

Environmental Effects On Probiotic Prevalence

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Introduction

Probiotics

“Probiotics”, meaning “for life”, make up a form of beneficial bacteria centrally located in the intestinal tract of organisms. These bacteria aid in the digestion of food and killing of harmful microorganisms. Probiotics are readily available in supplement form, such as powders, tablets, or capsules. However probiotics are also obtainable via food forms including a variety of yogurts and other fermented food products (e.g., milk, sour cream, butter). More recently, probiotics have been added to other ingestible products; children’s juice-boxes and various brands of beverages (Juice Beverages with Probiotic Bacteria; n.d.). These probiotics are usually added to the food mixture before they are prepared, if they were not originally present in the food. From the moment of birth, already having probiotics in their body and starting off with a healthy intestinal tract, children are continually infused with added probiotics through the natural act of breastfeeding and the intake of formula. The earlier the probiotics are released to the baby, the more effective they will be in strengthening the immune system and delaying the formation of asthma. When taken in large amounts, the consumption of probiotics will affect the composition of the microflora; living microorganisms that are so small that they can be seen only with a microscope and that maintain a more or less constant presence in a particular area (Microflora; n.d.). Microflora is located in the nasal, skin, oral cavity, vaginal, and gut areas of the human body. The main functions of microflora in the human body are: to provide a barrier to infection by other bacteria species which can cause illness. Secondly, they help the immune system, especially since the largest portion of it is found in the beginning sections of the small intestines. The human microflora signal the immune system to strongly stay in its default mode of tolerance, so it does not go off on a tangent wasting its resources and reacting to something

that is not really threatening until something worthy of mounting an offensive comes along. This prevents inappropriate inflammatory and allergic responses as well as autoimmune reactions.

The third function of the microfloras is that they are generators of nutrients, specifically some B vitamins, short chain fatty acids, and amino acids (Kasdorf; n.d.). Microfloras work with probiotics to keep the intestinal tract of the human functioning and maturing properly.

Probiotics differ from prebiotics, non-digestible food ingredients that selectively stimulate the growth and/or activity of beneficial microorganisms already in colons (An Introduction to Probiotics; n.d.). Prebiotics are not bacteria; they are nutrients that can be found in foods containing whole grains and fruits. Prebiotics possess the nourishment needed for the probiotics to be able to colonize and work with the body. However when prebiotics and probiotics are mixed together, they form a synbiotic. A synbiotic is used in reproducing and improving the amount of microflora in a person. An example of a synbiotic is a yogurt, where live and active cultures are mixed with inulin and produce a synbiotic effect. Inulin is a prebiotic that acts as a soluble fiber and resists digestion, so almost all of it is able to move from the small intestine to the large intestine (Probiotic, Prebiotic & Synbiotic; n.d.).

A vast amount of probiotics supplements, and those forms used in modern-day foods, are especially similar to those bacteria found originally in the human body. Many of these bacteria can be divided into one of two groups: *Lactobacillus* or *Bifidobacterium*. Among these genera are the species: *L. acidophilus*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. helveticus*, and *B. bifidum*, *B. longum*, *B. breve*, *B. infantis*, and *B. animalis*. Depending on the food or supplement taken, the species of bacteria will differ; as different species are more viable than others when combined with certain ingredients or when subjected to varying temperatures. One study, done by Luther Black, proved this when he monitored the amount of viable organisms in a broth

culture being prepared for distribution. At the time of manufacture, the prepared broth was said to contain 200,000,000 viable organisms and one month later, the date of expiration, to contain 100,000,000 viable organisms. However, when tested three days after manufacture, the broth contained a maximum of 48,000,000 per cc (Black, Luther; 1931). The amount of viable organisms significantly decreased because of the interactions taking place between the different substances in the broth and the temperatures at which the broth was stored.

Probiotics Health Purposes

When a baby is born, their intestinal tract is filled with helpful bacteria, to help in the absorption of calcium, the breakdown of milk sugar and the prevention of diarrhea (Probiotics For Children; n.d.). Probiotics, however to ensure the proper development of their immune system throughout life, they will need to constantly be consuming these bacteria. Found in many of the brands of formula are these probiotics. But before these brands can sell the formula, the probiotics being put into the formula must be of a stable strain, where no mutations will occur, and they must be tested to guarantee the safety of the product. However if opposed to the benefits of these formulas and foods for babies, breastfeeding has also been shown to contain a substantial amount of probiotics, which emit benefits as well (How Can Probiotics Help My Baby While Breastfeeding; n.d.). Probiotics found in breast milk hold the ability to synthesize vitamins and carry out the same health benefits as probiotics in other foods would, as well as aiding in the absorption of calcium. Probiotics, when absorbed by babies, have even been shown to decrease the effects of diaper rash, which is common among young infants. Having a strong immune system would help the baby after birth greatly, as a weakened immune system would be more susceptible to disease and various other problems. For this reason, when a mother is

dealing with an unstable or compromised immune system, breastfeeding the infant these probiotics is not recommended (How Can Probiotics Help My Baby While Breastfeeding; n.d.).

Continuing on in life, children are also recommended to have a regular intake of probiotics. These probiotics are available in everyday foods that children would willingly consume, such as Dannon[®] yogurts, ice creams, frozen yogurts, and fermented milk (Sanders, M. E.; n.d.). Having a strong immune system that is able to readily fight off disease and pathogens would be beneficial to everyone.

Similar to babies, older aged adults also have to take more specialized care when dealing with their immune systems and health. Probiotics also benefit this age group, as it continues to provide the benefits of strengthening the immune system and improving the composition of the microflora. The intake of probiotics also improve the nature of the gastrointestinal tract and to help restore the bacteria lost when taking antibiotics (Acidophilus and Other Products; 2009). As older aged people are more likely to be consuming a larger quantity of antibiotics, which destroy beneficial bacteria, as well as harmful bacteria, the ingestion of probiotics adds yet another benefit.

Probiotics have long been advertised to fend off harmful infections and diseases including: infectious diarrhea, irritable bowel syndrome, inflammatory bowel disease (ulcerative colitis and Crohn's disease), infection with *Helicobacter pylori* (*H. pylori*), a bacterium that causes most ulcers and many types of chronic stomach inflammation, tooth decay and periodontal disease, vaginal infections (yeast infections), stomach and respiratory infections that children acquire in daycare, and skin infections (An Introduction to Probiotics; n.d.). Yet there have been a limited number of scientific studies done on the actual benefits from probiotics. It is known that they are helpful, as the name portrays, but only a few proper, fully carried-out studies

have been completed on the effects of these beneficial bacteria. It is, however, known that the ingestion of probiotics will aid in some areas, such as diarrhea (Teitelbaum, J.; 2005). Even further, the products containing probiotics have yet again been ill-tested for their true benefits. It has been brought up that the life of probiotics on store shelves are significantly decreased after they undergo environmental changes during manufacturing. While probiotics are packaged, whether it be in a fermented food form (milk, sour cream, and yogurt) or in a table form, they all undergo some sort of environmental change. These changes include increased heat or cold in the temperature or if they undergo freeze-drying or centrifugation they are at a higher risk to die sooner, if not already dead after the change (Code, B.; 2005). Therefore the actual benefit of these bacteria is difficult to predict, as a substantial amount of testing on the products after manufacturing has not been completed yet. However many companies should start looking into completing more research on these bacteria and the effects of manufacturing before they advertise the immense amount of benefits they have. Similarly, many products on the market today contain all dead, or very few living probiotics, when they are still advertising that millions of beneficial bacteria can be located in their products (Anderson, S.; n.d.). Additional research and experimentation should be completed on products such as these before people start buying into everything they are subjected to. Although companies do take part in false advertising, there is still a degree of truth in them, as probiotics do have substantial benefits.

One of the most known benefits of the ingestion of probiotics is the natural balance it creates in the digestive system, which helps to fend off diarrhea. Typically people experience diarrhea when under extreme amounts of stress or if uncomfortable, nevertheless the effects of diarrhea can be reduced, if not diminished by the intake of probiotics. Probiotics help to regulate

the digestive system and the intestinal tract, causing the breakdown of foods and antibiotics to be more easily completed (Probiotics & Probiotic Supplements: Uses and Safety; n.d.).

Another benefit to consuming probiotics is to help protect a woman's body against yeast infections. The amount of beneficial bacteria, especially in a woman, needs to stay relatively high in order to help fight off infections and harmful bacteria. Therefore when the level of helpful bacteria is too low, a yeast infection is a possibility. An effective way to cure yeast infections is to take a table form of probiotics encapsulated inside a coating; therefore the beneficial bacteria will actually make it to the intestinal tract without being broken down by the acids present in the body (Subhasree, Bhakayaraj, & Vidhyalakshmi; 2009).

Along with other benefits, probiotics have been shown to help increase the absorption of nutrients including: B vitamins, calcium, iron, and zinc. More of the unproven side of the benefits of probiotics include the promotion of health bacteria in the colon and reduces the amount of bile transferred into cancer causing substances, and may increase the amount of lymphocytes as well as antibodies in a human. Yet again these benefits have not been thoroughly scientifically proven; therefore these benefits are based on rough hypotheses and small studies.

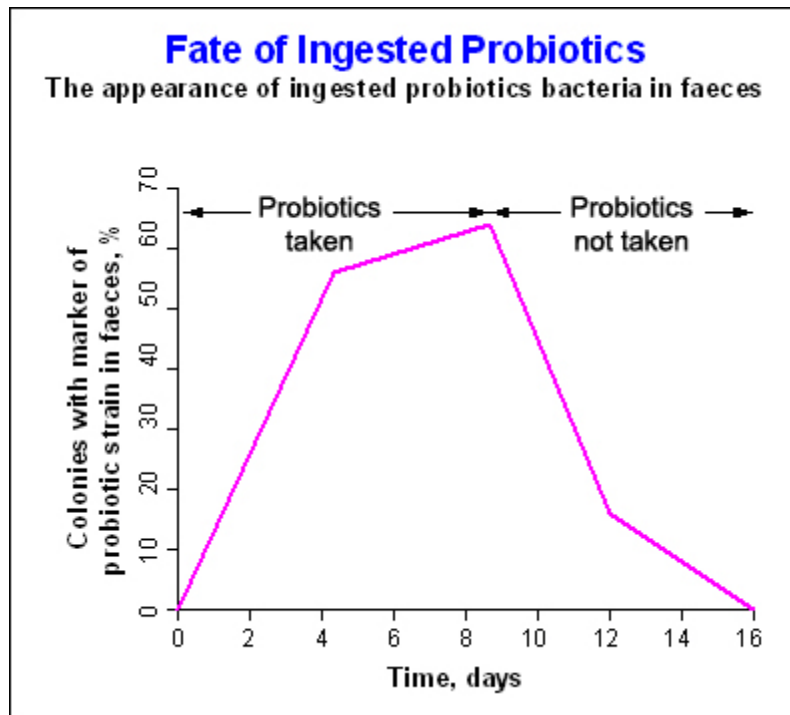


Figure 1(http://www.customprobiotics.com/_images/probiotic_fate.gif)

Small scale studies proving the benefit of probiotics have been completed (refer to Figure 1), however the accuracy of these studies on a large scale are significantly low.

Potential Problems with Probiotics

Although probiotics seem to be all benefit – no downfall, there is another side to these “friendly bacteria.” As more and more companies begin to process and manufacture probiotics and integrate them into an immense amount of everyday foods consumed by humans, more problems also begin to arise. For most of the commercial foods produced, the probiotics are usually mixed amongst each other rather than separately placed into the food; therefore the actual rate of benefit is significantly decreased. The range of foods currently containing probiotics is quite large; however as time goes on more foods will begin to incorporate these beneficial bacteria as well. Since the future holds even more for probiotics, the need for stronger regulations to be implemented now is greater. As of this moment, restrictions and regulations on

the manufacturing and packaging of probiotics is very lenient and essentially almost nothing. However the regulations vary by country. For example in Japan there are little to no regulations on claims regarding probiotics. China follows the same pattern, but is working on establishing guidelines. Australia and New Zealand have divided their system into high and general level claims. And in India claims are told to not be misleading (Starling, S.; 2008). Because of this, the future foods and their benefits will become even more of a gray area, as the mass manufacturing will decrease the stability of the bacteria by the time they reach the customer. The life of the bacteria cells after being subjected to harsh environmental conditions, such as change in temperature, is certainly depleted. In the future, or even now, when manufacturing foods with probiotics, companies need to start paying more attention the viability and stability of the bacteria after undergoing various conditions and if they even hold any benefit by the time they reach the consumer(Matilla-Sandholm , Myllarinen, Crittenden, Mogensen, &Saarlea; n.d.).

Many foods containing probiotics are manufactured with ingredients that may actually kill off all of the present probiotics. For example some yogurts are made with ingredients that give off hydrogen peroxide, therefore killing off the *Lactobacillus acidophilus*, which hold benefits to the human body.

Especially noticeable in fermented foods, it is less expensive for the manufacturer to produce foods by using a starter plate, consisting of many forms of probiotics. The problem with starter plates in manufacturing is that they may create possible antagonisms between the probiotics, therefore reducing their benefits. However the growth of probiotics in non-fermented foods is not desirable because of the unappealing flavor they produce.

When packaging foods containing probiotics it is essential to store them in a packaging material that is a high-quality oxygen barrier, this will promote the life of the bacteria. When

these bacteria are subjected to high-oxygenated areas they are easily killed, as many of the bacteria are anaerobic. One anaerobic probiotic is *Bifidobacterium bifidum*, which are commonly found in yogurts and make up the gut flora in the colon (Probiotic Ingredients Glossary; 2009). Even throughout the storage process, it is paramount to store the foods containing probiotics in an area with a constant temperature, as probiotics cannot be efficiently grown or sustainable in certain temperatures. Storage includes that of a store, when the temperature is too high or too low, the bacteria are more likely to die in massive quantities.

Testing for Probiotics

Probiotics are supposed to be available in many food products today; however whether or not they are truly there and of substantial value is the problem. After being hampered around during manufacturing and packaging procedures, the bacteria have already been subjected to various degrees of temperatures and any other methods they have to endure. These “hamperings” may include freeze-drying or centrifugation and during these processes the viability of the bacteria are significantly decreased. Testing for the presence of probiotics has been done and there are various ways in which to test. One recent report[®] has been conducted by ConsumerLab[®], in which the results showed that the probiotics that had been advertised to contain a certain amount of probiotics, specifically *L. acidophilus* and *Bifidobacterium*, are false claims.

The test completed by ConsumerLab[®] discovered that about one third of the products tested contained less than 1 percent of the expected number of viable bacteria - with several products containing ten thousand times less than expected (Dairy products failing probiotics test; n.d.). The lab tested twenty five various products including nineteen general use products; three children based products and three yogurt products. Of the twenty five tested products only

sixteen had contained the originally recommended one billion organisms or greater per daily serving (Dairy products failing probiotics test; n.d.). The companies producing these products have also claimed, on some products, that the amount of bacteria they advertise is “at time of manufacture”, if they indicate an amount at all.

Another increasingly popular probiotics product, the enhanced beverage, is facing harsh reviews. These enhanced beverages are primarily sold over the Internet and do not specifically claim the amount of – or the type of – bacteria they contain. It is very easy to get a consumer to buy into your product, especially since probiotics have become a huge hit. Replenishing the “healthy bacteria” in the body in order to improve a life, would appeal to many people and big companies are easily gaining customers. Many of these drinks contain around thirty five strains of bacteria, however only about twelve actually survive long enough to make it through to the intestinal tract. It has also been concluded that out of about fifty commercial products on the market today, about half of them contain non-beneficial bacteria, therefore being sold at higher prices for nothing (Hall; n.d.).

The Future of Probiotics

Probiotics and their supposed benefits have been around for thousands of years, however only recently are they gaining true recognition. Since new studies have been conducted, truly revealing the potential benefits of these bacteria, people are rapidly catching on to the products. Probiotics are already, supposedly, available in a wide array of products from yogurt, baby formula, milk, sour cream, to pizza. The possibilities of food that can contain these bacteria are endless, conversely with the number of products containing probiotics, regulations and rules need to become more intact. The large manufacturing companies are starting to become careless

with the procedures they use to package and handle these bacteria, often killing off the bacteria. If the future for probiotics is going to go anywhere, it needs to start at the root of the products, and the viability of the bacteria needs to become more stable. It is known that probiotics have health benefits, although all of them are not yet identified, and in order for them to be spread manufacturers of all probiotics products need to take into consideration the amount of people they are reaching and the impact of wrong bacteria in foods on their customers. The probiotics revolution is just beginning and with the correct protocols and advertisement the amount of benefits they can effuse into the public are infinite.

Abstract

One consistently increasing problem in our country today is the lack of quality put into manufactured goods. An example of this is seen in many food products sold commercially such as foods containing probiotics. Our country, among many others, has set an absurdly low standard for evaluating and testing probiotic foods on the market today. Therefore something needs to be done to be able to demonstrate to consumers that they are not just paying for the supposed ingredients in a product; but that they are indeed there. This project tested for the presence, and quantity, of probiotics in designated food products said to contain probiotics (yogurt, frozen yogurt). This experiment was placed a color indicator in the food sample which turned green to indicate lactic acid – formed when probiotics are present in the food. From here the amount of probiotics present in each food sample was approximated. After the amount was found the sample underwent an environmental change (extreme heat or cold). After the sample underwent the environmental change the amount of probiotics was again tested. This testing is beneficial because it has a practical application in identifying the viability of probiotics in a food product after it has undergone environmental changes, similar to those it will encounter during manufacturing. Consumers will now be able to purchase products based on the true amount of probiotics inside and not just by the fancy advertising the companies can do. Being able to know this information will allow the consumers to purchase products based on the amount of probiotic intake they need for the various benefits probiotics contain.

Materials/Procedure

<i>Chemicals/Consumables</i>	<i>Supplies</i>	<i>Equipment</i>
Trypticase soy powder Agar powder Distilled/deionized water Bromocresol green Crystal violet Ethyl alcohol Ammonium oxalate Stonyfield organic vanilla yogurt Frozen yogurt	Petri dishes Weighing boats Bunsen burner Test tubes/covers Test tube rack 200mL/ 500 mL glass bottles	Autoclave sterilizer Shaking water bath

Testing Presence of Probiotics in Food Samples

In order to test for the presence of probiotics in the given food samples, a trypticase soy broth will need to be made. This broth will be made by mixing together 200mL of distilled water with 6g trypticase soy powder. Place this broth in a glass bottle, then into the autoclave sterilizer. After the broth has been autoclaved, add .08g of bromocresol green indicator into the broth and fill small test tubes with broth solution. Invert the small test tubes into the larger test tubes and then fill about a half of an inch more with broth into the larger test tube. Place the test tubes with covers) into a breaker and autoclave. Place yogurt samples into the test tubes with broth and inverted test tubes using a scoopula. Put test tubes into shaking water bath at 42°C overnight. If test tubes turned green, probiotics are present.

Creating Environmental Changes

Take a sample from the test tubes with probiotics growth, subject the sample to extreme heat (anything over 55°C) or extreme cold (anything under 15°C). Test samples for presence of probiotics, using microscope or gram-stain. Use dilution and plating in order to quantify the amount of living bacteria before and after environmental changes.

Dilution and Plating

Use a clean, sterile, dry pipet to remove 0.1 ml from the bacteria sample and blow it into the 9.9 ml of dilution fluid in tube #1 and mix thoroughly by blowing lots of bubbles with the pipet for a couple seconds. Discard the pipet into the used jar for later cleaning. Notice tube #1 now contains 1/100 the concentration of bacteria in the original sample because 0.1 ml is 1/100 of 10 ml. Using another clean, sterile, dry pipet remove 0.1 ml from Tube 1, wipe pipet, blow contents of pipet into Tube 2, continue blowing bubbles for a second or two for good mixing. Using another clean, sterile, dry pipet remove 0.1 ml from Tube 2, wipe, blow contents of pipet into Tube 3, continue blowing bubbles for a second or two for mixing. Plate 0.1 from Tube 3. Suppose 125 colonies grow in the petri plate. Multiply the dilution factors by the count to obtain the titer of the bacteria sample: $100 \times 100 \times 20 \times 125 \Rightarrow 250,000,000$ bacteria per milliliter in the original sample. $250,000,000 = 250$ million = 2.5×10^8 bacteria per milliliter (Eddleman 1998). Sterilize a wire loop over Bunsen burner, then obtain a small sample from the test tube, after dilution, and place the sample on the plate with agar. Then incubate the plates for 24 hours at 37°C .

Conclusion

After all of the various testing methods were completed and results were gathered, the experiment had finally come to a close. Since the purpose of this experiment was to test for the presence of probiotics in store-bought vanilla yogurt in various forms, specific tests were conducted to gather the results. The experimentations included creating a Trypticase soy broth (to allow for bacterial growth) with bromocresol green added to the broth to indicate the presence of lactic acid. This broth was added to several test tubes, as well as a small amount of the plain yogurt sample. These test tubes were placed in a shaking water bath at 42°C overnight to allow for bacterial growth. After gather observations from the test tubes, it was concluded that the presence of lactic acid (probiotics) was existent in the test tubes. Since there was a presence of lactic acid in the plain yogurt sample, further tests were conducted using other samples and to calculate an estimated amount of probiotics present in each yogurt sample.

The subsequent test involved several dilutions of each yogurt sample to find more accurate results. Dilutions were conducted by obtaining 0.1 g of the food sample - and adding to that sample – 1000 µl of PBS buffer solution. From this mixture, 100 µl were plated onto a sterilized petri dish, with Trypticase soy agar already on the bottom as a growing medium. Petri dishes of each food sample were created at ten-fold dilutions (10^{-6} , 10^{-5} , 10^{-4} , ...). These petri dishes were placed in an incubator at 37°C for two days. At the end of the two days, the petri dishes were observed for bacterial growth of colonies. The results provided the amount of colonies that were present on the petri dishes of each food sample and its diluted plates. The only plates that were used in the data collection were plates 10^{-6} , 10^{-5} , and 10^{-4} , as they were the only plates with a countable number of colonies (shown below).



Plain yogurt plate 10^{-4}



Plain yogurt plate 10^{-5}



Plain yogurt plate 10^{-6}

The picture above shows the amount of bacterial colonies present on the petri dish of the plain yogurt sample. After obtaining the amount of bacterial colonies from each different food sample, the numbers were used to create an approximate probiotic cells per gram of food sample. The calculations completed for the plain yogurt sample dishes are shown below.

$$\begin{array}{r}
 6.00 \text{ colonies} \\
 + 8.90 \text{ colonies} \\
 \hline
 14.90 \\
 + 4.16 \text{ colonies} \\
 \hline
 19.06 \text{ total}
 \end{array}
 \Rightarrow 19.06 \div 3 = 6.35 \text{ average probiotic cells}$$

↓

$$6.35 \text{ cells} \times 1,000,000 \text{ dilution} = 6,350,000 \text{ cells}$$

↓

$$6,350,000 \text{ cells} \times 10 \mu\text{l PBS dilution} = 63,500,000 \text{ cells}$$

↓

$$6,350,000 \times 10 \mu\text{l PBS} + \text{food sample} = 635,000,000 \text{ probiotic cells/g}$$

After completing the calculations for each food sample and its petri dish colonies, the data chart shown below was created to as an organized conclusion of the data collected.

Food Substance	10 ⁻⁴ Plate Dilution Bacterial Growth	10 ⁻⁵ Plate Dilution Bacterial Growth	10 ⁻⁶ Plate Dilution Bacterial Growth	Estimated Amount of Living Probiotic Bacteria
Plain Yogurt (Stonyfield Organic French Vanilla Yogurt)	416	89	6	6.35x10 ⁸ probiotic cells/g
Plain Yogurt (Open for 1 Week)	No growth	No growth	No growth	-
Frozen Yogurt (Hood Frozen Yogurt)	-	37	4	3.85x10 ⁸ probiotic cells/g
Hot Yogurt (Heated to 62°C)	No growth	No growth	No growth	-

The data collected from the experiments conducted proves that companies do partake in false advertising. Several companies claim that the amount of probiotics present in their products are much higher than they possibly can be. After the food undergoes manufacturing and shelf-time and time exposed to oxygen, the amount of probiotics alive in the product significantly decreases. After being left open for a week in at room temperature, the plain yogurt sample produced no trace of probiotics – the same data was collected for the yogurt that was heated to 62°C. However the plain and frozen yogurt petri dishes did produce several bacterial colonies which were then converted into an approximation of probiotic cells per g. The plain yogurt had an estimated 6.35×10^8 cells/g, while the frozen yogurt had a mere 3.85×10^8 cells/g.

Companies producing these foods should not be allowed to advertise an abundance of probiotic cells contained in their products, until rightful testing has been completed. The regulations on probiotic-containing products need to become more strict so that companies are not engaging in false advertising and leaving the customers at a loss.

The validity of these experiments cannot be completely verified, as multiple trials were not conducted. After multiple trials are conducted for each experiment and food sample, the conclusions will be more accurate and valid.

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