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Antimicrobial Activity of Natural Inhibitors Against *Salmonella typhimurium*

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نشاط المضادات الطبيعية المقاومة للجراثيم ضد السالمونيلا

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خلاصة: عدد من التوابل والأعشاب لديها مقدرة مضادة للجراثيم نتيجة تركيبتها الكيميائية. وقد تم تقدير حساسية ال السالمونيلا (*S. typhimurium ATCC* ١٤٠٨٢) لهذه المضادات الطبيعية بقياس المنطقة الخالية (التضاد) لكل نوع من هذه المضادات كل على حده. كل العينات المختارة اظهرت مستويات مختلفة من التصدي لهذا النوع من البكتيريا. عموما اظهرت السالمونيلا (*S. typhimurium ATCC* ١٤٠٨٢) مقاومة واضحة لهذه المضادات الطبيعية. فقط القرفة والثوم أوجدوا منطقة تضاد واضحة. الطاقة التضادية للثوم كانت ثابتة خلال التسخين الحراري الطبقي وكذلك باستخدام الموجة الصغرى. من جهة أخرى كانت الطاقة التضادية للقرفة منخفضة خلال الموجة الصغرى ومفقودة كليا باستخدام التسخين الحراري الطبقي. لم يكن الثوم على تركيز ٥ % قاتلا لهذه البكتيريا ولكنه كان مؤثرا في خفض معدل تكاثر السالمونيلا (*S. typhimurium ATCC* ١٤٠٨٢).

ABSTRACT: A range of herbs and spices are known to possess antibacterial activity as a consequence of their chemical composition. The sensitivity pattern of *S. typhimurium* ATCC 14082 to a range of these natural inhibitors was determined by measuring the zones of growth inhibition produced by individual herbs or spices. The herb and spice samples caused different levels of growth inhibition with *S. typhimurium* ATCC 14082. In general, *S. typhimurium* ATCC 14082 demonstrated considerable resistance to the antimicrobial activity of these inhibitors. Only cinnamon and garlic produced zones of growth inhibition. The antimicrobial activity of the garlic remained stable during mild heating, either on a hot plate or by exposure to microwaves. This was in contrast to the inhibitory activity of cinnamon which was reduced following microwave exposure, and which was completely lost after heating on a hot plate. Garlic at a concentration of 5% was not bacteriocidal, but considerably reduced the rate of replication of *S. typhimurium* ATCC 14082.

Keywords: *Salmonella typhimurium,* antimicrobial, natural inhibitors, herbs, spices.

The resistance of foods to microbial degradation can, in part, be related to the presence of naturally occurring antimicrobial substances. Antimicrobial agents can occur in foods of both animal and vegetable origin (Gould *et al.*, 1986). The most important antimicrobials of vegetable origin include essential oils, tannins, glycosides and resins (Haas and Barsoumian, 1994). Inducible resistance responses in plants for protection against pathogenic bacteria have been documented (Caruso and Kuć, 1979; Goodman, 1978). A consequence of these responses may be the production and accumulation of compounds, within the plants and fruits, which inhibit bacterial growth.

Herbs and spices have been used for centuries by many cultures to improve the flavor and aroma of foods. Also, it has long been recognized that they have a value in the preservation of foods. Scientific studies have identified the active antimicrobial agents of many herbs

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and spices (Shelef, 1983; Zaika, 1988). These include eugenol in cloves, allicin in garlic, cinnamic aldehyde and eugenol in cinnamon, allyl isothiocyanate in mustard, eugenol and thymol in sage, and isothymol and thymol in oregano (Table 1).

Illnesses due to food contaminated with either chemical or biological agents, are one of the most widespread problems throughout the world. Salmonellae are one of the most common causes of human food poisoning. Salmonellosis caused by ubiquitous *Salmonella* organisms can have enormous personal and economic consequences.

To be a successful pathogen, a bacterium must be highly adapted, being able to detect and respond to changing environments, and must also be able to out-compete other microbes to enable their own proliferation and colonization. Shortage of any essential nutritional requirement, or extreme physical or chemical conditions can restrict the growth of bacteria, (both rate and yield), and these effects, can be bacteriostatic or bacteriocidal. The aim of this study was to investigate the potential of a range of herbs and spices to inhibit the growth of *S. typhimurium* (ATCC 14082) in foods.

Materials and Methods

A range of vegetable products were used in this study, including garlic, cinnamon, mustard oil, sage, tea, black pepper, cloves and onion. A comprehensive list, with suppliers, is shown in Table 1. The antimicrobial activity of the different, individual products was determined against *S. typhimurium* (ATCC 14082). The activities of the selected natural inhibitors were assessed by measuring inhibition of bacterial growth.

TABLE 1

Vegetable products, commercial suppliers and active ingredients.

Inhibitor	Manufacture	Active Ingredient	Proximate Essential Oil Content (%)
Garlic	Local	Allicin	0.3 - 0.5
Cinnamon	Ducros, France	Cinnamaldehyde	0.5 - 2.0
Mustard oil	RR, LTD, U.A.E.	Allyl isothiocyanate	0.5 - 1.0
Sage	GYMA, France	Thymol, Eugenol	0.7 - 2.0
Black pepper	Majdi, Stove 6, India	Piperine	
Cloves	Local	Eugenol	16 - 18
Onion	Local	Numerous organic sulphur compounds	
Tea	Sainsbury' s different countries	Tannins or polyphenols	20 - 30
Oregano	GYMA, France	Thymol, Caracol	0.8 - 0.9

Cultures of *S. typhimurium* (ATCC 14082) were prepared in 5 ml volumes of peptone water (Oxoid, Basingstoke, Hampshire, UK), incubated, static, in air at 35°C for 24 hours. Purity was confirmed on selective medium, Hektoen Enteric agar (H.E.) (Oxoid). These cultures were used to prepare bacterial lawns on plate count agar (PCA) (Oxoid), into which wells had been cut. To prepare bacterial lawns, approximately 0.5 ml of overnight culture was spread over the surface of an agar plate. The plate was rotated to ensure that a complete, even covering of culture was obtained. After coating the surface of the agar, the excess volume of culture was removed using a sterile pipette and discarded. A sterile cork borer was used to make the wells in the PCA. Four wells were made in each plate, a separate well was used for each inhibitor. The wells were filled carefully to ensure that they were completely full, but that the area of agar around the well was not contaminated with the inhibitor and no cross-contamination between the inhibitor samples occurred.

The onion and garlic samples were crushed with a garlic press and the cloves were powdered using a sterile pestle and mortar; disruption of the tissues enhances the liberation of the antimicrobials (Block *et al.*, 1992) and also facilitates the complete filling of the well. Dry black tea leaves were used and were compacted to obtain a good contact between the tea and the surrounding agar. When all the wells were filled, the plates were incubated aerobically at 35°C for 24 hours. Following incubation, the plates were examined for zones of growth inhibition around the wells. The experiment was repeated on three occasions and was also repeated using the selective medium H.E. agar instead of PCA.

To determine the effect of mild cooking on the antimicrobial activity of garlic and cinnamon, samples were heated on a hotplate or exposed to microwaves. One clove of garlic was crushed (~3 g) and then exposed to 220 W microwaves for 15 s. Another was heated on a hotplate for 3 minutes. The heat exposure times were selected on the basis of the time required for the texture of the product to soften. Cinnamon (~3 g) was also treated in the same way. After heating the samples, they were then tested for antimicrobial activity using the growth inhibition test outlined above.

The effect of garlic on the growth characteristics of *S. typhimurium* (ATCC 14082) was determined by measuring changes in cell density with time. Crushed garlic was added to buffered peptone water (Oxoid, UK) to give a final concentration of 5% (w/v). Cell growth was followed spectrophotometrically (Milton Roy) at a wavelength of 540 nm. Cell growth in control samples containing no garlic was also determined. The initial cell density of all cultures was constant, this was achieved by inoculating 50 ml volumes of buffered peptone water with 0.1 ml of an overnight culture. However, due to the addition of 5% garlic the initial optical density of test cultures was higher (Figure 1). The cultures were mixed continuously and incubated at room temperature (25°C). Repeat experiments were performed and means and standard deviations calculated; the mean change in absorbance against time was plotted for test and control samples (Figure 1).

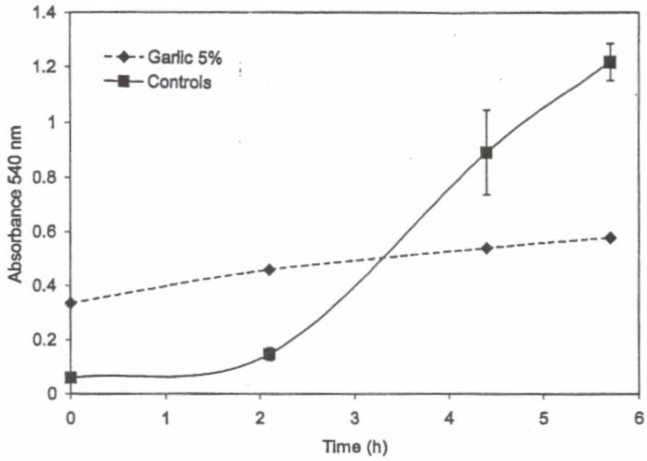

Figure 1. Effect of 5 % crushed garlic on the growth curve of *S. typhimurium* ATCC 14082 at 25°C, in buffered peptone water measured as the change in cell density at 540 nm.

Results and Discussion

Areas of diffusion into the PCA plates from the test wells were observed, especially around the cinnamon, cloves and tea. This indicates that the method is appropriate for detecting the inhibitory affects of the different herbs and spices. Good reproducibility was obtained in repeat experiments. In this study no significant antimicrobial activity was observed with black tea. More activity could have been detected if unfermented green tea had been used (Chou *et al.*, 1999).

Clear zones of growth inhibition were obtained around the wells containing cinnamon or garlic. The mean radii are shown in Table 2. Garlic showed the greatest inhibitory effect on *S. typhimurium* (ATCC 14082), producing an average inhibition zone of 1.6 ± 0.27 cm. Repeat experiments using H.E instead of PCA agar gave the same results. The selective medium is slightly less optimum for growth of *S. typhimurium*. However, this additional environmental stress was not sufficient to influence the inhibitory activity of the test products.

In the experiments there was a suggestion of very limited growth inhibition by onion and cloves. However, no regular, measurable, clear zones of inhibition were obtained. This suggests that the antimicrobial activity of the cloves and onion samples were just on the limit of inhibiting growth of *S. typhimurium* (ATCC 14082). As the samples diffused through the agar the concentration was reduced sufficiently to allow the organism to grow. Concentrating the samples or ensuring that the products are as fresh as possible could result in some inhibition being detected with these products. The antimicrobial activity of onion has been reported to be relatively weaker than that of garlic (Al-Delaimy and Ali, 1970). Although the method of preparation also has a significant effect, freshly reconstituted dehydrated onion showed greater inhibition of *S. typhimurium* than did freshly reconstituted dehydrated garlic (Johnson and Vaughn, 1969). Cinnamon, cloves, and mustard are reported to be amongst the most effective microbial herbs and spices (Zaika, 1988). However, in this study no anti-bacterial activity was detected from mustard oil.

TABLE 2

Detected areas of growth inhibition.

Natural Inhibitor	Inhibition Zone (cm)
Garlic	1.6 ± 0.27 [5]
Cinnamon	0.5 ± 0.10 [5]
Mustard oil	NDI
Sage	NDI
Black pepper	NDI
Cloves	NDI
Onion	NDI
Tea	NDI
Oregano	NDI

NDI represents no detectable inhibition.
Values are means ± standard deviations.
The number in square parentheses is the sample size.

As eugenol is also present in cloves and in a higher proportion than in cinnamon, the results indicate that cinnamaldehyde may have an important role in the antimicrobial activity of cinnamon. It may be the combined activity of eugenol and cinnamaldehyde that results in the inhibition of *S typhimurium* growth, or alternatively it could be that the eugenol is in a more active form in the cinnamon sample used in this experiment than in the cloves. Without further studies it is difficult to speculate on the specific activities of the active components of the herbs and spices. The aim of our study was to consider the antibacterial activity of the herbs and spices in the form in which they are most frequently used in food preparations e.g. garlic crushed, onion chopped.

The *S. typhimurium* cells which were used to prepare the bacterial lawn were grown in peptone water which allows sub-maximal growth of the organism. This was to model the situation in foods where bacteria usually grow

TABLE 3

Effect of mild cooking on detected areas of growth inhibition.

Treatment of the Natural Inhibitor	Radius of Inhibition Zone (cm)	
	Garlic	Cinnamon
Control	1.9 ± 0.14 [2]	0.2 ± 0.03 [2]
Heated	1.6 ± 0.35 [2]	NDI [2]
Microwaves	1.7 ± 0.28 [2]	0.1 ± 0.09 [2]

NDI represents no detectable inhibition.
Values are means ± standard deviations.
The number in square parentheses is the sample size.

at restricted rates due to nutrient limitation or an extreme factor in the surrounding environment. The previous growth conditions (Peters *et al.*, 1991; Lin *et al.*, 1995; and phase of growth (Foster and Spector, 1995; Humphrey *et al.*, 1995) have a considerable effect on the ability of the cells to survive inhibitory challenges.

The antimicrobial activity of cinnamon was more heat-labile than that of garlic (Table 3), with direct heating destroying the detectable activity of cinnamon. Inhibition of *S. typhimurium* (ATCC 14082) was still observed with garlic that had been exposed to mild cooking (Table 3).

When *S. typhimurium* (ATCC 14082) was exposed to 5% crushed garlic an increase in cell density was observed (Figure 1), indicating that this concentration of garlic was not bacteriocidal or bacteriostatic. The shape of the growth curves differed considerably between control cultures and those where garlic was present. A distinct lag period (first 2 hours) was seen in the control cultures before a more linear increase in cell density is observed (Figure 1). This lag was not apparent in cultures with 5% garlic. In this case the rate of increase in cell density was linear over the entire time period of the study, giving a Pearson's correlation coefficient of 0.990. Repeat experiments gave close values, as indicated by the low standard deviation values. A visual indication of this is shown by the inclusion of error bars on the control samples in Figure 1.

The rate of change in cell density was markedly higher in the control cultures being 0.22 ± 0.05 $\Delta A_{540}h^{-1}$, compared to 0.042 ± 0.01 $\Delta A_{540}h^{-1}$ when 5% garlic was included (Figure 1). Therefore, 5% garlic reduced the rate of increase in cell density of *S. typhimurium* (ATCC 14082) growing at room temperature by approximately 80%. These data indicate that the incorporation of 5% garlic would provide a beneficial inhibitory affect on the growth of *S. typhimurium* (ATCC 14082), during food preparation at room temperature, and also, possibly, following mild cooking.

Although the antimicrobial activity of vegetables represents a promising area of research, to date their use as food preservatives has been limited. The normal amount of spices and herbs added for flavor enhancement is insufficient to completely inhibit microbial growth (Giese, 1994), as demonstrated in this study. On the other hand, modern consumers demand food products with reduced artificial chemical additives, providing an increased demand for alternative natural microbial inhibitors. The addition of herbs and spices in combination with other techniques for microbial inhibition, such as refrigeration, would be expected to aid in preservation and to improve the safety of foods. Identification of natural antimicrobials that inhibit the growth of salmonellae and consequently reduce their competitiveness is useful.

An enhanced resistance to the adverse chemical composition of herbs and spices would confer a selective advantage to a bacterium in many foods. As more resistant bacteria are better able to increase or maintain their numbers over long time periods in fluctuating environments, the chances of their numbers exceeding the infective dose when a host is encountered are increased.

This study could be continued to address some of the questions that have arisen from this preliminary investigation. It may be that spices cannot be added to foods in sufficient quantities to act as preservatives alone. Therefore their activity when in combinations with each other, or with other factors such as low temperature or low pH should be determined. Also, it would be beneficial if the test samples could be distilled so that concentration gradients of the inhibitors could be compared. It would also be pertinent to extend the range of organisms used to include most of the common causative agents of food poisoning, as it is anticipated that the tolerance patterns of the different organisms will vary. In addition, it would be pertinent to determine the antimicrobial activity of spices against salmonellae when they are incorporated into food products following traditional recipes.

Conclusions

In this study a preliminary investigation of the sensitivity of *S. typhimurium* to the antimicrobial activity of selected herbs and spices was made. The findings demonstrate that in general, *S. typhimurium* (ATCC 14082) has considerable resistance to the growth inhibitory effects of herbs and spices. The study shows that the tolerance of *S. typhimurium* (ATCC 14082) to different herbs and spices varies. The most inhibitory agents to the growth of *S. typhimurium* (ATCC 14082) were cinnamon which contains the active ingredients eugenol and cinnamaldehyde, and crushed garlic, where the antimicrobial agent is allicin. The antimicrobial activity of the garlic was resistant to mild heating, whereas a reduction in the inhibitory activity of cinnamon was observed following heating. Garlic at a concentration of 5% caused a significant reduction in the rate of growth of *S. typhimurium* (ATCC 14082). Many factors concerned with the sensitivity of salmonellae to the inhibitory effects of herbs, spices and other antimicrobials present in foods remain unknown. This is an ideal topic for further research.

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