Production of anti-microbial substances by probiotics

Charumati Mishra, MSc and John Lambert, MBBS, FRACP, PhD

Gastrointestinal Research Group, Dept of Medicine, Monash Medical Centre and Mornington Peninsula Hospital, Melbourne, Victoria, Australia

Bacterial antagonism has been recognised for over a century but in recent years this phenomenon has received more scientific attention, particularly in the use of various strains of lactic acid bacteria (LAB). Antimicrobial compounds produced by LAB have provided these organisms with a competitive advantage over other microorganisms. Lactic acid bacteria have a natural ecological niche in many foods as well as in the intestinal tract. The efficacy and spectrum of antimicrobial products of lactic acid bacteria are broad and include lactic and acetic acid, hydrogen peroxide, carbon dioxide, diacetyl as well as bacteriocins or bacteriocin-like substances. Further screening for agents with a broad spectrum of activity is required. This will involve genetic or protein engineering of such compounds to commercialise these agents.

Introduction

The gastrointestinal tract is a site for proliferation of microorganisms with the domination of different strains being influenced by various factors such as changes in tissue surface chemistry (by hormonal fluctuation), administration of antibiotics, age and diet. The impact of antibiotic treatment includes reduction in colonisation resistance, implantation of new and pathogenic micro-organisms and development of resistant strains in the normal microflora. The process whereby microflora resists invasion by pathogens has been referred to as colonisation resistance. Selective colonisation of the intestinal flora by using dietary supplements has been attempted since the work of Metchnikoff [1] who showed that cholera could be prevented by the presence of 'desirable' microflora such as those found in cultured milk products.

Interest in biocontrol that involves the incorporation of lactic acid bacteria (LAB or lactics) in food supplements (or 'probiotics'), is presently experiencing a resurgence in the food and pharmaceutical industries. A probiotic is defined as a cultured product or live microbial feed supplement which beneficially affects the host animal (by improving its intestinal balance)[2]. The most commonly used lactics in probiotic preparation, either singly or in a mixture of different organisms, include *Lactobacillus bulgaricus, Lactobacillus acidophilus, Streptococcus thermophilus* and *Bifidobacterium*. The use of other organisms for example *Enterobacter faecalis, Escherichia coli* and *Bacillus subtilis* is questionable because of public health concern.

Fermentation with LAB is one of the oldest known methods of food preservation. However, it was not until the 1970s that the mechanism(s) of their action was/ were investigated. The inhibition of many pathogenic bacteria in fermented foods is believed to be the result of anti-microbial substances produced by LAB resulting in natural preservation through bacterial antagonism. The principle of bacterial antagonism applies to the preparation of new probiotics as well.

Desirable characteristics of organisms used as probiotics

There are several desirable characteristics for organisms to be used as dietary adjuncts. Organisms should be a normal inhabitant of the human intestinal tract, non pathogenic, non toxic and be capable of surviving passage through the gastrointestinal tract. Within the gut it must produce the desired effects. Furthermore, it must maintain viability and activity in the carrier food before consumption. Finally, the organism should be sensitive to antibiotics used to treat infection and should not harbour plasmids resulting in antibiotic resistance. It is also important to know the number of organisms needed to colonise human subjects to estimate the effective therapeutic dose.

Antimicrobial compounds produced by LAB have provided these organisms with a competitive advantage over other microorganisms. Exploitation of antibiosis of LAB is the best choice for not only improving the microbial safety of the food products but as a probiotic preparation because of their natural adaptation to the gut environment. Lactics need to be acid tolerant bacteria and exhibit resistance to lysozyme present in the saliva and other enzymes, gastric juice and duodenal fluids. Many lactics are resistant to the bile salt present in the gut and survive the intestinal motility and adhere well to gastric mucosa.

Possible modes of action of probiotics are: suppression of viable count by production of antibacterial compounds; competition for nutrients and adhesion sites; alteration of microbial metabolites and stimulation of immunity. Lactics

Correspondence address: Mrs. Charumati Mishra, Dept of Medicine, Monash Medical Centre, 246 Clayton Road, Block E, Level 5, Clayton 3168, Victoria, Australia
Tel: +613 9550-5521/ 23 Fax: +613 9550-5524

are known to produce a wide range of antimicrobial substances which will be discussed in the following section.

Inhibitory compounds produced by lactic acid bacteria

Antimicrobial activity of LAB isolated from food has been the subject of intensive research due to the potential application of these bacteria as protective cultures in biological preservation[3]. The major groups of inhibitory compounds produced by LAB are:

1) lactic and other volatile acids with a resulting decrease in pH.
2) other primary metabolites such as hydrogen peroxide, carbon dioxide and diacetyl.
3) bacteriocins-- special antimicrobial compounds.

Each of these groups of compounds, especially a combination of them, can be used to extend the shelf life and safety of food products. Antibiosis of LAB has been extensively reviewed[4,5]. The mechanism of antibiosis produced by LAB are summarised in Table 1.

Table 1. Inhibitory compounds produced by lactic acid bacteria and their mechanism of action

Inhibitory compound	Mechanisms of action
Lactic and other volatile acids	Disruption of cellular metabolism[7]
Hydrogen peroxide	Inactivation of essential biomolecules by superoxide anion chain reaction[11], activation of lactoperoxidase system[12]
Carbon dioxide	Anaerobic environment and/or inhibition of enzyme decarboxylation and/or disruption of the cell membrane[14]
Diacetyl	Interference with arginine utilisation[16]
Bacteriocin (secondary metabolites)	little is known, disruption of cytoplasmic membrane (in the case of nisin)[34-39]

Lactic acid is the major metabolite produced by LAB and depending on the substrate and microorganisms, lactic acid has been reported as having good, average or poor antimicrobial properties. Acetic acid is another organic acid produced by LAB. Both lactic and acetic acids and their salts are generally regarded as safe by the United States Food and Drug Administration. Acetic acid and its salts assert their antimicrobial activity up to pH 4.5 and the effect is due to undissociated molecules[6]. The inhibitory effect of lactic acid produced by LAB has been most extensively investigated. Low pH affects every aspect of cellular metabolism, with retardation of the growth of unwanted microbes in culture media. Undissociated lactic and acetic acids penetrate the cell membrane and disturb the transmembrane potential, resulting in inhibition of substrate transport and membrane-bound FoF_1 ATPase activity[7]. The minimum inhibitory concentration of undissociated lactic acid shows strain specificity.

Hydrogen peroxide produced by LAB is inhibitory to both Gram-negative *Pseudomonas* sp.[8] and Gram-positive *Staphylococcus aureus*[9]. Because LAB do not possess catalase,[10] H_2O_2 accumulates in the surrounding medium, resulting in anaerobic conditions. The lethal effect of H_2O_2 may be due to the inactivation of essential biomolecules by the superoxide anion chain reaction[11]. It may also function via the lactoperoxidase-thiocyanate system. The H_2O_2 oxidises the thiocyanate to release toxic oxidation products that are detrimental to foodborne pathogens[12]. The H_2O_2 is more effective as a sporicide than as a bactericide[13]. Carbon dioxide may exert its antimicrobial effect in several ways such as by rendering the environment more anaerobic, by inhibiting enzymatic decarboxylation and by disrupting the cell membrane with the accumulation of the gaseous phase in the lipid bilayer[14].

Diacetyl (2,3 butanedione) is synthesised by certain species of LAB from pyruvate. It inhibits the growth of Gram-negative bacteria and Gram-positive bacteria other than LAB and yeasts[15]. Diacetyl interferes with arginine utilisation by reacting with arginine-binding proteins of Gram-negative organisms[16].

Bacteriocins produced by LAB are the subject of intense research because of their antimicrobial activity against foodborne bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Clostridium botulinum* and several others[17-21]. Bacteriocins have considerable promise for application as natural food preservatives. Bacteriocin producing strains of LAB may be very important in competing with other organisms in the intestine. They consist of a biologically active protein moiety, have a bactericidal mode of action and attach to specific cell receptors. Wide variation exists in their chemical composition and specific mode of action. The effects of bacteriocins have been elucidated in the food systems, however, a prophylactic role in the intestine has yet to be proven conclusively. In the following section, our knowledge of bacteriocins produced by LAB is reviewed.

General characteristics of bacteriocins

Although not exactly defined, bacteriocins differ from classical antibiotics. They are a heterogeneous group of bacterial antagonists that vary considerably in molecular weight, biochemical properties, range of sensitive hosts and mode of action[18]. Klaenhammer redefined them as follows: "Bacteriocins are proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium"[18].

In the past few years many bacteriocins or bacteriocin-like compounds have been reported and characterised[21-25]. Many of these substances do not comply with the definition proposed above. Bacteriocins are generally active against closely related species and thus the inhibitory spectrum is very narrow (especially bacteriocins from LAB). However, nisin from *Lactococcus lactis*[25] and pediocin A from *Pediococcus pentosaceus* B61 and L-7230[26] are active against a broader range of foodborne pathogens. Recently it was claimed that bacteriocins from *Pediococcus damnosus* and *P. pentosaceus* inhibited the growth of Gram-negative organisms such as *Yersinia enterocolitica, Pseudomonas fragi* and *Pseudomonas fluorescens*[24].

Exceptions exist with regards to Klaenhammer's definition of bacteriocins, including their bactericidal mode of action, the pure protein nature and the narrow spectrum of antibacterial activity. Bacteriocins from *P.*

pentosaceus 43200 and 43201 are insensitive to proteases and are not protein in nature[22]. Most bacteriocins produced by LAB are bactericidal. However, lactocin 27 from *Lb. helveticus*[27], bacteriocin from *Lb. sake* 148[28], and bacteriocin from *Leuconostoc gelidum*[29] have a bacteriostatic effect. The term 'bacteriocin-like substance' was suggested[3] for those antagonistic substances that do not fit the traditional definition of a bacteriocin.

There is no clear cut boundary between antibiotics, bacteriocins or microcins. Like antibiotics, bacteriocins therefore may be bacteriostatic or bactericidal with narrow or broad ranges of activity, that could be included in a family of peptide antibiotics. Antibiotics are synthesised nonribosomally by multi-step enzyme pathways. There are many ribosomally synthesised bacteriocins. While many antibiotics promote the development of resistant strains, development of resistance to bacteriocins is rare. However, the possibility of resistance to bacteriocins may have been overlooked[25,30,31]. Many antibiotics can be chemically synthesised. There is no report to date in which bacteriocins are chemically synthesised. However, it is appropriate to expect that, with genetic engineering, analogues of bacteriocins may be designed and constructed.

There is considerable overlap in the definition of antimicrobial substances. It is thus generally accepted that bacteriocins are a heterogeneous group of proteinaceous compounds that may vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties. Their ability to inhibit other bacteria and the fact that all are proteinaceous may be the only common features of this mixed group of substances[21].

Lipid and/or carbohydrate moieties can be associated with these proteinaceous compounds and be part of the bacteriocin complex. Bacteriocins can be either cell bound or released extracellularly and may be produced early or late in the growth cycle. They are susceptible to proteases and have variable stability at different pH and temperature.

The first detailed characterisation of bacteriocinogenic activity of lactobacilli was reported in 1961[32]. Since then, considerable research has been continuing on bacteriocins from various LAB, notably on nisin. The important properties of some well characterised bacteriocins are shown in Table 2.

Nisin is produced by *Lactococcus lactis* subsp. *lactis* of dairy origin. Nisin is the only bacteriocin that is accepted as a food preservative. It has a broad spectrum of activity against Gram-positive organisms including sporeforming bacteria. Nisin occurs as subtypes, A, B, C, D or E, that differ in amino acid composition and biological activity[25]. It is a pentacyclic cationic polypeptide, referred to as a lantibiotic. Nisin contains 34 amino acids and is synthesised by posttranslational processing of ribosomally synthesised precursors. Processing events include dehydration of serine and threonine to dehydro forms, some of which react with cysteine residues to form thioether cross linkages. In addition, a leader peptide is cleaved and the mature bacteriocin is exported from the cell. The modified peptide bacteriocin is characterised by the occurrence of the sulphur-containing amino acids lanthionine and β-methyllanthionine. The unusual amino acids of nisin, dehydroalanine (DHA) and dehydrobutyrine (DHB), are thought to inactivate sulphydryl groups in germinated bacterial spores[33]. The biochemistry, genetics and mode of action of nisin A has been extensively reviewed[18,25,34-38].

Table 2. Properties of some well characterised bacteriocins

Bacteriocin	Producer organism	Properties
Nisin	*Lactococcus lactis* subsp. *lactis* ATCC 11454	Lantibiotic, broad spectrum, chromosome/ plasmid mediated, bactericidal, produced late in the growth cycle[25]
Pediocin A	*Pediococcus pentosaceus* FBB61 and L-7230	Broad spectrum, plasmid mediated[26]
Pediocin AcH	*Pediococcus acidilactici* H	Broad spectrum, plasmid mediated[41]
Leucocin	*Leuconostoc gelidum* UAL187	Broad spectrum, plasmid mediated, bacteriostatic, produced early in the growth cycle[28]
Helveticin J	*Lb. helveticus* 481	Narrow spectrum, chromosomally mediated, bactericidal[43]
Carnobacteriocin	*Carnobacterium piscicola* LV17	Narrow spectrum, plasmid mediated, produced early in the growth cycle[42]

Mechanisms of action of bacteriocins

Little is known about the mechanism of action of bacteriocins. For nisin, it was reported that the cytoplasmic membrane is the main target, because treatment with nisin causes rapid, nonspecific efflux of amino acids and cations, and rupture of the cell membrane, resulting in the death of sensitive cells[34,39]. The phospholipid composition of the membrane may be influential in the effectiveness of nisin[39]. The combined results obtained in cells, vesicles and liposomes, suggest that the specificity of lactococcin A from *Lactococcus lactis* may be mediated by a receptor protein associated with the cytoplasmic membrane[40]. The treatment of the cell walls to remove lipoteichoic acid prevented the binding of pediocin AcH from *P. acidilactici*[41]. It has also been suggested that lipoteichoic acid molecules, that are present only in Gram-positive organisms may be one of the binding sites for pediocin AcH. This may be the reason why LAB bacteriocins are adsorbed to Gram-positive bacteria and not to Gram-negative bacteria. The cell lysis is associated with cell death, and may depend on the strains of sensitive bacteria, presence of nonspecific receptor sites, such as lipoteichoic acid, and specific receptors[41].

Genetic determinants for bacteriocins

The gene or genes encoding bacteriocin production may be located on the chromosome or on plasmids. The production of pediocin A[26], leucocin A[29] and carnobacteriocins[42] are plasmid-mediated; while production of helveticin J[43] and lactacin B from *Lb. acidophilus* N2[44] are chromosomally-mediated. In the case of nisin, it is not clear whether the gene is located on a plasmid or on the chromosome, because no physical evidence linking this phenotype to a distinct plasmid has been obtained. The conjugal transfer of plasmid encoded genes could not be detected in lysates of transconjugants of *L. lactis* 11454[45,46].

Little attention had previously been directed towards the chromosome of these industrially important organisms.

Recently, the application of transposable elements in genetic analyses has received attention in the genus *Lactococcus*. A broad distribution of the insertion elements has been observed in many lactococcal plasmids and chromosomal DNA, encoding lactose fermenting ability[47], proteinase activity[48], and bacteriophage resistance[49]. Many researchers observed the involvement of insertion elements (IS) in the nisin gene. Multiple copies of IS904 within the lactococcal chromosome downstream of the nisin gene were observed[50]. The nucleic acid sequence of the nisin precursor gives rise to a prepropeptide of 57 amino acids, including a 23 amino acid leader region and a 34 amino acid structural region (*spaN*). No promoter or rho-independent terminator was found, leading to the conclusion that the *spaN* gene is translated from polycistronic mRNA.

The genes for nisin production in *L. lactis* ATCC 11454 are thus located on the chromosome[51]. The restriction patterns indicate that the size of *L. lactis* genome is about 2,500 kb. The fact that nisin gene lies on a DNA restriction fragment that is one-half the size of the genome rules out the possibility of it being plasmid encoded, unless it is a plasmid that rivals the size of the chromosome which is unprecedented[51].

To date, antibiotic resistance vectors have been used to study the genetics of LAB because of the ease with which the desired clones can be selected, but these markers are unsuitable for food-grade bacteria. Ideally, a food-grade cloning vector should be constructed from DNA derived from a microorganisms that are approved for food use and contain a selectable marker that does not compromise human drug therapy[52]. A major objective at present is the development of homologous vector systems based only upon LAB DNA with metabolic or otherwise acceptable selection phenotypes for food use, such as genes associated with carbohydrate metabolism, bacteriocin production and resistance. Recently, a food-grade cloning vector, pFM011, employing the Nis^r phenotype as a selectable marker was developed[52] and this may lead to a better understanding of LAB bacteriocin genes.

Nisin which is produced by lactococci of dairy origin, has been approved for use as a food preservative in over 45 countries. In 1988, it was approved by the US Food and Drug Administration for use in pasteurised cheese to inhibit outgrowth of spores of *C. botulinum*. Nisin is of particular interest because of its effectiveness against a broad range of Gram-positive organisms including *L. monocytogenes, C. botulinum and S. aureus*. Although normally resistant to nisin, Gram-negative organisms can be sensitised when the outer membrane is weakened in the presence of chelating agents[52]. Nisin is being exploited as a food preservative mainly in the dairy foods. It is nontoxic and digested by intestinal enzymes. It is heat stable and does not contribute to off-flavours. It has no value in medical therapy because it is practically insoluble in blood at physiological pH[25]. The large size of the molecule precludes absorption if intramuscular injection is used. However, nisin can be used for topical applications.

The role of bacteriocins in the suppression of pathogens in the intestine is questionable, mainly because of their susceptibility to proteolytic enzymes within the gut. However, the search for strains which produce bacteriocins and bacteriocin-like substances resistant to degradation continues because of the potential importance of these agents.

Conclusion

To derive the optimum human benefit from this bacterial antagonist requires the selection of strains with desirable properties. The LAB are not only crucial to the manufacture of fermented products, but they have been reported to be effective in the treatment of a variety of disorders, including colitis, prevention of recolonisation of the intestine with pathogens after antibiotic treatment, gastroenteritis, hypercholesterolaemia, hepatic encephalopathy, lactose maldigestion, urogenital infection and in prevention of tumours.

Although claims for health benefits have been made for LAB in fermented dairy products for nearly a century, the nutritional and therapeutic value of these organisms remains controversial. Clarification of these claims will require further scientific evaluation of antimicrobial products of LAB with stringent strain selection criteria and conduct of well controlled clinical trials. Further research is also required in the area of host specificity, methods of assessing bacterial adherence and bacterial labelling so that quantification, localisation and identification of microbial species can be performed as they pass through the gastrointestinal tract. The future of beneficial interference will depend on a greater understanding of the genetic mechanisms involved.

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原生菌（Probiotics）的抗生素物質的產生

摘要

細菌的桔抗作用已被公認有一世紀，但最近這一觀象更受到科學界的注意，特別是在不同乳酸杆菌菌株的應用上。乳酸杆菌產生的抗生素化合物使這些細菌具備超越其他微生物的競爭條件，很多食品以及腸道是乳酸杆菌的天然生態小境。乳酸菌的抗菌產物有廣泛的效能和抗菌譜，它們包括乳酸、醋酸、過氧化氫、二氧化碳、二乙酰和細菌素或細菌素類物質。進一步篩選廣譜活性物有其必要性，這將牽涉到上述化合物的遺傳或蛋白工程，將這些物質商品化。

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