Antimicrobial Properties of Commercial Annatto Extracts against Selected Pathogenic, Lactic Acid, and Spoilage Microorganisms

VERONICA GALINDO-CUSPINERA,1 DENNIS C. WESTHOFF,1 AND SCOTT A. RANKIN2*

1Department of Animal and Avian Sciences, University of Maryland, College Park, Maryland 20742-2311; and 2Department of Food Science, 1605 Linden Drive, University of Wisconsin, Madison, Wisconsin 53706-1565 USA

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ABSTRACT

Annatto preparations are used to impart distinctive flavor and color to foods and are a primary colorant in dairy foods such as cheese and butter. There are several reports indicating that certain fractions of the annatto plant have biological activities against microorganisms of significance in food fermentation, food preservation, and human health. However, little is reported describing the nature of the antimicrobial compound(s) or their potential presence in commercial annatto colorant preparations. This study was conducted to determine whether commonly available annatto extracts are capable of influencing the outgrowth of selected lactic acid, spoilage, and pathogenic microorganisms. Disk diffusion and tube macrodilution techniques were used to determine the MICs and MBCs of double-strength water-soluble annatto extracts. Standard antibiotic disks were used as controls for the disk diffusion assay. The results demonstrate that annatto has an inhibitory effect on Bacillus cereus, Clostridium perfringens, and Staphylococcus aureus, with MICs of 0.08, 0.31, and 0.16% (vol/vol) and diameters of inhibition of 9 to 10, 12 to 13, and 15 to 16 mm, respectively. A concentration of 0.63% (vol/vol) inhibited the growth of Streptococcus thermophilus, Lactobacillus casei subsp. casei, Lactococcus lactis, and Paenibacillus polymyxa. The MICs for Listeria monocytogenes and Enterococcus durans were 1.25 and 2.5% (vol/vol), respectively. No activity was detected against Lactobacillus plantarum, Bifidobacterium bifidum, yeasts, or selected gram-negative bacteria.

The term ‘annatto’ refers to a series of preparations containing the carotenoid-type pigments cis-bixin and nor-bixin. Annatto is considered essentially nontoxic; in the United States, annatto is classified as a natural colorant exempt from certification as such. Although annatto is used in many foods, the foremost use is as a colorant in cheese. Added directly to cheese milk at a rate upward of 0.06% (vol/vol) with typical usage rates of 0.01 to 0.02% (vol/vol), annatto provides the distinctive orange hue of Cheddar, Colby, Cheshire, and many other colored cheeses (22). Additionally, in some Muenster-type cheeses, annatto may be applied directly to the finished cheese to provide a distinctive orange/red surface color.

In African and Latin American countries, annatto has long been used for medicinal purposes. Annatto is used as a folk remedy in the form of an oil suspension for the treatment of diabetes in the West Indies (14). South American natives use annatto to promote the healing of wounds, to treat skin infections, to heal burns, to act as an antipyretic (23), to treat measles, (1) and to subdue diarrhea and asthma symptoms (16).

Annatto plant extracts have demonstrated some specific antimicrobial activities. The leaves of Bixa orellana exhibit an inhibitory effect against Neisseria gonorrhoea (4). Leaf extracts have been shown to exhibit significant antimicrobial activity against standard strains of gram-positive bacteria including Bacillus subtilis, Streptococcus faecalis, and Staphylococcus aureus (14). A recent study of the volatile profile from commercial annatto extracts identified several mono- and sesquiterpenes (10), some of which have been reported as having antimicrobial activities (17, 18).

Although no specific reports are available in the literature, it is generally considered that the addition of annatto to cheese does nothing more than influence the color (22). Given the anecdotal and research-based evidence of its antimicrobial activity and the complex microflora of dairy foods, there is sufficient cause to warrant a more thorough investigation of the potential antimicrobial properties of commercial annatto extracts against pathogenic, lactic acid, and spoilage microorganisms of significance to dairy foods.

MATERIALS AND METHODS

Microorganisms and culture media. Seven pathogenic microorganisms were tested in this study and include S. aureus (ATCC 25923, American Type Culture Collection, Manassas, Va.) (17), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Bacillus cereus (ATCC 11778), Listeria monocytogenes (ATCC 19115), Clostridium perfringens (ATCC 13124), and Salmonella Typhimurium (donated by Dr. Jianghong Meng, University of Maryland, Department of Food Science). Lactic acid bacteria included Lactobacillus plantarum (ATCC
700210), Lactobacillus casei subsp. casei (ATCC 39539), Streptococcus thermophilus (ATCC 14485), Lactococcus lactis (ATCC 11454), and the probiotic strain of Bifidobacterium bifidum (ATCC 15696). Spoilage bacteria included Pseudomonas fluorescens (ATCC 13525), Paenibacillus polymyxa (ATCC 842), Acinetobacter baumannii (ATCC 19606), and Enterococcus durans (ATCC 11576). Spoilage yeast included Candida parapsilosis (ATCC 22019), Debaryomyces hansenii (ATCC 10623), Kluyveromyces marxianus (ATCC 8554), Saccharomyces cerevisiae (ATCC 2601), and Yarrowia lipolytica (ATCC 9773). All culture media were purchased from Difco (Sparks, Md). Microorganisms were grown overnight on deMan Rogosa Sharpe (MRS) broth (lactic bacteria), reinforced clostridial broth (anaerobes), Emmons Sabouraud agar (fungi), and Trypticase soy broth (all other microorganisms) under optimal conditions. The standard inoculum was adjusted following the principles developed for the standard disk diffusion technique (2). Overnight cultures were adjusted to match a 0.5 McFarland standard (BBL; Becton Dickinson, Cockeysville, Md.) and further diluted 1:100 with Mueller-Hinton broth, Yeast Nitrogen Base with glucose and asparagine (for yeasts), or Anaerobe Broth (for C. perfringens and B. bifidum). The dilution obtained served as the inoculum for disk diffusion tests and broth dilution tests. Standard plate counts were performed for this inoculum. See “Results” for the actual amount of bacteria present in the tubes and plates tested; the original inocula were corrected to account for the final dilution.

Disk diffusion technique. The diffusion test was performed following standard procedures (3, 26). The objective was to measure any halo of inhibition caused by a specific annatto solution. In practice, 0.10 ml of the inoculum was spread with glass sticks onto either agar antibiotic 1 (most bacteria), MRS (lactic bacteria), Wilken-Chalgren anaerobes, or Emmons Sabouraud (fungi) plates. Sterile 6-mm paper disks were soaked with 25 µl of a 5.0% double-strength annatto (DSA: 2.8% norbixin, DSM Food Specialties, Menomonee Falls, Wis.) solution and placed on the previously inoculated plates. Plates were maintained at optimal conditions, and the halo of inhibition was measured after 18 to 24 h of incubation. A disk containing known amounts of penicillin, gentamicin, or chloramphenicol (BBL) was placed in one quadrant of the plate as a comparative standard.

Antimicrobial dilution test. A stock solution of annatto was prepared by diluting 5.2 ml of DSA (2.8% norbixin) with 99 ml of phosphate buffer (pH 7.0; VWR, Westchester, Pa.). This solution was adjusted to pH 7.4 with 2 N HCl and filtered through a 0.2-µm pore-size membrane (ZapCap, VWR, Bridgeport, N.J.), yielding the annatto stock solution. The stock solution was used to prepare serial twofold dilutions to a final concentration of 0.08% of annatto. A 2.0-ml aliquot of the microbial inoculum was placed in each tube containing annatto. A positive blank containing 2.0 ml of the microbial inoculum and 2.0 ml of medium was prepared for each series. At least three replicates were performed for each microorganism. Tubes were incubated for 18 to 24 h at optimal conditions, after which the MIC was determined. The MIC was defined as the lowest concentration of annatto in a tube with no visible growth when viewed against standard fluorescent light. To corroborate the results, the microbial density was measured with a Novaspec II spectrophotometer (Pharmacia, Cambridge, UK) at 540 nm (absorbance >0.05 was considered a positive).

After the MIC was determined, each tube showing no visible evidence of microbial growth was then subcultured to determine whether viable organisms were present. Using a calibrated loop, 0.01 ml was subcultured to a quadrant of Mueller-Hinton agar plate (MRS agar for lactics, Wilken-Chalgren agar for anaerobes, or Emmons Sabouraud agar for yeasts) to determine the MBC. After 18 to 24 h of incubation under optimal conditions, the plates were examined, and the number of CFUs was determined. The MBC was defined as the lowest concentration that resulted in 99.9% kill (2).

Data analysis. To correct for variation among replicates and outlier values, the median MIC and MBC were calculated by SAS version 8 statistical software. For this analysis, the median was chosen because it reflects the actual concentration necessary to inhibit growth. With twofold dilutions, the concentration range between tubes is different, and the arithmetic mean tends to increase the MIC and MBC when the overall tendency is for a smaller value.

RESULTS AND DISCUSSION

Pathogenic bacteria. According to a report released in 1990 on the microbiological safety of cheese made from heat-treated milk (15), between 1948 and 1988, the most frequent causative factor in U.S. and Canadian cheese-related outbreaks was postpasteurization contamination. Three organisms, Salmonella spp., L. monocytogenes, and enteropathogenic E. coli, were considered high-risk threats to the cheese industry, while S. aureus was considered low risk because growth and toxin production are readily suppressed by adequate refrigeration and pH control. In a recent study based on data collected from 1970 to 1997, Salmonella spp., S. aureus, and L. monocytogenes were the most common organisms in cheese-related disease outbreaks (9). The current study evaluated the activity of annatto against common foodborne pathogens (Table 1). Annatto did not affect the growth of E. coli, Salmonella Typhimurium, or P. aeruginosa. L. monocytogenes outgrowth was weakly inhibited. A 1.25% solution was capable of inhibiting the growth of L. monocytogenes; however, no bactericidal effect was detected at the concentrations tested. The outgrowth of S. aureus, B. cereus, and C. perfringens was substantially inhibited in the presence of annatto extracts. The highest activity was detected against B. cereus, where an 0.08% solution was sufficient to inhibit growth and a 0.16% solution exhibited a bactericidal effect. It is important to note that in the case of B. cereus, the inoculum used was smaller than the one used with other bacteria. S. aureus was inhibited with a 0.16% DSA solution and showed the largest zone of inhibition among all microorganisms tested.

Cheese commonly presents anaerobic conditions and pH levels favorable for clostridial growth, although clostridia outbreaks in cheese are rare (9). In this study, C. perfringens, another well-known food pathogen, was inhibited with a 0.31% annatto solution, and a 0.55% solution was capable of killing vegetative cells.

Lactic acid bacteria. It is widely recognized that the flavor of cheese results from the interaction of starter bacteria, milk and rennet enzymes, and secondary microflora (25). The microorganisms involved in cheese making and ripening can be divided into starter lactic acid bacteria and nonstarter lactic acid bacteria. L. lactis and S. thermophilus are common starter cultures, while typical nonstarter lactic

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Table 1. Antimicrobial activities of double-strength water-soluble annatto

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Average inoculum (log CFU/ml)</th>
<th>MIC (% DSA)</th>
<th>MBC (% DSA)</th>
<th>5% DSA disk inhibition (mm)</th>
<th>Antibiotic disk inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogenic bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>4.40</td>
<td>0.08</td>
<td>0.16</td>
<td>9–10</td>
<td>P 9–12</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>5.23</td>
<td>0.31</td>
<td>0.55</td>
<td>12–13</td>
<td>P 30–32</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>6.98</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>ni</td>
<td>C 13–15</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>5.91</td>
<td>1.25</td>
<td>nb</td>
<td>12–13</td>
<td>P 18–21</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>6.89</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>ni</td>
<td>G 13</td>
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<tr>
<td>Salmonella Typhimurium</td>
<td>5.29</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>ni</td>
<td>G 8–9</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>5.98</td>
<td>0.16</td>
<td>1.60</td>
<td>15–16</td>
<td>P 30–31</td>
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<td>Lactic acid bacteria</td>
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<td></td>
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</tr>
<tr>
<td>Bifidobacterium bifidum</td>
<td>5.91</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>8–9</td>
<td>P 22–25</td>
</tr>
<tr>
<td>Lactobacillus casei subsp. casei</td>
<td>5.46</td>
<td>0.63</td>
<td>0.94</td>
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<tr>
<td>Lactococcus lactis</td>
<td>6.80</td>
<td>0.63</td>
<td>1.04</td>
<td>9</td>
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<tr>
<td>Lactobacillus plantarum</td>
<td>5.30</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>9</td>
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</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>5.08</td>
<td>0.63</td>
<td>2.5</td>
<td>9–10</td>
<td>P 18–20</td>
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<tr>
<td>Yeasts</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Candida parapsilosis</td>
<td>4.97</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>ni</td>
<td>N 22–25</td>
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<tr>
<td>Debaryomyces hansenii</td>
<td>4.68</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>ni</td>
<td>N 15–17</td>
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<tr>
<td>Kluyveromyces marxianus</td>
<td>4.78</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>ni</td>
<td>N 29–31</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>4.66</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>ni</td>
<td>N 21–26</td>
</tr>
<tr>
<td>Yarrowia lipolytica</td>
<td>4.67</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>ni</td>
<td>N 18–20</td>
</tr>
<tr>
<td>Spoilage bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>5.62</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>ni</td>
<td>G 11</td>
</tr>
<tr>
<td>Enterococcus durans</td>
<td>5.38</td>
<td>2.5</td>
<td>nb</td>
<td>8–10</td>
<td>P 17–19</td>
</tr>
<tr>
<td>Paeonibacillus polymyxa</td>
<td>4.85</td>
<td>0.63</td>
<td>1.25</td>
<td>8–9</td>
<td>P 16–18</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>5.72</td>
<td>&gt;2.5</td>
<td>nb</td>
<td>ni</td>
<td>G 9–13</td>
</tr>
</tbody>
</table>

*DSA, double-strength annatto in water; pH 7.4, % (vol/vol); nb, nonbactericidal at 2.5% DSA; ni, no inhibition.

a P, penicillin 2 U; C, chloramphenicol 5-U disks; G, gentamicin 10 U; N, nystatin 2 U.

Probiotic bacterium was included in this classification because of its association with fermented dairy foods.

acid bacteria include several lactobacilli species such as L. casei, L. plantarum, L. paracasei, and L. curvatus (8). In this study, four strains of lactic acid bacteria were tested. Additionally, because fermented dairy foods are recognized as a source of health-promoting or probiotic bacteria (21), B. bifidum, a common probiotic strain, was also included.

Annatto exhibited limited activity against L. lactis, L. casei subsp. casei, and S. thermophilus. A 0.63% DSA solution was sufficient to inhibit the growth of these organisms (Table 1). The MBC concentration had more variation among these three organisms; S. thermophilus proved to be the most resistant at 2.5%. No antimicrobial activity was detected against B. bifidum or L. plantarum.

Influence on yeasts. Yeasts fulfill an important role in the manufacture and spoilage of many dairy foods. In pasteurized dairy products, yeasts typically originate from postpasteurization contamination and are common contaminants of cheeses (7). Among the most frequently occurring yeasts in dairy products are D. hansenii, S. cerevisiae, K. marxianus, C. parapsilosis, and Y. lipolytica. These five strains have been isolated from cheese and yogurt and occasionally have been found in ice cream (7, 20). Annatto did not inhibit the growth of any of the five yeasts tested at the concentrations used (Table 1). Growth was consistent throughout the dilution series. Annatto even appeared to promote the growth of yeasts. Some growth was detected on the disk itself, and the tubes that contained annatto appeared slightly more turbid than the positive blank.

Spoilage bacteria. Psychrotrophic bacteria affect the quality and shelf life of numerous milk products (13, 24). Pasteurized dairy products can be spoiled by gram-negative bacteria resulting from postprocess contamination or by gram-positive organisms, especially spore-forming bacilli, that survive pasteurization. Psychrotrophs have proteolytic and lipolytic activity and may produce malodorous metabolites in milk. In a study made to classify the spoilage flora of raw and pasteurized milk, Pseudomonas spp. and Bacillus spp. were the most frequently encountered spoilage bacteria. P. fluorescens and P. fragi are commonly associated with raw and pasteurized milk spoilage (24). Bacillus polymyxa and B. cereus are described as the second most important bacteria in the spoilage of pasteurized milk. Bacilli spores withstand pasteurization and can grow at low temperatures, thus becoming a limiting factor in the shelf life of pasteurized milk products (6). B. cereus is also a well-known foodborne pathogen. It is capable of rapid growth during the manufacture of Cheddar cheese between the end of cooking and the curd-milling step and has been isolated...
from cheese samples (5). Some psychrotrophic strains of *B. cereus* have been shown to produce toxins and thus are of importance from a public health standpoint (6).

Other bacteria important in dairy food spoilage are *Acinetobacter* and enterococci. *Acinetobacter* spp. have low spoilage potential because they lack important food spoilage biochemical activities such as proteolysis or production of unpleasant odors. However, they are of particular note because of their potentially high levels in raw milk and their ability to produce a capsular polysaccharide resulting in ropy milk and whey as well as slimy surface defects in cheese (12). Enterococci fulfill many roles in dairy products as starter, probiotic, and spoilage bacteria (11). Some strains seem to play an important role in the development of cheese flavor; nevertheless, they are not considered GRAS (generally recognized as safe) organisms (11). Enterococci are thermoresistant bacteria that withstand lactic acid and are commonly found in levels ranging from several thousands to tens of millions per gram in a variety of cheeses produced from raw and pasteurized milk (19).

The antimicrobial activity of annatto was tested against four spoilage bacteria commonly found in dairy products (Table 1). There was no inhibitory effect detected against *A. baumannii* or *P. fluorescens*, both gram-negative bacteria. Conversely, annatto showed activity against gram-positive *E. durans* and *P. polymyxa* (also referred to as *B. polymyxa*). A 0.63% solution of annatto was capable of inhibiting the growth of *P. polymyxa*, while a 1.25% solution had a bactericidal effect. In the case of *E. durans*, a more concentrated solution of 2.5% annatto was necessary to inhibit growth; no bactericidal activity was detected at the concentrations tested. Because of the spreading characteristic of bacilli, it was not possible to detect a consistent halo of inhibition with the disk diffusion technique. Also, it was difficult to obtain an inoculum higher than 10⁶ because of the turbidity caused by the capsule. However, growth inhibition was clearly detected against both bacilli strains in the tube macrodilution technique.

Although annatto is generally considered an inert coloring agent in dairy products, these results suggest that annatto may also have some ability to influence microflora of significance to dairy foods, namely certain gram-positive species. In each case, the inhibitory effects were manifested at substantially higher concentrations than those found in cheese manufacturing practices. However, little is known about the properties of the antimicrobial agent(s) in annatto or its fate during the cheese making process. It is possible that the inhibitory influence is ultimately increased in cheese because of the concentrating effect manifested during the separation of curd and whey. Conversely, the active agent(s) may be entirely water soluble and partition with the whey, thus further diminishing its potentially antimicrobial effect in the curd. When annatto is applied directly to the outer surface of cheese, as in the case of Muenster cheese, annatto may be present at concentrations sufficient to serve as a surface-associated antimicrobial barrier. Further research is necessary to identify the compound(s) responsible for this activity and to determine the effectiveness in an actual food matrix.

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