal, and (D) *trans*-2-decenal

the lipid bilayer of the microorganism (21). The chain length of *trans*-2-decenal was composed of C10 as shown in Figure 3. In the previous study of the same researchers (22), geranyl acetol with the same chain length (C10) also exhibited activity against *S. aureus* while linalool, of which the chain length composed of C6, did not show any activity.

The aldehydes from green leaf volatiles used in this study did not show antimicrobial activity against *E. coli* or *S. Typhimurium*. *Trans*-2-decenal was ineffective on *E. coli* up to 800 µg/mL (21), which was similar to the present study. Cho et al. (11) also reported that a higher concentration of *trans*-2-nonenal, at 1.000 ppm, was required to kill *E. coli* O157:H7 and *Salmonella* Typhimurium. On the other hand, *trans*-2-hexenal and *cis*-3-hexenal, at doses of 6.25-100 µg/mL, inhibit *E. coli* O-157:H7, according to a report of Nakamura and Hatanaka (27), which is contrary to our results. The strain of *E. coli* in the present study was O1:K1:H7. The reason for these variable results might be related to the different types of strains of bacteria.

Green leaf volatile aldehydes moderately promote the growth of some beneficial gut bacteria like *B. bifidum* and *L. acidophilus* at lower concentrations. In a previous study, low concentrations of *trans*-2-hexenal and *trans*-2-decenal also stimulated the growth of pure cultures of *Fibrobacter succinogenes*, which contribute to pre-gastric cellulose fermentation in ruminants (13). Saponins, one of the phytochemicals, promoted *in vitro* bacterial growth and feed utilization in the rumen at low doses while they exhibited inhibition at high doses (30). The researchers indicated that gut bacteria can hydrolyze some plant metabolites, such as flavonoids and phenolic acids, to protect themselves against the toxic effects of these compounds (2, 23). Bacterial degradation products are usually more bioactive than precursors, hence, they can stimulate the enzymatic activity of certain groups of bacteria in the gut (2, 23).

In conclusion, in the present study, green leaf volatile aldehydes moderately promoted the growth of some beneficial gut bacteria at lower concentrations. *Trans*-

2-hexenal, *cis*-3-hexenal, and *trans*-2-nonenal did not inhibit beneficial intestinal bacteria, with the exception of *B. infantis*; nevertheless, their antimicrobial activity on pathogens was weaker than that of *trans*-2-decenal. *Trans*-2-decenal also protected beneficial bacteria at the dose at which it inhibited pathogenic ones. All the used aldehydes have a potential to diminish *F. nucleatum* as one of the agents of colorectal cancer. Further studies are required on the effects of green leaf volatile aldehydes in mixed cultures of intestinal microbiota and *in vivo* to evaluate the interplay between different bacterial species and to determine the value of green leaf volatiles as food supplements and enteric infection inhibitors.

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