Beneficial Effects of Natural Antibodies During Times of Stress in Puppies
子犬のストレスに対する自然抗体の有効性について

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Thank you for having me here this afternoon. Before I get started, I'd like to do a survey of the audience. Could you raise your hand if you are a veterinarian? Or, are you a veterinary or animal science student? Perhaps you are just a passionate animal lover? Those of you who haven't raised your hand, are you from another pet food company? The reason I ask this question is that it is always nice to know who my audience is. Then we can be sure we are communicating at the same level. The other thing I would like you to know before I talk about the science is that this dog [photo] is my newest puppy. So this is a topic that is near and dear to my heart right now.

There are three main things I wish to communicate today. The first is: I want to make certain that we all agree that a healthy immune system is central to starting a healthy life and key to overall longevity. That is the case, not only in dogs, but certainly in humans as well. Another thing I want to talk about is that the immune system can be effectively modulated, and explain the different ways that we can modulate it, remembering, of course, that modulation is not just stimulating the immune system, but also dampening the inflammatory response as well. Finally, I would like to share some data with you related to milk derived proteins and their effects on, not only the immune system, but on overall gut health.

[Slide 3] This slide is just a reminder for those of you who are veterinarians, and a quick tutorial for those of you who are not, about the immune system. The first thing is that it is the job of the immune system to act both locally at the level of the gut and the skin (and sometimes at the level of the kidney as well as systemically or over the entire body) in order to repel potential pathogens or invaders. Thinking of the immune system as if it were a corporation [slide 4] helps us understand how it works a little bit better. In this picture I have organized the immune system as a chart, in the same way as you would put the names of people within a corporate organization.

So, if there are two arms to the immune system, the innate arm and the adaptive arm, and if, under both arms, there are processes and cells carrying out those processes, from a modulating point of view, we want to affect both the process and the cells themselves and the way they react. (One point I forgot to mention on this slide is that 70% of the immune system resides in the gut). There is a very close association between gut health and overall immune status. So if you have an unhappy or upset gut there is a potential for immune compromise because the primary placement for immune cells is in the gut.

[Slide 5] This slide lists for you all of the different components of the immune system. I have made this slide for two reasons: the first is to remind you that the immune system is all over the body – it's not in one concentrated place in the body. The second is to show you a picture of this lovely cat even though this is a discussion primarily about dog gut health. The reason I'm showing you this lovely cat is because she is my cat and her name is Lymph Node. (My other two cats are named Protein and Fat but my husband says that if I'm going to name my cats with stupid nutrition names, I can't have anymore cats. Right now we're at an impasse. We're stuck at three cats).

[Slide 6] As I said, there are two arms to the immune system. The 'innate arm' is responsible for non-specific activity so it's going to react the same way regardless of whether it is facing a really virulent pathogen or a mild pathogen. It will use the same set of cells and reactions to do that. [Slide 7] On the other hand, the 'adaptive arm' of the immune system, requires knowledge of the pathogen. This part of the immune system therefore takes a few hours, or even a few days, to understand what the pathogen is and then develop an antibody against it. The adaptive arm uses a combination of not only B and T cells (which is primarily what we think of when we think about the adaptive immune system), but it also uses a combination of all these other cells and byproducts of these cells, like cytokines. Of course, you all know that once the adaptive immune system

has an antibody for a particular pathogen (or group of pathogens), it can respond very quickly when facing those pathogens again.

So, that was a five-minute course on how the immune system works. What I'd like to do now is switch topics and talk specifically about what growing up means to puppies from both a physical and a physiological point of view.

[Slide 7] Growing up is fast. And there are three major organ systems that we tend to think of as the protective organ systems at Nestle Purina. The skin and coat is a protective organ system. The gastrointestinal tract is a protective organ system. And the immune system also helps to protect the body as well. When puppies are growing so quickly, there are lots of changes in all these protective systems. When we talk about the skin and coat what we know is that this is the second largest organ in the body and that it requires rapid development during this growth phase. Just as side information, puppies don't lose their puppy coat when they grow up – they just gain their adult hair – because puppies are born with simple hair follicles, like humans. They have one hair coming out of every hair follicle but, as they grow up and gain their new hair, they add multiple hairs coming out of the same follicle. That is true for most breeds of dogs.

[Slide 9] Now, switching to talking about the gastrointestinal tract and the immune system, I will talk in detail about the changes that happen there. Other than the lungs, the digestive tract makes more changes than any organ system in those first 24 hours after the puppy is born. Suddenly the digestive tract has to go from processing amniotic fluid across the placenta to, instead, needing to digest milk that's being ingested. It has to rapidly adjust and start making enzymes in order to assimilate these nutrients. So, in the first three weeks, cell thickness in the gut doubles to allow for increased surface area to assimilate the nutrients and to increase enzyme production. At this same time the gut is also being inoculated with micro flora.

[Slide 10] As you all know, the first 24 to 48 hours is when colostrum is produced by the mother, and this colostrum passes on a variety of different things. Firstly it's going to pass on passive immunity from the mother. So, antibodies are being transferred from her during that time period. At the same time the colostrum is passing on growth factors to help the gut to begin to grow. It is also passing along resident micro flora from mother to puppy. The puppy is also getting micro flora from the environment. The final transition for the gut it at weaning. So the gut has to go from processing what is essentially a highly digestible liquid diet to digesting a very complex matrix of nutrients from either a complete balanced dog food or a homemade diet. Sometimes that transition can take up to 22 weeks in order for it to happen fully. My point here is that the immune system of the gut, although functioning when puppies are born, is not fully functioning as it will when the dogs become adults. So we need to take some care in the way we feed a naive gut compared to the way we would feed an adult gut.

[Slide 11] Now I want to switch and talk about things that happen in the immune system in puppies. The first thing to know is that it takes several weeks, or even months, for the immune system to fully develop in a puppy. The reason for this is that, because they have naive immune systems, everything is a 'first response'. That is to say, it is the first time for the immune system to develop an appropriate reaction. The next thing is that maternal antibodies last between 10 and 16 weeks and provide protection during that period. But there are cases when maternal antibodies aren't enough for the puppy. Listed on the slide are four different incidences where maybe colostrum doesn't provide enough antibodies. Everything from, the 'mother doesn't produce enough' to the 'puppy doesn't have the ability to take in milk' (for whatever reason) – that is, they have compromised absorption –, or finally, that the puppies have been 'moved during this critical transition time', something which compromises the puppy's ability to absorb those antibodies.

I have a good example of puppies probably not getting enough colostrum. I was in Australia in October and a breeder of Mastiffs came up to me and very proudly showed me a picture of her Mastiff bitch. She said, "Look at my beautiful Mastiff, she just had 20 puppies." But dogs aren't built to have 20 puppies in one litter so I can guarantee that not all of those puppies have received enough colostrum. This is a case where, because of the pressure we're putting on our dogs, we are actually setting them up to be less healthy than they could. It could be reproductive pressure, as in this case, or environmental pressure. My point here is that, like humans, there is a lot of stress associated with growing up and building all of these organ systems to maturation. That stress can make puppies

vulnerable and, as with humans, there is an 'immunity gap' in puppies that lasts from about week three (when they start to transition to eating complex food matrices) up until about five months of age.

This is why, of course, we vaccinate our puppies during that time frame to help protect them against potential infections. Although I have already established that there are stressful things that can happen to puppies as they're growing up, this slide [Slide 12] lists a variety of commonplace activities that are actually stressful for puppies. I can tell you this with some certainty because we did a study to determine if these really are stressful activities. We used statistically validated fecal scores as a way to evaluate stress in the puppies. We were looking to see if, when doing certain things, there was a change in the puppies' fecal consistency and quality. If there was a change, the conclusion was that some form of stress must be causing changes in the microbial population.

[Slide 13] In this slide the three bars represent a 48 hour time period around a puppy being bathed. The reason we measured 48 hour time periods is that it takes 24 hours for dogs to assimilate a meal. Taking two measurements over a 48 hour time period allowed us to evaluate the effect of the associated stress event over a two meal period. Based on the fecal scoring chart, a score of 75 or above is considered normal feces. A score of 75 or below is considered abnormal. Typically the feces is soft and may have a change in color. Looking at this graph, we see that in the 48 hour time period associated with the bathing stress, the puppies had non-normal fecal quality. This told us that the bathing was a stressful event for them. To explain this better, I need to back up and explain what is happening in the small intestine.

[Slide 14] There are essentially two groups working in the small intestine. There are the good guys, which in this case I represent with ladybug images (because I hear these are very lucky bugs). And there are the bad guys, which I represent with spiders.

The good guys, such as bifidobacteria, do positive things in the gut. They provide energy for the cells and help prevent diarrhea. They make the environment more acidic which increases nutrient absorption, particularly of minerals. The bad guys do bad things such as make enterotoxins and potential carcinogens as well as creating the substances that make feces smell.

[Slide 15] The first thing I have to tell you about this slide is that this Labrador is not my dog. How many of you own a Labrador? (Nobody – probably that's because people in Japan tend to have smaller dogs?) Labradors will do this [climb into dish washers] if given the opportunity. This is a good representation of potential stress in the gut of a puppy.

What happens when there is stress in the gut is that it shifts the microbial population. The result can be increased shedding of bad bacteria as well as a decrease in the functional barrier of the gut (because of this shift to bad bacteria). The results and negative implications include a negative effect on growth and a negative effect on nutrition absorption. And, as I mentioned to you earlier, because so much of the immune system is based in the gut, if there is a negative impact on the gut there can subsequently be a negative impact on the immune system. I will give you a personal example of this – I have a dog that, one year at Christmas time, ate a lemon, some coffee beans and wrapping paper. Needless to say there was a negative shift in his gut bacteria and his stomach was making all kinds of noise for the next 12 hours. That is an example of a negative thing that can happen in the gut that causes stress.

[Slide 16] This is a little summary, before I go on and talk about the data, of the points I have mentioned so far. The first is that, it takes the gut a few weeks to become fully functioning and that there are many things that have to change in the gut for it to function properly. The second is that there are many things that we, as humans, would not consider stressful but which, for our puppies are stressful. This does not necessarily mean 'bad stressful', because playing with puppy friends is not a bad thing but, nonetheless, it is stressful from a physiological point of view. The last thing is based on data, not just for puppies, but for other species as well, that there is a gap in the body's ability to protect itself during the growth phase. In puppies this gap is between three weeks and five months of age.

And just to give you one more example of the immune system being important for overall health and longevity. There was a lovely study done that looked at the incidence of allergies in children. What they found is that, if they inoculated the children by day three with a certain subset of probiotics or bacteria, they could prevent the incidence

of allergy in children genetically pre-disposed to allergies. However, if they instead gave children a placebo they tended to have the same incidence of allergy as their mothers. [Slide 17] My point, once again, is that setting the immune system up to function properly at a very young age is important for overall health and longevity. That is a key component for making sure that the gut works properly.

I will now talk about different ways that we can modulate the immune system through nutrition. I don't think I mentioned that I have five dogs in addition to the three cats. So in this presentation you will see every dog I own. The only thing you won't see is the husband and the horses. Other than that I'm showing you my whole life. [Slide 18] So this is one of my dogs and I use him as my example for modulating the immune system. There are three things that can change the way the immune system works. The first are the genes but we, as scientists, can't do anything about those genes right now. However, they certainly affect how his immune system is going to initiate itself, at least in the beginning.

The second thing is 'life stage'. We can't make dogs younger or older than they are, and they age at a certain rate. But what we can all affect as caregivers, and what I can affect as a nutritionist, is lifestyle. Lifestyle includes everything from what environment we expose our animals to, to the diet they eat. There are certain health implications associated with modulating the immune system and they are both long and short term. The first one is that if we can improve the way the innate arm of the immune system, the non-specific arm, works we can improve how quickly that arm of the immune system can respond. If we can improve how the adaptive arm of the immune system works, we can prime the cells that make antibodies so that they are ready to work quicker. We can increase the activity of certain specialized cells like B and T cells so that the adaptive arm can react quicker.

[Slide 19] The result would be increased and faster antibody production in the face of a potential pathogen and therefore priming of the acquired adaptive immune system. All of this priming results in an increase of the natural defenses. It increases the gut defense system, the skin and coat immune system and, overall, increases immune reactivity. There would be a decreased incidence, or potential decreased incidence, of autoimmune disease and allergies to create the foundation for a healthy life.

[Slide 20] I will now show a study, conducted three or four years ago, in which we were evaluating different ingredients that have a potential to modulate the immune system. One of the ingredients we evaluated had been used in the human food sector. It was called 'hyper immunized egg powder' which is a powdered egg component made from the eggs of hens which are fed a specialized diet and which are immunized. In this, a certain set of hens are selected and immunized against certain pathogens, namely, the ones listed on the slide. The eggs are processed to maintain the immunoglobulins, and then applied to certain foods.

[Slide 21] For our project, we applied them to a puppy food and conducted a 40 week-long study to evaluate the efficacy of hyper immunized egg powder on the puppies. We did this study at our facility in Alaska with Alaskan sled dog puppies. We wanted to know if we could affect gut, or local immune response at the gut. The puppies were fed for 40 weeks during which they had an exercise protocol because they were growing up to be sled dogs. We took sequential fecal swabs from the puppies as well as blood samples. [Slide 22] What we found is that hyper immunized egg powder decreased fecal PH. As I said before, decreasing fecal PH is a good thing because it increases nutrient absorption and decreases the ability of pathogenic bacteria to reproduce. It also increased IgA in the gut and decreased stress-induced and related diarrheas. That was a great ingredient and we really thought that that was the best ingredient for us to use at the time.

[Slide 23] However, the next step is to find out if we can improve on that. We thought about how we could improve on immune modulation in two ways. The first concerns the fact that hyper immunized egg powder is very expensive and very difficult to produce. Colostrum, on the other hand, is a readily available and under-utilized organic ingredient. There appeared to be a lot of data showing that colostrum had a benefit in other species. [Slide 24] For those not familiar with what colostrum is, this is the first milk from cows. The pie chart data lists the composition of bovine colostrum in comparison to mature milk from cows – it is much higher in immunoglobulins and specific bioactive molecules like lactoferrin and glycomacropeptide. [Slide 25] Based on studies in other species we know that colostrum has antimicrobial activity, specifically for lactoferrin. In some species it has anticancer activity. It also has some mild prebiotic activity, based on one of the peptides, and it can

promote satiety – that is – it can make animals feel full. The other thing that we know about colostrum, based on other studies, is that it has a direct effect on how the gut matures. As I mentioned earlier if there is insufficient colostrum the guts of puppies don't develop properly. [Slide 26] On this slide I have listed all the different specific growth factors that come from colostrum and which affect the way that cells mature. We must keep in mind that gut cells turnover every three days and that, if there is not enough of these growth factors (which help promote this turnover), then gut health will be compromised. [Slide 27] Specifically, when it comes to the immune system, we know that there are both immune stimulating molecules and immune modulating molecules. I said earlier that it is important to have both. We don't want to constantly stimulate the immune system – we only want to be able to modulate it and have it down-regulate, if needed. At the same time, colostrum is also a really great source of certain amino acids that are used directly by immune cells as fuel. They are also used by muscle cells.

There were 2,205 studies on the benefits or potential benefits of colostrum and colostrum's components in the last 10 years. Of all those studies only one of them was done with dogs. [Slide 28] It was a very small study looking at the effect of feeding bovine colostrum to puppies after they arrive at a pet shop. They found that the puppies fed colostrum were less likely to have diarrhea than the puppies that weren't fed colostrum. They concluded that there was that something in colostrum helping to prevent diarrhea in the puppies. These were puppies that had just gone through a very stressful event. They are being weaned, so their digestive tracts are assimilating to a complex food matrix, and they have just left their mother – highly stressful events for them.

[Slide 29] Now I will show you the data results from a study we did to compare colostrum to a control diet and whether we saw any beneficial effects of adding colostrum. This study was set up in a very similar way to the study explained earlier regarding hyper immunized egg powder. [Slide 30] There were 24 dogs on 2 diets, for a 40 week trial, again carried out in Alaska. In addition to looking at changes in gut immune health and gut digestive health, we were interested to know the systemic immune response or overall body immune response. [Slide 31] This is the schematic for the study. All dogs were fed the control diet for the first four weeks of the study. We did that because we wanted them all to be using the same set of enzymes and it takes between 2 and 3 weeks to change enzyme levels in a dog's gut when you switch diets. We also wanted the dogs to have similar bacterial populations because they were eating the same diets for a four week period before we added the colostrum.

At week zero we changed diets to either the control or the control with colostrum and we vaccinated the dogs against distemper. After that we took serial blood samples every four weeks in order to monitor a variety of immune factors and we also arranged a stress event for the dogs. There were 12 dogs per treatment and this slide [Slide 32] lists all of the different things we evaluated. I didn't mention this earlier but our company policy is a non-invasive policy. This means that we don't do anything to dogs and cats that isn't normal routine veterinary care unless they need it. To us scientists it means that we must find alternative, non-invasive, ways to evaluate changes in certain organ systems. So one of the things we have done is develop a protocol that allows us to evaluate systemic immune response by using vaccines that are part of normal veterinary care, at least for dogs in the U.S.

We use a protocol that was validated in 2003. What we do is to select a vaccine that the dogs would normally require and give that vaccine singly. Then we monitor the antibody levels in response to that vaccine. That tells us the magnitude of the systemic response in relationship to a nutritional change, (keeping in mind that nutritional change is a physiological change, not a pharmacological change). It is a very subtle change and we have to develop sensitive ways to evaluate it. [Slide 33] This graph shows the fold change in canine distemper vaccine antibodies in the dogs that were either fed the control diet or the diet with colostrum.

So what we see is that the dogs fed colostrum had a much stronger antibody production and that the increased level of antibody production lasted longer than it did in the dogs that were fed the control diet. This told us that the immune system is experiencing a system-wide body response. We knew we were making a change in the entire body because that is what the data was showing us. So we were also interested in seeing if we could make a change in the local immune system or the gut associated lymphoid tissue because the gut is so closely tied to the immune

system. [Slide 34] To have a positive response in the gut will usually predict for us a positive response in the body. From this data we see that secretory IgA (which is produced in the gut) was statistically higher in the dogs fed the colostrum than in the dogs that were not fed colostrum. These results beg the question; "Was the immune system over-stimulated? Is that is why there is such a large response?" [Slide 35] So, in order to evaluate this possibility, we looked at a variety of factors. If you are a veterinarian you will recognize that 'C-reactive protein' is a way to monitor the amount of inflammation in the animal gut. To those who are not veterinarians I need only say that there are ways to evaluate if there's inflammation in the body and that we carried out one such test. When there is inflammation in the body the C-reactive protein levels will be over 50 micrograms per milliliter. Normal levels of the protein (which is indicative of inflammation) range from 8 ~16 ml. So, based on these values, neither group of these dogs was experiencing an inflammatory reaction.

[Slide 36] So the immune system was only responding when challenged – which is what the vaccine was providing with a specific challenge. Listed here are the other things we evaluated. We looked at plasma IgG, IgM and IgA, which are all antibodies found in the body which will increase when there's inflammation. There is no statistical difference between the dogs fed the different diets, which reiterates my point that the immune system was not overreacting.

[Slide 37] So now I will summarize everything I just put together about the immune system. Based on the protocol that we used to evaluate the changes in antibody level (associated with the administration of a vaccine) there was an enhanced systemic immune response and this response was stronger and lasted longer when dogs were fed colostrum, and it enhanced the local gut immune response. The benefits are not only helping to provide an overall better systemic response, or full body response but, by increasing local gut response, we are protecting the body from other pathogens that could potentially be ingested. Finally, based on evaluation of certain proteins and certain antibodies in the blood, we confirmed that we were not causing any over-reaction or inflammatory reaction in the dogs by adding colostrum to the diet.

[Slide 38] The second component of this study was to look at gut health measurements. And what we looked at in these studies was fecal quality, because that's what nutritionists do – we like to look at 'poop' and our lives as nutritionists revolve around poop! The other thing we were interested in was whether there were any changes in the microbial populations. I can report that there was no statistical difference in fecal quality, so I will not bore you with all the graphs. As I said, we also looked at changes in microbial population and we find that the gut microflora are constantly in flux – they are changing all the time, [Slide 39]. There are a variety of different reasons why these changes occur. These can be everything from fast cell turnover (discussed a few slides ago) to the introduction of new foods (which can certainly affect bacteria population) as well as an interaction of the microenvironment. By that I mean 'how the bacteria are responding to certain foods'. An example of that would be (in people) the eating of yogurt, which promotes interactions within the microenvironment of the human gut and, of course, we humans are certainly promoting the health benefits of yogurt and such prebiotic or probiotic foods because we know that they have positive effects.

There can also be negative effects if we eat bad things. I say this because we wanted to evaluate if there are changes in the different species of bacteria in the guts of dogs and how stable such populations are. What we did, as I said already, was to create a stress event for the dogs in the study. All the dogs were trained to run 14 miles every other day (which is their job, being sled dogs). We then gave them a five day rest period and then had them run 14 miles again. As any regular runners knows, if you take 5 days off and then run again, you feel bad the next day if you have run at the same pace for the same distance. There are also changes in your gut health associated with that first new run. So what we did in this study was to take rectal swabs a day before the dogs ran, then again after their rest, then right before the dogs ran, again after they ran, and then a day after they ran. We took the swabs so that we could collect small pieces of feces to evaluate the bacteria present.

[Slide 40] What this slide shows is a 'gel electrophoresis' picture. Each of the lines represents a species of bacteria. We were looking for consistency from sampling period to sampling period. We wanted to know how many different species of bacteria were present in the dogs fed with the controlled diet compared with dogs fed the diet containing natural immunoglobulins, and if those bacteria remained the

same. If the species remain the same from sampling period to sampling period this tells us: one, the diversity of the microbial population and, two, the stability of the population when facing stress. The slide shows that consistency between the sampling periods is not that great for the dogs fed the control diet. There are some bands that are fairly consistent (from sampling period to sampling period) but when you look at the dogs fed the natural antibodies diet there was more consistency (from sampling period to sampling period). We also found that there were significantly more stable bacteria populations in the dogs fed the colostrum. [Slide 41] There was about 35% stability in the control dogs versus 68% digestibility in the dogs fed the colostrum. So, in other words, there was nearly double the neighborhood stability in dogs fed colostrum versus dogs fed a control diet.

[Slide 42] If we put this in different terms, what the data means is that feeding dogs with colostrum appears to increase gut micro-diversity. It increased the different numbers of bacteria species in the guts of both puppies and dogs. This means that when there are many different kinds of bacteria available, there is a far less likelihood that one virulent pathogen will take over. The second thing we learn from this is that dogs fed colostrum have more stable populations. All the different bugs stayed there regardless of stress, the potential benefit of which means fewer incidences of loose stools – something which is stressful for both puppies and their owners. Gut upsets are also less likely because there is no shift in the microbial population (as discussed earlier). I mentioned about my own dog eating bad things which caused him a gut upset. We are preventing this from happening.

The final thing we looked at were changes in body weight as well as blood profile changes – CVC's and blood chemistries. There were no significant differences so I will not bore anyone with that data. But if I take all of the information from this study and summarize it for you in six key points, what we know is that:

[Slide 44] Colostrum in this study appeared to enhance systemic immune status based on the vaccine model. Based on increases in antibody production in the local gut we know that it enhanced local immune response. When we looked at potential inflammatory proteins we did not see any changes. This means there was no overt immune activation – so the body wasn't over reacting, it was just reacting appropriately. It also appears that the bioactive ingredients in colostrum promote gut micro-diversity and decrease shifts in microbial population under stress.

[Slide 45] This photo shows both my dogs. The black dog is 18 years old and the little guy is 6 months old. I tell my husband that, if he's nice to me, I can make certain that he lives to be as old as that black dog. But if he's not nice to me, being a nutritionist, I know ways to make his life significantly shorter. In summary, when pet food companies do such extensive research we do it for a reason. That reason is to make product that has some benefit to the dogs or the cats we are targeting. Here is the end result of all of that data that I just showed to you. We have re-launched our puppy foods and the reason we have done that is because we have added colostrum into the diets of those puppy foods. We believe very strongly in the science I have just shown to you and we want to make that available to people who have puppies so that we can help set up their immune systems and their gut health in a very positive manner while the puppies are growing. So, that is everything that I wanted to cover in my talk, but I am happy to answer any questions. We don't have to go through all my 'frequently asked questions' [slides] but I'm happy to answer any questions you may have