Volatile Compounds of Therapeutic Importance Produced by Leuconostoc paramesenteroides, a Native Laboratory Isolate

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Abstract: Probiotics are a community of nutraceuticals. Their potential in functional foods serves to promote health or to prevent diseases. Ramping up of flavor profile is an ongoing trend in modern dairy, so designer products are at the forefront towards desired health, wellness and sensorial enjoyment of everyday life. In the present work, volatile compounds of a potentially beneficial culture, *Leuconostoc paramesenteroides*, isolated from cheddar cheese were studied for 21 days. Culture grown in MRS broth was taken out periodically and was extracted in dichloromethane in order to study the formation of volatile compounds over time. The results showed the presence of a large number of short chain fatty acids, namely pentadecanoic acid (12.21 mg %), known to have antiarthritic property; nonadecanoic acid (13.99 mg %), which has anti-inflammatory action; benzoic acid, used as antiseptic (1.26 mg %), which showed a maximum yield after 16 h of incubation at 37 °C in MRS broth; nonacosane, having antibacterial activity (50.62 mg %); hexacosane, known for hair pigmentation (34.4 mg %); and nonacosanol, known to reduce cholesterol (8.62 mg %), which was found to be maximum after 7 days of incubation. All these compounds are known to be therapeutically important.

Key Words: Flavor profile, therapeutic application, GC-MS spectra, Leuconostoc paramesenteroides

Introduction

With the rise in consumer awareness of individual health, nutrition and well-being, the interest and demand for value-added foods and beverages have expanded (1). Probiotics serves as one such means, where it is known that ingestion of probiotic microorganisms exerts health benefits beyond inherent basic nutrition (2). Probiotics are defined as "live microorganism which when administered in adequate amounts confers a health effect on the host" (3). Lactic acid bacteria (LAB) are indigenous probiotic microflora of the mammalian gastrointestinal tract and can play an important role in host microecology. These have been credited with an impressive list of therapeutic and prophylactic benefits (4).

With the current focus on disease prevention for optimum health at all ages, the probiotic market potential is enormous. Health professionals are in an ideal position to help and guide their clients towards appropriate prophylactic and therapeutic uses of probiotics.

A large amount of credible scientific evidence in human studies has shown that regular consumption of probiotic products helps in maintaining good health (5). Nutritional and therapeutic aspects of fermented dairy products are well known (6). These cultures are known to inhibit pathogenic microorganisms (7), have antimutagenic and anticarcinogenic activity (8), increase immune response (9,10), and reduce cholesterol level in serum (11). It is known that LAB added as starter cultures are able to transform lactic acid, citrate, lactate, protein and fats into volatile compounds that together with amino acids and other products play a critical role in the development of flavors (12,13). Short chain fatty acids (SCFA) from Bifidobacterium spp. are known to exert a wide range of antimicrobial activity against yeast and molds as well as bacteria (14). SCFAs are absorbed and enter the portal vein and appear to make a significant contribution to energy through hepatic metabolism. They lower colonic pH and protect against colonic carcinogenesis and overgrowth of pathogens (15). SCFAs absorbed in the colon are known as possible therapeutic agents for the cure of colitis, radiation proctitis, pouchitis and antibiotic-associated diarrhea (16). Earlier, Alonso and Fraga (17) analyzed the volatile flavor compounds in yogurt by headspace gas chromatography-mass spectrometry (GC-MS), wherein important compounds were acetone and acetic acid. Fermented products formed from starter mixture of Lactococcus lactics ssp lactics and Lactococcus lactic ssp cremoris are known to produce volatile compounds like acetaldehyde, acetone, 2-butanone, diacetyl and ethanol (18).

Agrawal et al. (19) studied the flavor profile of idli batter prepared from Pediococcus pentosaceus and Candida versitalis as starter culture, wherein desirable flavor compounds such as ketones, diols and acids were found up to 8 days of storage. According to the literature, therapeutically important compounds are produced by LAB (20,21). With this in mind, this work was undertaken to find out and identify the volatile compounds of therapeutic importance produced by this culture. According to WHO, a dose of 10⁶ cfu/g is known to give the desired beneficial effect (22). Hence, this study was carried out to investigate the formation of functional volatile compounds produced by Leuconostoc paramesenteroides, a laboratory culture isolate, at different stages of growth. This LAB culture was isolated from cheddar cheese and characterized by standard methods.

Materials and Methods

Microorganism. Leuconostoc paramesenteroides was initially isolated from cheddar cheese locally in the laboratory at Central Food Technological Research Institute (CFTRI), Mysore, India. The culture was identified and characterized according to Bergey's Manual (23) and by 16SrRNA analysis. Primers were designed to anneal to highly conserved regions of the 16S rRNA gene and to amplify a 16S rRNA gene. The primers used for PCR amplification were 5'AGAGTTTGATCCTGGCTCAG 3' and 5'GTCTCAGTCCCAATGTGGCC 3' (Genei, Bangalore). The thermal cycler was programmed as follows: 10 min at 94°C; 25 cycles of 1 min at 94°C, 2 min at 61°C, and 5 min at 72°C. The amplified product was analyzed by electrophoresis in 1% (w/v) agarose gels after ethidium bromide (0.5 g/ml) staining. The amplified PCR product was sequenced, and on multiple sequence alignment on BLAST search the culture was identified as Leuconostoc paramesenteroides.

The culture was studied for its probiotic properties like tolerance to low pH, resistance to high bile salt concentration, viability in alkaline conditions, and non-hemolytic characters by standard methods. The culture was grown and maintained in MRS (deMan Rogosa Sharpe) broth media and maintained by subculturing into fresh media every 15 days.

Bacterial growth. The growth pattern of the culture was studied in MRS broth (pH 6.5) containing peptone (10g/l), beef extract (10g/l), yeast extract (5g/l), Tween 80 (0.5g/l), triammonium citrate (2g/l), sodium acetate (5g/l), magnesium sulphate (0.1g/l), manganese sulphate (0.05g/l) and di potassium hydrogen phosphate (2g/l). The culture was inoculated into MRS broth (1% v/v) and incubated at 37 °C. At each time interval an aliquot of sample was taken, serially diluted and after appropriate dilution plated on MRSA plates. Plates were then incubated at 37 °C for 24 hours. The colonies grown were counted and expressed as colony forming units per ml (CFU/ml).

Analysis of volatile compounds. Formation of volatile compounds was analyzed at different stages of growth. A cell pellet was obtained after centrifugation (10,000 rpm; 30 min) and the volatile compounds were extracted thrice in dichloromethane (2g/10ml) till complete extraction. This filtrate was dried over anhydrous sodium sulfate. The sample was concentrated and analyzed by GLC (gas liquid chromatogram) and GC-MS (19). The fragmentation pattern was then compared with the GC-MS library data base.

Results and Discussion

Bacterial growth. An exponential growth was observed from 3 h till 48 h (2 d) followed by a stationary phase till 96 h (4 d) and then initiation of decline by 144 h (6 d) as shown in Figure 1.

Analysis of volatile compounds. The major compound produced was pentadecanoic acid, which is used in the cure of arthritis and also used as an antiinflammatory agent. The yield (0.11% at 8 h) reached maximum (12.2%) in 16 h of incubation, which later gradually decreased to 1.47% at 21 days. Nonadecanoic acid, which is used for anti-inflammation, was produced (0.11%) in the early exponential phase (8 h), which increased (13.99%) till 16 h and then gradually declined to 0.56% on the 21st day. Benzoic acid, which is used as an anti-rheumatic and antiseptic agent, was produced at 16 h (1.26%) but declined (0.33%) on the 21st day. Nonacosane, which has antibacterial property, was formed after 8 h (0.99%) and reached a maximum on the 7th day (29.30%); the yield declined (1.65%) after 21 days. Hexacosane, which is known to promote growth

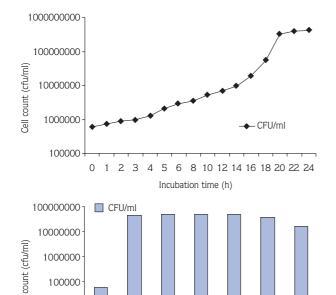


Figure 1. Growth of culture in MRS broth at 37 °C.

Incubation time (days)

5

6

Cell

10000

0

and black pigmentation in hair, was formed after 8 h (0.93%) incubation, reached a maximum yield after 7 days (17.17%) and then declined (1.65%) after 21 days. Nonacosanol, which is a constituent of policosanol known to reduce low density lipoprotein (LDL) cholesterol,

showed a maximum yield after 7 days' incubation (4.31%). Hentriacontane, which is known to reduce LDL and high density lipoprotein (HDL) cholesterol, reached maximum (from 1.04% to 29.30%) in 7 days and then gradually decreased (1.65%) after 21 days. Xylazine, used as a sedative and analgesic agent, showed a yield of 1.15% after 16 h of incubation, and propoxyphene, used as a narcotic analgesic drug, was formed after 16 h (1.49%) of incubation period. The fragmentation patterns of all compounds formed and the yields over time are given in Table 1 and Figure 2.

The compounds formed during growth in Leuconostoc paramesenteroides have been worked out earlier by various investigators using microorganisms. Pentadecanoic acid, which formed maximum (12.21%) at 16 h of growth in MRS medium in the present study, has also been reported by Nel et al. (24), where it was formed at the end of alcoholic fermentation by Saccharomyces bayanus Saccharomyces cerevisiae. In our study, the yield was much higher and was formed in less time, which shows the advantages of the culture. Hirschler et al. (25) showed the formation of pentadecanoic acid by carboxylation at C_3 of ketone and the removal of two terminal carbon atoms at C₁ and C₂ position of hexadecane.

Table 1. Yield of volatile compounds at different time intervals.

COMPOUND	MOL WT	YIELD (mg %)						
		8 h	16 h	7 d	14 d	21 d	FRAGMENTATION PATTERN	USES
Pentadecanoic acid	242	1.1	12.21	-	1.99	1.47	43,60,73,41,55	Used as marker for intake of milk Constituent of manaca plant used for arthritis and rheumatism and has anti-inflammatory action
Nonadecanoic acid	298	1.1	13.99	2.7	0.75	0.56	43,73,57,29,69	Anti-inflammatory action
Benzoic acid	278	-	1.26	-	-	0.33	105,122,77,51,50	Anti-rheumatic and antiseptic property
Nonacosane	408	9.9	24.77	50.62	2.17	1.65	57,43,71,85,99	Anti-bacterial activity
Hexacosane	366	9.3	15.83	34.4	2.17	1.65	57,71,43,85,99	Promotes growth and blackness in hair
Nonacosanol	424	3.4	1.21	8.62	0.71	-	43,57,97,83,69	Constituent of policosanol- reduces LDL and increases HDL cholesterol
Xylazine	220	-	1.15	-	-	-	205,220,177,145,130	Used as sedative, analgesic, muscle relaxant in veterinary medicine
Propoxyphene	325	-	1.49	-	-	-	58,83,91,117,208,265	Narcotic analgesics drug

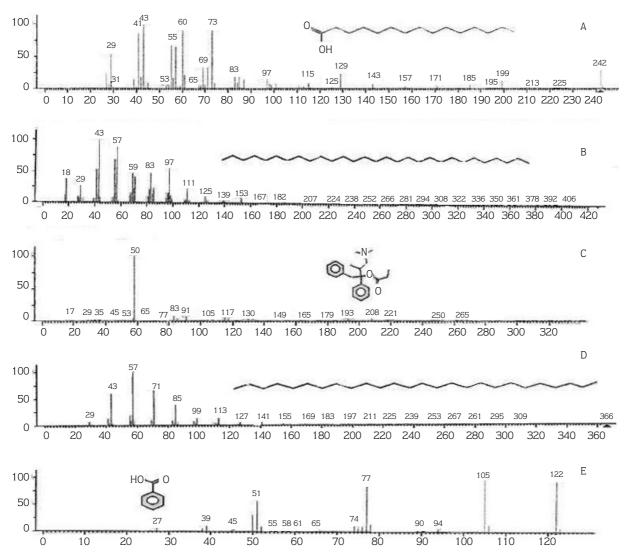


Figure 2. Standard GC-MS fragmentation pattern of A) pentadecanoic acid B) nonacosanol C) propoxyphene D) nonacosane E) benzoic acid.

Quiding et al. (26) studied the analgesic effect of codeine, dextra propoxyphene and paracetamol, and found a pronounced pain reduction and the highest proportion of pain-free patients with dextra propoxyphene preparation. In the present study, propoxyphene was formed (1.49%) after 16 h of incubation.

Another important compound is xylazine, used as a sedative, analgesic, and muscle relaxant in veterinary medicine. When xylazine is used as a sedative agent, it is shown that there is no adverse effect on echocardiographically derived load-dependent index. In the present work, 1.15% of xylazine was formed after 16 h of incubation.

Nonacosanol, which is a constituent of policosanol, was formed on the 7th day of incubation with a maximum yield of 8.62%. Policosanol is known to be effective in lowering LDL cholesterol (27,28). The authors have shown that policosanol decreased LDL cholesterol by 11-31% and raised HDL by 7-9%. This has not been reported in any other microorganism. Fernandez et al. (29) and Ortensi et al. (30) compared the cholesterol lowering effect of policosanol with pharmaceutical drugs and showed that in both cases results were similar. An average dose of 10-20 mg/day of policosanol is recommended for the treatment of hypercholesterolemia.

Benzoic acid, which is a natural antibiotic, is known to be produced earlier by *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (31), but the yields have not been mentioned. In the present work, *Leuconostoc paramesenteroides*, the native starter culture showed a yield of 1.26% after 16 h of growth in MRS broth. The present culture has its importance as it can also be used for food preservation. According to Masja et al. (32), benzoic acid is formed by aminotransferase reaction of keto acids.

In the present work, three types (straight chain, branched and aromatic or cyclic) of fatty acids are formed. According to White et al. (33), biosynthesis of saturated fatty acids requires acetic acid in the form of co-enzyme A ester and malonyl Co A for chain elongation. This reaction is catalyzed by a multifunctional enzyme complex of type II. At first, acetyl Co A and malonyl Co A attach to carrier protein (ACP) that extends to form 3-oxobutanoate acyl by a reaction catalyzed by β -ketoacyl-ACP synthetase. Later, it dehydrates and is reduced to butanoic acid.

For a branched chain fatty acid synthesis, bacteria require two types of primer sources. The first type includes branched chain $\alpha\text{-keto}$ acids that are known to be used by nearly all bacteria. The second type includes branched short chain carboxylic acids which are exogenously supplied and are used by only a small proportion of bacteria, mainly those incapable of utilizing branched chain $\alpha\text{-keto}$ acids as primer sources (34).

Fatty acid synthesis is found in different microorganisms when supplemented with different substrates. Chi Ming So et al. (35) observed the anaerobic transformation of alkanes to fatty acids. They proposed that a sulphate-reducing bacterium anaerobically transformed an alkane to fatty acids through a mechanism, which includes subterminal carboxylation at $\rm C_3$ —position of alkane and elimination of the two adjacent terminal carbon atoms that the culture incorporates as cellular lipids.

Thierry and Maillard (36) showed that when amino acids are used as a substrate it is catabolized by several

reactions like deamination, decarboxylation, oxidative deamination and transamination, leading to keto acids that enter the pathway of fatty acid synthesis. Fatty acid synthesis is also the result of aminotransferase, amino acid oxidases and dehydrogenases resulting in phenyl pyruvic acid, a keto acid that is further subjected to chemical reaction leading to benzaldehyde.

Conclusions

In the present study, all these short chain fatty acids are produced by *Leuconostoc paramesenteroides* and are known to have therapeutic value in various ailments via their anti- inflammation, anti-rheumatic, antiseptic, anti-bacterial and anti-tumor activity. As these compounds were formed in a shorter time with higher yields, the present work is very important.

Today, the flavor industry has come a long way in terms of making nutraceutical products more acceptable to the consumers, wherein this potent probiotic culture may play a very important role. With the present status of increasing drug-resistant microorganisms and the side effects caused by these drugs, there is an immense need for the development of alternative natural food products with health-promoting properties that are free of side effects. Food formulations with addition of such culture or byproduct or metabolite of the culture may be of significant benefit with an immense health effect in humans and can prove to be good functional food.

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